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Role of Intrinsic Coagulation Pathway in the Pathogenesis of Idiopathic Pulmonary Fibrosis

INAUGURALDISSERTATION zur Erlangung des Grades eines **Doktors der Humanbiologie**
des Fachbereichs Medizin der Justus-Liebig-Universität Gießen

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I. Table of contents

II. List of figures

III. List of tables

IV. List of abbreviations

APS	Ammonium persulfate
ATII	Alveolar epithelial type II cells
BAL fluid	Bronchoalveolar lavage fluid
BK	Bradykinin
B1	Bradykinin receptor 1
B2	Bradykinin receptor 2
BSA	Bovine serum albumin
C1q	First complement component
cDNA	Complementary deoxiribonucleic acid
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin immunoprecipitation
CK1	Cytokeratin 1
CTGF	Connective tissue growth factor
CTI	Corn trypsin inhibitor
DAPI	4’,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
EDTA	Ethylendinitrilo-N,N,N’,N’ tetra acetate
EGTA	Ethylene glycol-bis (2-amino-ethyleter)- N,N,N’,N’-tetraacetic acid
EGF	Epidermal growth factor
ERE	Estrogen responsive element
ERK	Extracellular signal-regulated kinases
FAK	Focal adhesion kinase
FITC	Fluorescein-5-isothiocyanate
FCS	Fetal calf serum
FSB	First strand buffer
FVII	Factor VII
FX	Factor X
FXI	Factor XI

FXII	Factor XII
gC1qR	First complement component receptor
HepG2	Human hepatoma cell line
HAE	Hereditary angioedema
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLF	Human lung fibroblasts
HMWK	High molecular weight kininogen
HMWKa	Kinin free protein
HNF-3	Hepatocyte nuclear factor-3
IgG	Immunoglobulin G
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
JNK	c-Jun NH_2-terminal kinase
KLK	Kallikrein
LH	Lung homogenate
LMWK	Low molecular weight kininogen
MAPK	Mitogen-activated protein kinase
MNL	Multinuclear leucocytes
NIH3T3	Mouse embryonic fibroblasts
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor-1
PARs	Protease activated receptors
PBS	Phosphate-buffered saline
PCK	H-D-Pro-Phe-Arg-chloromethylketone
PCR	Polymerase chain reaction
PCRP	Serine protease prolycarboxypeptidase
PK	Prekallikrein
PMSF	Phenylmethylsulphonyl fluoride
SDS	Sodium dodecyl sulfate
SBE	Smad binding element
pro-SP-C	Surfactant protein C precursor

TBS	Tris buffered saline buffer
TBS-T	Tris buffered saline buffer + 0.1% Tween 20
TEMED	*N*,*N*,*N*′,*N*′-tetramethyl-ethane-1,2-diamine
TF	Tissue factor
TGF-β	Transforming growth factor-β
TβRI	TGF-β receptor type I
TβRII	TGF-β receptor type II
TNF-α	Tumor necrosis factor-α
t-PA	Tissue-type plasminogen activator
u-PA	Urokinase-type plasminogen activator
uPAR	u-PA receptor

V. Summary

Idiopathic pulmonary fibrosis (IPF) is a rare, chronic, progressive interstitial lung disease characterized by abnormal and excessive deposition of fibrotic tissue in the pulmonary interstitium. Elevated procoagulant and decreased fibrinolytic activities have been observed in bronchoalveolar lavage (BAL) fluid from IPF patients. Alterations of alveolar haemostatic balance, mainly due to increased expression of tissue factor (TF), factor VII (FVII) and plasminogen activator inhibitor 1 (PAI-1), and decreased synthesis of urokinase (u-PA) promote fibrin deposition in the alveolar compartment. Moreover, cellular activities of coagulation factors also potentiate fibrotic responses in the lungs through stimulation of fibroblast proliferation and differentiation, production of profibrotic cytokines and increased deposition of extracellular matrix components.

Coagulation factor XII (FXII) is a key component of the intrinsic blood coagulation pathway involved in coagulation, fibrinolysis and inflammation. Active FXII (FXIIa) converts factor XI (FXI) into activated FXI (FXIa) and prekallikrein (PK) into kallikrein (KLK). Consequently, FXI activation culminates in a series of proteolytic reactions resulting in thrombin generation and the release of the proinflammatory and vasodilatory bradykinin (BK).

The implication of the extrinsic coagulation pathway in the pathogenesis of pulmonary fibrosis has been well described, however the potential role of intrinsic coagulation factors, namely FXII, FXI and high molecular weight kininogen (HMWK), has never been reported in the pathomechanisms of chronic fibroproliferative lung diseases. The present study was undertaken to evaluate the contribution of the intrinsic coagulation pathway in the pathogenesis of IPF.

Increased expression of FXII, FXI and HMWK and elevated activity of FXIIa were detected in the lungs of bleomycin-treated mice as well as of IPF patients. The strongest immunoreactivity of FXII was observed in fibroblasts and on the surface of alveolar epithelial type II cells (ATII). *In vitro* experiments identified FXIIa as a potent mitogen for primary murine lung fibroblasts. FXIIa mitogenic activity was mediated by the α5β1-integrin and the u-PA receptor (uPAR), since a blockade of these molecules abolished FXIIa-induced cell proliferation. Moreover, FXII-dependent induction of lung fibroblast proliferation was attenuated by the pharmacological blockade of the extracellular signal-

regulated kinase (ERK) 1/2 pathway. In line with *in vitro* data, FXII knockout mice were found to be protected against bleomycin-induced fibrosis and intratracheal application of FXIIa inhibitor strongly reduced a fibrotic response after bleomycin administration. The lack in reduction of fibrotic responses in bradykinin receptor 1/2 deficient mice indicated that BK did not mediate FXII profibrotic properties.

Although regulation of FXII expression by estrogen in hepatocytes is well described, no data are available about regulation of FXII synthesis in cells other than hepatocytes. Interestingly, human lung fibroblasts (HLF) were found to express FXII in a regulated manner. Treatment of HLF with Transforming growth factor-β1 (TGF-β1) induced FXII production in a time-dependent manner. The intracellular mechanism by which TGF-β1 stimulates FXII expression was investigated and the respective FXII promoter region necessary for TGF-β1 mediated FXII production was characterized.

In conclusion, these findings identified FXII/FXIIa, apart from its possible role as coagulation factor in the alveolar compartment, as a novel profibrotic factor that may contribute to the development of lung fibrosis by potentiating proliferation of lung fibroblasts. Therefore, FXII and its downstream signaling pathway in lung fibroblasts should be considered as a novel target for therapeutic intervention in pulmonary fibrosis.

VI. Zusammenfassung

Die idiopathische Lungenfibrose (IPF) ist eine seltene, chronisch progressiv verlaufende, interstitielle Lungenerkrankung, die durch übermäßige Deposition von fibrotischem Gewebe im Interstitium charakterisiert ist. In der bronchoalveolären Lavageflüssigkeit von IPF Patienten finden sich erhöhte prokoagulatorische und erniedrigte fibrinolytische Aktivitäten. Veränderungen der alveolaren Homöostase, hauptsächlich verursacht durch vermehrte Expression von Gewebsfaktor (Tissue factor, TF), Faktor VII und Plasminogen Aktivator Inhibitor 1 (PAI-1), sowie verminderte Synthese von Urokinase (u-PA), verstärken die Fibrinablagerungen in den Alveolen. Zusätzlich steigern die Gerinnungsfaktoren die fibrotische Reaktion der Lunge durch Stimulation der Dedifferenzierung und Proliferation von Fibroblasten, Produktion von pro-fibrotischen Zytokinen und extrazellulären Matrixkomponenten.

Gerinnungsfaktor XII (FXII) spielt eine Schlüsselrolle in der intrinsischen Gerinnungskaskade während der Koagulation, Fibrinolyse und Inflammation. FXIIa aktiviert Faktor XI (FXI) zu Faktor XIa (FXIa) und Prekallikrein (PK) zu Kallikrein (KLK). In der Folge kommt es zu einer Serie von proteolytischen Reaktionen, die zur Synthese von Thrombin und zur Freisetzung des proinflammatorisch und vasodilatorisch wirkenden Bradykinins (BK) führen.

Die Bedeutung des extrinsischen Gerinnungssystems für die Pathogenese der Lungenfibrose ist relativ gut erforscht. Eine potentielle Rolle für intrinsische Gerinnungsfaktoren, wie FXII, FXI und hochmolekulares Kininogen (HMWK), wurde dagegen noch nicht im Hinblick auf den Pathomechanismus chronisch fibroproliferativer Lungenerkrankungen untersucht. Ziel dieser Arbeit war daher die Evaluation der Bedeutung des intrinsischen Gerinnungssystems für die Pathogenese der IPF.

Sowohl in den Lungen Bleomycin-behandelter Mäuse als auch von IPF Patienten war die Expression von FXII, FXI und HMWK, sowie die Aktivität von FXII erhöht. Die stärkste Reaktivität von FXII fand sich in Fibroblasten und an der Oberfläche von Alveolarepithelzellen vom Typ II (ATII). *In vitro* Experimente identifizierten FXIIa als potentes Mitogen für primäre murine Lungenfibroblasten. Da die Inhibierung von α5β1-Integrin und dem Urokinaserezeptor (uPAR) ausreichte, um die FXII-vermittelte Proliferation von Fibroblasten aufzuheben, konnten diese Moleküle als übergeordnete

Mediatoren identifiziert werden. Daneben konnte auch eine pharmakologische Inhibierung des extracellular signal-regulated kinase (ERK) 1/2 Signalweges die FXII-abhängige Proliferation pulmonaler Fibroblasten hemmen.

In Übereinstimmung mit den *in vitro* Daten, waren FXII Knockout-Mäuse geschützt gegen eine Bleomycin-induzierte Fibrose. Daneben konnte auch eine intratracheale Applikation von FXIIa Inhibitor in Bleomycin-behandelten Mäusen die fibrotische Reaktion signifikant hemmen. Da bei Bradykininrezeptor 1/2-defizienten Mäusen keine Verringerung der fibrotischen Reaktion auftrat, scheint FXII auf diesem Weg keine Wirkung zu entfalten.

Während in Hepatozyten die Regulation der FXII Expression durch Östrogen beschrieben ist, gibt es zurzeit keine Daten für andere Zelltypen. Interessanterweise exprimieren humane Lungenfibroblasten (HLF) FXII, weshalb hier die Regulation der FXII Expression genauer untersucht wurde. Die Behandlung von HLF mit TGF-β1 führte dabei zu einem zeitabhängigen Anstieg der Expression von FXII. Durch weitere Untersuchungen zum intrazellulären Mechanismus konnten die spezifischen Stellen in der Promoterregion charakterisiert werden.

Zusammengefasst identifizieren diese Ergebnisse FXIIa als neuen pro-fibrotischen Faktor, der, unabhängig von seiner möglichen Gerinnungsaktivität im Alveolarraum, über eine verstärkte Proliferation von Lungenfibroblasten entscheidend zur Entstehung der Lungenfibrose beiträgt. Daher bieten sich FXII und seine nachgeschalteten Signalwege als neue Angriffspunkte in der Therapie der Lungenfibrose an.

1. Introduction

1.1. Blood Coagulation Pathways

Blood coagulation is a defense mechanism which prevents blood loss from damaged vessels. One can distinguish two pathways of blood coagulation: intrinsic and extrinsic. Extrinsic pathway activation takes place when TF is exposed to plasma during vessel injury. TF is a non-enzymatic lipoprotein expressed on the surface of cells that normally have no contact with plasma. The interaction of TF with FVIIa, which circulates in plasma, triggers the activation of Factor X (FX). This leads to conversion of prothrombin to thrombin, followed by fibrin formation.

The intrinsic coagulation pathway can be triggered *in vitro* when blood has contact with negatively charged surfaces like kaolin (1), sulfatides (2), dextran sulfates, phospholipids, urate crystals and glass (3). It is believed that the activation of this pathway occurs when FXII binds to artificial surfaces and autoactivates. FXII can be also activated by KLK (4,5). Activation of FXII is enhanced by HMWK, which serves as a docking protein (6). Activated FXII triggers the activation of FXI, followed by FXa generation and thrombin formation (Figure 1.1).

1.1.1. Structural and functional characteristics of the intrinsic coagulation pathway factors

1.1.1.1. Factor XII

FXII is produced in the liver as a single chain 78 kD zymogen. Activated FXII consists of a heavy and a light chain (7). The heavy chain contains the following domains: leader peptide, fibronectin type II domain, the epidermal growth factor (EGF) domain, fibronectin type I domain, the kringle domain, and the proline reach domain, which is unique for FXII (Figure 1.2) (8). FXII has a similar domain organization as the serine protease family members tissue-type plasminogen activator (t-PA) and u-PA, but is different from other coagulation factors (8). The heavy chain contains artificial surface binding regions localized at the amino terminus, fibronectin type I region (9), and

possibly localized at the second EGF-like domain or kringle domain (10). The light chain contains the catalytic domain typical for serine proteases. The active site of FXII consists of canonical His40, Asp89 and Ser191 residues (8,11). This site is also a target for the major intrinsic coagulation pathway inhibitor, C1 inhibitor (12). Cleavage of FXII by KLK or its autoactivation results in the splitting of the Arg353-Val354 bound in the FXII zymogen, leading to the generation of the active alphaXIIa form, which contains a heavy and a light chain bound by a disulfide bond (11). Hydrolysis of two more peptide bonds in alphaXIIa form generates 30 kDa betaXIIa, which consists of a light chain and a small fragment of a heavy chain. AlphaXIIa is able to bind to negatively charged surfaces and activates FXI and PK. BetaXIIa has no surface-binding ability but can activate PK (13).

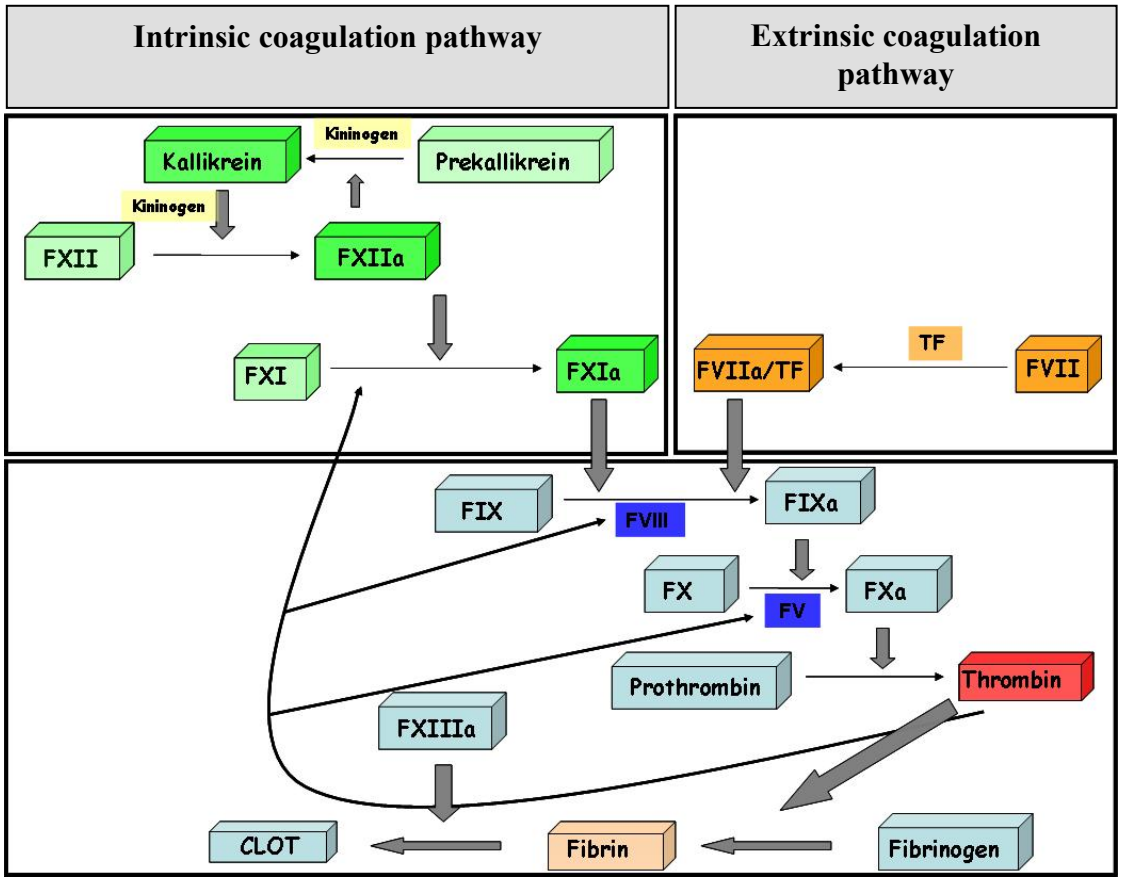

Figure 1.1. Blood coagulation pathways.

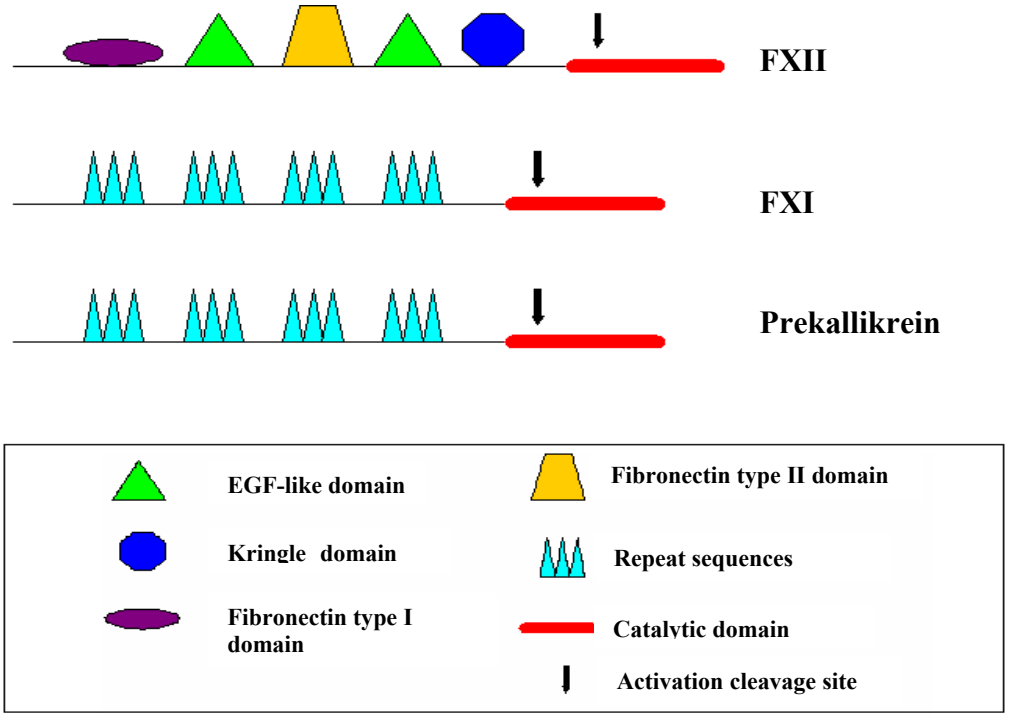

Figure 1.2. Structure of intrinsic coagulation pathway protease zymogens.

1.1.1.2. Factor XI

FXI is synthesized in the liver as a glycoprotein with a molecular weight of 143 kDa (14). This serine protease consists of two identical peptide chains bound by disulfide bonds and therefore contains two active sites. Each of the two peptide chains consists of a heavy and a light chain, which are connected to each other by disulfide bonds. Each light chain contains an active site, which consists of His44, Asp93, and Ser188 . FXI domains are homologous to domains of PK (Figure 1.2) (14). FXI is activated by FXII in the presence of HMWK after cleavage of the disulfide bond in each polypeptide chain (15,16). Activated FXI cleaves further FIX. Inherited FXI deficiency, also called Hemophilia C, is an uncommon autosomal recessive disorder, which is associated with a variable bleeding tendency that usually occurs after trauma or surgery (17). There are reports showing that FXI may be activated independently of FXII, by thrombin (18). In view of opposing results (19) it is still questionable, whether thrombin activates FXI *in vivo*.

1.1.1.3. High molecular weight kininogen

There are two forms of plasma kininogens, HMWK and low molecular weight kininogen (LMWK). Both molecules are the products of the same gene (20,21). HMWK is mainly synthesized in the liver as a 120 kDa glycoprotein (20). Moreover, HMWK was found in human umbilical vein endothelial cells (22), primary fetal murine fibroblasts, human fibroblast cell line WI-38 (23,24), platelets (23), granulocytes (24), renal tubular cells (25,26), and skin (27).

The single polypeptide chain of HMWK consists of a heavy chain (domains 1-4), a BK region (domain 4) and a light chain (domain 5-6) (28). Each domain of HMWK possesses physiological activities. Domain 1, with unclear function, contains Ca^{2+}-binding site (29). Domains 2 and 3 contain highly conserved pentapeptide QVVAG, responsible for cysteine protease inhibitory activities of HMWK (30,31). Domains 3 and 5 bind in a Zn^{2+} dependent manner to thrombocytes (32,33), neutrophils (34,35) and endothelial cells (38,39). HMWK bound to thrombocytes inhibits thrombocytic calpain, attenuating binding of thrombin and subsequent aggregation of thrombocytes. HMWK acts as an anti-adhesive molecule after binding to multinuclear leucocytes (MNL) (36). HMWK is also required for the KLK stimulated activation of MNL (37). Domain 4 contains BK sequence. BK delivery is the major activity of HMWK. The cleavage of HMWK by plasma KLK leads to generation of BK and kinin free protein (HMWKa) (20,21). HMWKa consists of a 62 kDa heavy and a 56 kDa light chain. The functions of BK will be described later.

The light chain of HMWK is involved in binding to hydrophilic and anionic surfaces and contains regions binding PK and FXI (38). Thus HMWK has the ability to mediate interactions between FXII, FXI and PK and the vascular wall (39). Therefore, HMWK as a nonenzymatic cofactor is involved in the forming a complex and is involved in the activation of intrinsic coagulation pathway protease zymogens. HMWKa has been reported to induce apoptosis of proliferating endothelial cells and to inhibit angiogenesis (40,41). Domain 5 inhibits endothelial cell migration towards vitronectin (42). There are reports suggesting that HMWKa inhibits endothelial cells adhesion on vitronectin (42-44).

1.1.1.4. Kallikrein

PK is a glycoprotein produced as a zymogen in hepatocytes (45). The amino acid sequence has a high homology to FXI (46). PK circulates in plasma in a complex with HMWK (47) and has a molecular weight of 85 kDa or 88 kDa (45). Two forms of FXII (alphaXIIa and betaXIIa) have the ability to activate PK (48). Cleavage of PK generates KLK (Figure 1.3) which consists of a heavy and a light chain bound by a disulfide bond. The light chain contains an active site, which consists of His, Asp and Ser residues (49). The heavy chain is responsible for the binding of HMWK and FXII (50). On the endothelial cell surface, activation of KLK occurs in the absence of FXII (6,51). C1 inhibitor has been recognized to be a major inhibitor of KLK (52-54). α2-Macroglobulin is also able to bind and block KLK activity, although to a much lesser extent (55). KLK has many bioregulatory functions. KLK activates u-PA potentiating fibrinolysis on the surface of endothelial cells. Moreover, KLK is able to activate prorenin (56,57) and FXII (4). Purified KLK has been shown to aggregate human blood neutrophils (58,59).

The major role of KLK is cleavage of HMWK and delivery of BK, which has various physiological activities (Figure 1.3). BK is a peptide with vasodilatory and proinflammatory properties. BK stimulates synthesis of prostacyclin by endothelial cells, thereby inhibiting platelets and inducing vasodilation through elevation of intracellular cAMP level. It induces nitric oxide (NO) synthesis in endothelial cells, which acts as a vasodilator, inhibitor of platelet function and smooth muscle cell proliferation. BK is the strongest stimulator of t-PA release. Thus, BK generation plays a crucial role in the regulation of arterial pressure, modulation of vascular function and is responsible for classical signs of inflammation: redness, local heat, swelling, and pain. BK acts through two G-protein-coupled receptors: B1 and B2 (60). The B2 receptor is broadly and constitutively expressed, whereas the B1 receptor is weakly expressed under basal conditions but becomes upregulated during inflammation. Both receptors play important roles during inflammation and injury (61).

BK can also be generated in FXII independent manner by tissue kallikreins (62,63). Nevertheless, FXII is important for the proper rate of KLK generation and the release of BK and HKa, since in FXII deficient mice the plasma level of BK is supressed (64).

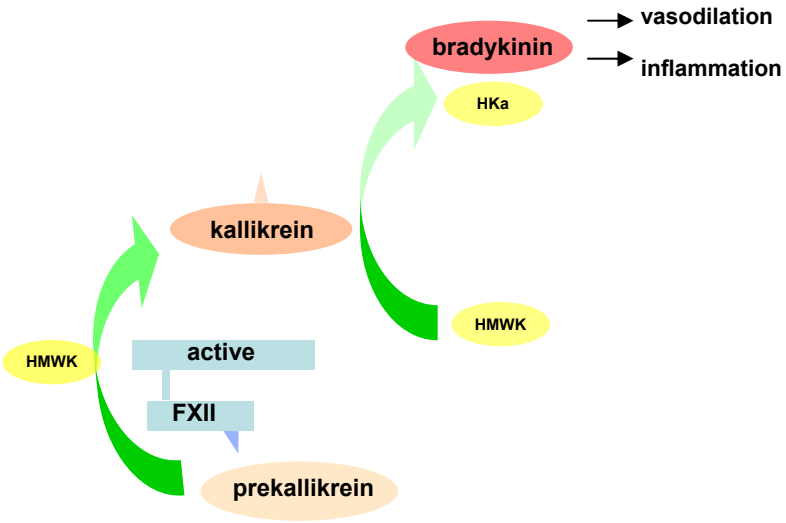

Figure 1.3. The KLK/kinin system.

1.1.2. Activation of FXII

1.1.2.1. Contact activation of FXII

FXII may be activated either by autoactivation on a negatively charged surface or by KLK. Many negatively charged physiological surfaces upon which FXII autoactivates have been described: articular cartilage, skin, fatty acids (65), endotoxin (66), amyloid protein (67), RNA (68), dextran sulfate, acidic phospholipids (69), sulfatides, glycocerebroside sulfates (2,70) and collagen (71).

Activation of platelets has been reported to promote fibrin generation in a FXII-dependent manner (72,73). A recent study has shown that polyphosphates (PolyP), secreted upon platelet activation, bound to and activated FXII *in vivo* (74). PolyP-activated FXII triggered not only plasma clotting, but also generation of proinflammatory BK. There are studies suggesting that acidic phospholipids and sulfatides expressed in platelets may activate FXII *in vivo* (69,75). It is worth mentioning that binding to and autoactivation of FXII on sulfatides has been reported to be Zn^{2+} independent, whereas autoactivation on other negatively charged substances has been demonstrated to be Zn^{2+} dependent (76-78). The activation of FXII *in vivo* may also be promoted by nucleic acids derived from damaged cells (68). Furthermore, it has been reported that misfolded protein

aggregates produced during systemic amyloidosis trigger FXII activation which results in KLK/kinin system activation without induction of the coagulation cascade (67).

1.1.2.2. Activation of the contact system on the endothelial cell surface

There are four components of the contact system: FXII, FXI, PK and HMWK. *In vitro*, FXII binds to negatively charged surfaces and undergoes autoactivation. Activated FXII cleaves PK into KLK (79). It has been shown that HMWK, FXII and PK can bind to cultured endothelial cells, platelets and granulocytes in a Zn^{2+} dependent manner. The specific binding sites for HMWK and FXII have been identified (6). Putative receptors of HMWK and FXII on endothelial cells are first complement component (C1q) receptor (gC1qR), uPAR and cytokeratin 1 (CK1). FXII and HMWK compete for the same binding sites on the endothelial cell surface (80-83). HMWK mediates binding of PK and FXI to the cell surface. PK binding sites other than HMWK have not yet been identified (6). FXI in contrast to PK has the ability to directly bind to platelets (84).

FXII binding to endothelial cells requires a much higher concentration of Zn^{2+} than a binding of HMWK/PK complex. Moreover, FXII is not activated on the endothelial cell surface in the absence of PK in plasma (6,51). These data suggest that FXII activation on endothelial cells follows PK assembly and activation. Assembly of contact phase proteins on the endothelial cell membrane leads to the formation of a multiprotein complex, that leads to PK activation by endothelial cell cysteine protease independent of FXII (83,85). PK activator on the endothelial cell surface has been identified to be a polycarboxypeptidase (PCRP) (85). Active KLK can further activate FXII into FXIIa.

1.1.2.3. Inhibition of FXII activity

1.1.2.3.1. Inhibition of FXII activity *in vitro*

Since FXII autoactivation occurs on negatively charged surfaces such as polyphosphates (74) and nucleic acids (68), phosphatases, RNases and DNases respectively, may be considered as possible FXII activation blockers. A specific Zn^{2+}

chelator could be another possible tool to control phospholipid-mediated FXII activation. Several native bioactive substances have been identified as FXIIa inhibitors.

Corn trypsin inhibitor (CTI) derived from corn seeds specifically inhibits FXIIa. It appears to inhibit only FXIIa among enzymes of the blood coagulation system (86,87). It is a 12 kDa polypeptide with no sequence similarity to other serine protease inhibitors (88). The exact mechanism of FXIIa inhibition by CTI is not clear. CTI was shown to bind to the precursor and activated forms of FXII (87,89). Most likely CTI binds reversibly to FXIIa and inhibits its activity by changing the conformation of FXIIa (87). CTI was also reported to block mitogenic activities of FXIIa (90,91).

Another FXIIa inhibitor isolated from pumpkin seeds (Pumpkin seed Hageman factor inhibitor) is a 3 kDa polypeptide. It also weakly inhibits human plasmin and bovine FXa (92). H-D-Pro-Phe-Arg-chloromethylketone (PCK) is the active-site directed inhibitor of activated FXII (93). It inhibits the amydolytic activity of FXIIa and plasma KLK-mediated activation of FXII (94).

1.1.2.3.2. Inhibition of FXII activity *in vivo*

C1 inhibitor is regarded as the most potent physiological FXII inhibitor, accounting for greater than 90% of the inhibition of this protease in plasma. C1 inhibitor binds irreversibly to alphaXIIa and betaXIIa (12,95). When associated with the kaolin surface, FXIIa is protected against inhibition by C1 inhibitor (96). FXII activity can also be attenuated, although to a lesser extent, by antithrombin (97), PAI-1 (98) and alpha 2-macroglobulin (99).

1.1.3. Physiologic activities of FXII

1.1.3.1. FXII and inflammatory reactions

1.1.3.1.1. FXII in hereditary angioedema

FXII is involved in inflammatory reactions since it enhances the generation of BK from HMWK by activating PK (100). Consequently, active KLK enhances the rate of FXIIa formation by reciprocal activation (4). Hereditary angioedema (HAE) is characterized clinically by episodes of angioedema with increased vascular permeability, particularly by recurrent acute skin swelling, abdominal pain, and potentially life-threatening laryngeal edema (101). Three forms of HAE have been described. The classic forms, HAE types I and II, are the consequence of mutations in the C1 inhibitor gene (102). The C1 inhibitor regulates the activity of both KLK (103) and FXII (99) by irreversible binding to these enzymes. Increased activity of FXII and KLK, due to the lack of inhibition by C1 inhibitor, leads to increased generation of BK, which results in increased vascular permeability and angioedema (102).

In contrast to HAE types I and II, HAE type III, characterized by increased activity of FXII (104), has been observed exclusively in women, where it appears to correlate with high estrogen levels, for example, pregnancy or the use of oral contraceptives. These conditions are characterized by elevated FXIIa plasma level, probably due to estrogen-induced FXII synthesis in hepatocytes.

1.1.3.1.2. FXII in sepsis

FXII and PK levels are low in sepsis, which may be a result of contact system activation on the bacterial surface followed by increased consumption of activated proteases (105). This can further lead to a release of BK, which promotes inflammation and a hypocoagulatory state (106). This mechanism may contribute to symptoms of sepsis like plasma leakage, hypotension and formation of microthrombi. FXIIa, through activation of the contact system, was shown to contribute to disease progression of severe

Salmonella lung infection in the mouse model. Accordingly, PCK, an inhibitor of FXIIa, was shown to prevent pulmonary lesions in mice infected with *Salmonella* (107).

In vitro, FXIIa activates C1q (93), downregulates a monocyte Fc receptor (108) and releases IL-1 and IL-6 from monocytes and macrophages (109). Purified plasma FXIIa is able to aggregate human neutrophils and causes their degranulation (110). FXII has been shown to activate chimerin, which is a ubiquitous plasma chemoatractant (111). Activated chimerin directs plasmatoid dendritic cells and macrophages to sites of infection and inflammation (112). Thus, FXII absorption and activation on the bacterial surface may also contribute to the host's defense system by regulating leucocyte function.

1.1.3.2. Factor XII in thrombosis

Deficiency of FXII, PK and HMWK is associated with no bleeding disorder. Therefore, the role of FXII in physiological haemostasis is not clear. All of these deficiencies are exceedingly rare. FXII deficiency is the most common one. Clinical investigations related to venous thrombosis risk or polymorphism of FXII and their influence on cardiovascular disease resulted in conflicting data. On the one hand, it has been postulated that there is an increased incidence of venous and arterial thrombosis in patients with FXII deficiency (113-115). Epidemiological studies showed that patients with a decreased FXII level have a greater risk of developing thrombotic disorders such as myocardial infarction and re-thrombosis of coronary arteries after thrombolytic therapy (116-118). On the other hand, the occasional venous thrombosis seen in patients with FXII deficiency seems to be due to other associated prothrombotic risk factors (119) and hypercholesterolemia (120). Finally, the most recent data indicates no correlation between FXII deficiency and an increased risk of thrombosis (121-124).

To elucidate FXII functions *in vivo* FXII knockout ($FXII^{-/-}$) mice were generated (125). $FXII^{-/-}$ mice are healthy and fertile and do not suffer from pathologic bleeding despite having prolonged activated partial thromboplastin time (125). FXII deficient mice have reduced arterial thrombosis risk in a mouse model of lethal pulmonary embolism (126). Moreover, FXII deficiency or inhibition of its activity protected the mice from ischemic brain injury in the transient middle cerebral artery occlusion model. The volume of infarcted brain in FXII deficient and FXII inhibitor-treated mice has been shown to be

reduced in comparison to wild-type animals. Targeting FXII reduced fibrin formation in ischemic vessels, and reconstitution of FXII deficient mice with human FXII restored fibrin deposition (127). Studies using intravital fluorescence microscopy revealed a severe defect in formation and stabilization of platelet rich occlusive thrombi induced by different means of injuries in $FXII^{-/-}$ mice (126,128).

In a mouse model of arterial thrombosis, pretreatment with RNase prior to ferric chloride administration to the adventitial vessel surface, delayed occlusive thrombus formation and was associated with the reduced thrombus material (68). These results indicated that RNase can exert a strong antithrombotic effect *in vivo* and should be considered as a new therapeutical agent.

1.1.3.3. Factor XII in fibrinolysis

KLK and FXIIa cleave plasminogen directly, but much slower than t-PA or u-PA (129,130). FXII participates in fibrinolysis by enhancing the activation of PK on the endothelial cells surface. Activation of PK initiates two pathways of fibrinolysis. Firstly, KLK cleaves HMWK, liberating BK which is a potent stimulator of t-PA release from endothelial cells (131,132). Secondly, KLK converts prourokinase into two-chain u-PA (133). Increased incidence of venous thrombosis (134-136), myocardial infarction (137-140) and re-thrombosis of coronary arteries after thrombolytic therapy in FXII deficient patients has been reported by some investigators (141,142). Further studies are required to determine whether FXII deficiency is associated with decreased fibrinolysis.

1.1.3.4. Mitogenic activities of FXII

FXII has been reported to be a potent mitogen for human hepatoma (Hep2) cells (91), smooth muscle cells, ATII cells and endothelial cells. FXII is able to stimulate cell proliferation in concentration ten times lower than its plasma concentration. CTI, a specific FXII protease inhibitor, has been shown to block FXII mitogenic activities. FXII-induced mitogenesis may be mediated via activation of the mitogen-activated protein kinase (MAPK)/ERK pathway (90), since exposure of A10 cells (primary rat aortic smooth muscle cells) to FXII resulted in ERK1/2 phosphorylation.

1.1.4. Characterization of FXII promoter

Thus far there is not much data on the regulation of FXII expression in the liver and other organs. FXII production in the liver is known to be regulated by estrogens. Previous reports demonstrated the induction of FXII gene transcription by 17β-estradiol in transiently transfected mouse NIH3T3 fibroblasts and human HepG2 cells. Furthermore, it was shown that a 230-bp fragment of FXII promoter, spanning nucleotides -181/49, conferred estrogen mediated transcription changes, suggesting that a functional estrogen responsive element (ERE) resides in this region. The lower response to estrogen was observed when longer fragments of FXII promoter were used, indicating the presence of a silencer located further upstream. The presence of ERE in the FXII promoter could explain estrogen-induced increase of FXII concentration in plasma (143-145). Four putative binding sites for the hepatocyte nuclear factor-3 (HNF-3) were recognized in the 5'-flanking region of the FXII gene (146,147).

Another study identified IL-6, a regulator of acute phase response, as a downregulator of FXII expression in HepG2 cells. Neither IL1-β nor TNFα changed FXII expression in HepG2 cells (148).

It has been reported that FXII mRNA can be detected not only in the liver, but also in the lung, brain, placenta (149) and porcine endometrium (150), but the role of FXII in these organs and the regulators of its synthesis have never been studied.

1.2. Idiopathic pulmonary fibrosis

IPF is defined as a distinct type of chronic fibrosing interstitial pneumonia of unknown cause limited to the lungs and associated with a histological pattern of usual interstitial pneumonia. IPF lungs are characterized by architectural destruction, dense scarring with honeycombing and scattered fibroblasts foci (areas of intensive fibroblast proliferation) (151). IPF has a progressive and usually fatal course with an average survival rate of 2–3 years following diagnosis (152,153). Patients with IPF are usually between 50 to 70 years old and the incidence is 7.4 cases per 100 000 for women and

10.7 cases per 100 000 for men per year. The incidence, prevalence and death increases with age (154).

To date, most treatment strategies have been based on eliminating or suppressing the inflammatory component. No pharmacological therapy has been proven to be effective in IPF treatment. All currently available therapeutic trials in IPF are severely limited by the lack of clear understanding of the disease etiology (151). The original hypothesis of the pathogenesis of IPF is that chronic inflammation in response to unknown etiological agents (idiopathic) leads to tissue destruction, initiation and propagation of wound healing responses and, subsequently, to progressive fibrosis. A recent proposal indicates that inflammation is necessary to trigger the initiation of the fibrotic process, but plays a minor role in the progression of the disease. In contrast to other forms of chronic interstitial lung diseases such as sarcoidosis and hypersensitivity pneumonitis, IPF is characterized by only limited inflammation. Recently, it has been suggested that IPF is mainly a disorder of alveolar epithelial injury, abnormal alveolar wound repair and remodelling (155,156).

1.2.1. Role of Transforming Growth Factor-β in the pathogenesis of lung fibrosis

TGF-β1 is a highly pleiotropic cytokine which plays a fundamental role in wound healing, embryonic development and disease states associated with inflammation and proliferation, for example tissue fibrosis. In the adult mice, TGF-β overexpression in the lungs leads to progressive pulmonary fibrosis (157). TGF-β is thought to promote fibrotic responses in the lungs mainly due to supression of alveolar epithelial cell proliferation, stimulation of fibroblast proliferation, activation of resident lung cells including epithelial cells, which differentiate into collagen-producing myofibroblasts. TGF-β1 enhances synthesis and inhibits degradation of extracellular matrix components (158,159). Moreover, recent studies suggest that TGF-β1 may contribute to fibrotic conditions by modulating procoagulant and fibrinolytic activities. In particular, TGF-β1 has been shown to upregulate the expression of TF (160), the key initiator of the extrinsic

coagulation pathway, and of PAI-1 in different cell populations including fibroblasts (161,162).

The cellular response to TGF-β1 involves ligand binding to TGF-β receptor type II (TβII) which phosphorylates TGF-β receptor type I (TβRI). Activated TβRI phosphorylates receptor associated Smads (Smad 1, 2, 3, 5, and 8), promoting their heterodimerization with common-mediator Smad (Smad 4) and their translocation from the cytoplasm to the nucleus. Within the nucleus, the Smad heterocomplex interacts with canonical smad-binding elements (SBEs) of target genes to activate their transcription (163,164). Human Smad 3 and Smad 4 have been shown to bind to SBE comprising CAGA box (161).

1.2.2. Bleomycin model of lung fibrosis

Different models of pulmonary fibrosis have been developed over the years, including radiation damage, instillation of bleomycin, silica or asbestos, gene transfer employing fibrogenic cytokines and transgenic mice (165). None of them mimic all features of human IPF. However, the standard agent for induction of experimental pulmonary fibrosis in animals is bleomycin. Bleomycin is a chemotherapeutic antibiotic, produced by the bacterium *Streptomyces verticillus* (166). It has been used as a drug in human cancer treatment.

Bleomycin induces the production of superoxide and hydroxide free radicals. Free radicals cause single and double-strand DNA breaks in tumor cells and thereby interrupt the cell cycle (167). Its use in animal models of pulmonary fibrosis is based on bleomycin pulmonary side-effects. Bleomycin causes lung fibrosis in 10% of patients treated with this drug. An overproduction of reactive oxygen species leads to epithelial cell injury, inflammatory response, activation of fibroblasts and as a consequence to fibrosis (168). The lungs poorly express bleomycin hydrolase, a bleomycin-inactivating enzyme, and therefore are more susceptible to bleomycin-induced tissue injury (169). Bleomycin as an inducer of experimental lung fibrosis was first described in dogs (170), later in mice (171). After intratracheal instillation bleomycin causes inflammatory and fibrotic

reactions within a short period of time. The switch between initial elevation of pro-inflammatory cytokines and fibrosis appears to occur around day 9 post application (168).

Despite mimicking typical histological hallmarks of the human disease, the bleomycin model also has many limitations. Firstly, bleomycin triggers an acute lung injury and inflammatory response, while inflammation in IPF is limited. Secondly, bleomycin-induced fibrosis is at least partially reversible (172), in contrast to irreversible progression of IPF (173).

1.2.3. Role of coagulation in the pathogenesis of idiopathic pulmonary fibrosis

Alterations of the alveolar haemostatic balance and excessive deposition of intraalveolar fibrin have been observed in the lungs of IPF patients. Intraalveolar fibrin accumulation, observed under these conditions, arises from the imbalance between locally produced pro- and anti-coagulant factors, in combination with the leakage of plasma proteins (including fibrinogen) into the alveolar space. Increased procoagulant activity in BAL fluids of patients with IPF is accompanied by a decreased fibrinolytic activity (174,175). Identical alterations of the haemostatic balance in the alveolar space have been observed in the bleomycin animal model of pulmonary fibrosis (176). In clinical and experimental lung fibrosis the procoagulant response is mainly attributable to TF associated with FVIIa, whereas the decreased fibrinolytic activity is ascribed to the inhibition of u-PA and t-PA by PAI-1 as well as the blockage of plasmin by α2-plasmin inhibitor (174-176). Although fibrin is required for reparative processes and normal wound healing, persistent and excessive deposition of extravascular fibrin is thought to contribute to the pathomechanisms of fibrotic lung diseases in several ways. Fibrin may serve as a reservoir of profibrotic growth factors (177). It incorporates and inactivates pulmonary surfactant, the lung lipoprotein complex critical for maintaining low alveolar surface tension (178,179). Surfactant dysfunction leads to atelectasis and loss of lung compliance. Moreover, inactivation of the surfactant system, in conjunction with "glueing" of the adjacent alveolar walls by fibrin, is thought to provide a provisional matrix on which fibroblasts proliferate and produce collagen (180).

In addition, the u-PA/PAI-1 system may contribute to the development of lung fibrosis by regulating of cell migration, cell adhesion and cell proliferation (181,182). Furthermore, various coagulation proteases such as thrombin, FXa and the TF-FVIIa complex exhibit cellular activities that may also contribute to fibrotic processes in the lung. Most of these functions are mediated via proteolytic activation of protease activated receptors (PARs). For instance, thrombin and FXa stimulate fibroblast proliferation and procollagen production in a PAR-1-dependent manner (183,184). Additionally, thrombin induces differentiation of normal lung fibroblasts to myofibroblasts via PAR-1 activation (185). Furthermore, activation of PAR-1 by thrombin, FXa and by the TF-FVIIa complex can increase the expression of profibrotic and proinflammatory cytokines (186,187). A potential role of PAR-1 in pulmonary fibrosis is underscored by the recent finding which demonstrated that PAR-1 deficient mice are protected against bleomycin-induced lung fibrosis (188). Additional evidence underlying the importance of cellular effects mediated by haemostatic factors in the development of lung fibrosis came from the recent observation which indicated no protection against bleomycin-induced lung fibrosis in fibrinogen-null mice (189). Despite a well described role of the extrinsic coagulation pathway in the pathogenesis of IPF, the impact of intrinsic coagulation factors on the development of this disease is not known.

2. Aim of the study

It is well known that dysregulation of alveolar haemostatic balance contributes to the development of chronic fibrotic interstitial lung diseases, such as IPF. Activation of the extrinsic coagulation pathway and inhibition of fibrinolysis are recognized as major factors responsible for the procoagulant state observed in IPF. The involvement of the extrinsic coagulation pathway in the pathogenesis of IPF is well known, however the role of the intrinsic coagulation pathway in this process has not been reported. The present study was undertaken to elucidate the contribution of FXII, the key contact phase protease, in development of lung fibrosis.

In this context, the research focus was:

1. to ascertain the expression pattern of FXII, FXI and HMWK in the lungs of IPF patients and bleomycin challenged mice
2. to investigate whether knockout or inhibition of FXIIa has a therapeutic effect on the development of bleomycin-induced lung fibrosis
3. to evaluate whether BK mediates FXII profibrotic activities
4. to elucidate the signaling pathways in the FXII-mediated proliferation of lung fibroblasts
5. to describe the molecular mechanism underlying FXII expression in lung fibroblasts.

3. Materials and methods

3.1. Materials

3.1.1. Equipment

Name	Company
Bacteria culture incubator	Heraeus, Germany
Cell culture incubator	Heraeus, Germany
Electrophoresis chambers	Biometra, Germany
Falcon tubes	Greiner Bio-One, Germany
Film cassette	Kodak, USA
Filter tips: 10; 100; 1000 µl	Eppendorf, Germany
Fluorescence and light microscope	Leica, Germany
Gel blotting paper	Amersham Biosciences, UK
Multifuge centrifuge	Heraeus, Germany
PCR-thermocycler	Biometria, Germany
Petri dishes	Greiner Bio-One, Germany
Pipetboy	Eppendorf, Germany
Pipets	Eppendorf, Germany
Power supply	Biometria, Germany
Radiographic film	Amersham Biosciences, UK
Sequence Detection System 7700	Applied Biosystems, USA
Tissue culture chamber slides	BD Falcon, USA
Tissue culture dishes	Greiner Bio-One, Germany
Transilluminator BioDocAnalyzer	Biometria, Germany
Ultra Microplate Reader EL 808	Bio-Tek Instruments, Germany
Water bath for cell culture	Medingen, Germany
Western blot chambers	Biometra, Germany
Vortex machine	VWR, Germany

3.1.2. Reagents

Product Name	Company
Ammonium persulfate	Sigma-Aldrich, Germany
Aprotinin	Sigma-Aldrich, Germany
1-butanol (n-butyl alcohol)	Merck, Germany
2-mercapto-ethanol	Sigma-Aldrich, Germany
2-propanol	Fluka, Germany
Acetic acid	Sigma-Aldrich, Germany
Acetone	Roth, Germany
Acrylamide solution, Rotiphorese gel 30	Roth, Germany
Agarose	Fluka, Germany
Albumine, bovine serum	Sigma-Aldrich, Germany
Ammonium acetate	Sigma-Aldrich, Germany
Ammonium formate	Sigma-Aldrich, Germany
Brillant blue G	Sigma-Aldrich, Germany
Calcium chloride	Sigma-Aldrich, Germany
DMEM	Gibco BRL, Germany
Dimethyl sulfoxide	Roth, Germany
Dithiothreitol (DTT)	Sigma-Aldrich, Germany
DNA ladder (100bp, 1kb)	Fermantas, Germany
Ethanol absolut	Roth, Germany
Ethidium bromide	Sigma-Aldrich, Germany
Ethylene glycol-bis(ß-amino-ethylether)-N,N,N',N'tetraacetic acid (EGTA)	Sigma-Aldrich, Germany
Ethylenediamine-tetraacetic acid (EDTA)	Sigma-Aldrich, Germany
ECL plus Western blotting detection system	Amersham Biosciences
Fetal calf serum (FCS)	HyClone, USA
Formaldehyde	Sigma-Aldrich, Germany
Glycerol	Roth, Germany
Glycine	Roth, Germany
Haemalaun	Roth, Germany

High fidelity DNA polymerase	Fermantas, Germany
2-(-4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES)	Roth, Germany
Hydrochloric acid	Roth, Germany
Leupeptin	Sigma-Aldrich, Germany
Magnesium chloride	Sigma-Aldrich, Germany
Methanol	Roth, Germany
Milk powder	Roth, Germany
MuLV reverse transcriptase	Applied Biosystems, USA
N,N'-methylene-bis-acrylamide	Sigma-Aldrich, Germany
Penicillin/streptomycin	Invitrogen, Germany
PCR nucleotide mix	Fermantas, Germany
Potassium chloride	Roth, Germany
Potassium phosphate monobasic	Sigma-Aldrich, Germany
Random hexamers	Applied Biosystems, USA
RNAse inhibitor	Applied Biosystems, USA
SDS	Sigma-Aldrich, Germany
Sodium chloride	Sigma-Aldrich, Germany
Sodium citrate tribasic dihydrate	Sigma-Aldrich, Germany
Sodium deoxycholate	Sigma-Aldrich, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Germany
Sodium hydroxide	Sigma-Aldrich, Germany
Sodium phosphate (monobasic, anhydrous)	Sigma-Aldrich, Germany
Sodium vanadate	Sigma-Aldrich, Germany
TEMED	Sigma-Aldrich, Germany
Tris	Roth, Germany
Triton X-100	Sigma-Aldrich, Germany
Trypsin/EDTA	PAA Laboratories, Austria
Tween 20	Sigma-Aldrich, Germany

3.2. Methods

3.2.1. Intratracheal bleomycin administration

Male mice (C57BL/6 strain) weighing between 20-22 g were used in all experiments. Mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were kept according to NIH guidelines and the experiments were undertaken with the permission of the local authorities. The animals were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride and xylazine hydrochloride. Bleomycin (Almirall Prodesfarma, Barcelona, Spain) in a dosis of 5U/kg body weight was given by aerosol. For bleomycin delivery, the animals were orotracheally intubated and mechanically ventilated. A microsprayer (Penn-Century Inc, Philadelphia, PA) was filled with 100 µl of saline solution containing bleomycin, introduced into the tracheal cannula, positioned slightly above the carina and aerosol generation was achieved under end-expiratory breath arrest by rapidly emptying the syringe. Control mice received vehicle only (0.9% saline). Five mg/kg body weight of CTI (Calbiochem, Darmstadt, Germany) and 8 mg/kg body weight of PCK (Bachem, Weil am Rhein, Germany) both diluted in 0.9% saline, were administered intratracheally on day 9, 12, 15 and 18. The mice were sacrificed after 21 days post application (if not otherwise indicated) with a lethal dose of ketamine and xylazine. FXII deficient mice were kindly provided by Dr. T. Renne (Karolinska Institutet, Stockholm, Sweden). Bradykinin receptor 1/2 knockout mice were kindly provided by Prof. M. Bader (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany).

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3.2.2. Pulmonary compliance measurements

The mice were tracheotomised and ventilated in a volume driven mode at a positive end-expiratory pressure of 0 kPa. Respiration rate was set at 20 breaths/min and ventilation pressure was recorded while inflating the lung at a tidal volume of 200 µl.

3.2.3. Lung preparation

After sacrificing the animals, the chest was opened and the lungs were flushed via a catheter that was placed into the pulmonary artery with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Once the effluent was clear of blood, the lungs were removed and placed in paraformaldehyde or nitrogen for further examination. For cell isolation the lungs were placed in a PBS buffer.

3.2.4. Isolation of murine and human lung fibroblasts and cell culture

Lung tissue specimens were obtained from IPF patients who underwent lung transplantation at the Department of Cardiothoracic Surgery of the Medical University of Vienna, Austria, director W. Klepetko. Non-utilized donor lungs or parts of donor lungs that were not implanted due to lack of compatibility (for instance oversized grafts) served as a control. All investigations were approved by the local ethics committee and written informed consent was obtained from all participants or their next-of-kin.

Human lung specimens of the pulmonary parenchyma and mouse lungs were chopped into < 1 mm^3 pieces. The minced pieces were washed twice with PBS and then plated in 100-mm dishes (Greiner-bio-one, Frickenhausen, Germany). The specimens were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10 % fetal calf serum (FCS; HyClone, South Logan, UT), and 1 % penicillin/streptomycin (Invitrogen) in a humidified atmosphere of 5 % CO_2 at 37 °C. The purity of isolated fibroblasts was verified by positive staining for vimentin, fibronectin and collagen IV. All experiments were carried out with lung fibroblasts from passages 3-4. The mouse NIH3T3 fibroblasts were cultured in DMEM supplemented with 10 % FCS, and 1 % penicillin/streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C.

3.2.5. Microdissection of lung tissue and alveolar epithelial type II cells

Frozen specimens of lungs were sectioned at 10 µm in cryostat, mounted on noncoated, thin glass slides and stained with haemalaun (Roth, Karlsruhe, Germany) for 45 seconds. The sections were then immersed in 70% and 96% ethanol until use. Not more than 10 sections were prepared at once to restrict the storage time. Chosen areas of lung specimen were laser-microdissected under visual control (PALM, Bernried, Germany). Tissue was harvested by a syringe needle and transferred into a reaction tube containing 10 µl fist strand buffer (FSB, 52 mM Tris pH 8.3, 78 mM KCl, 3.1 mM $MgCl_2$,). Samples were frozen in liquid nitrogen and stored for further preparation.

For ATII cell microdissection, cryostat sections (10 µm thick) were mounted on poly-L-lysine (0.01%, Sigma, Deisenhofen, Germany)-covered slides and stored in acetone for 5 minutes. For surfactant protein C precursor (proSP-C) staining a polyclonal rabbit anti pro-SP-C antibody was applied (1:100 in PBS; Chemicon, Temecula, CA) followed by incubation with FITC-labeled goat anti-rabbit IgG (1:40 in PBS, Santa Cruz Biotechnology, CA). Not more than two sections were prepared at once to restrict the storage time. ATII cells were selected according to their staining pattern and lasser-microdissected under visual control. Samples with 50 cell profiles each were snap frozen in liquid nitrogen and stored for further preparation.

3.2.6. RNA isolation and reverse transcriptase reaction

Total RNA was extracted using a PeqGOLD Total RNA Kit (PeqLab, Erlangen, Germany) according to the manufacturer's instruction. One µg of RNA obtained from lung homogenate, microdissected lung specimens, isolated fibroblasts or microdissected ATII cells was used in a reaction containing 4 µl 5x FSB, 2 µl dNTP (10 mM each, Fermentas, St. Leon-Rot, Germany), 1 µl random hexamers (50 µM), 1 µl DTT (0.1 M), 1 µl RNase inhibitor (40 U/µl) and 1 µl MuLV reverse transcriptase (200 U/µl, all from Applied Biosystems, Foster City, CA) in RNase-free water (final volume 20 µl). The reaction was incubated at 43ºC for 1 h and then at 94ºC for 2 min (TGradient Thermocycler, Biometra, Goettingen, Germany).

3.2.7. Real Time PCR

Real time PCR was performed by a Sequence Detection System 7700 (Applied Biosystems). Reactions were set up with Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) using 2 µl of cDNA. The β-actin gene was used as a reference gene. Cycling conditions were 95°C for 6 min, followed by 45 cycles of 95°C for 20 s, 55°C for 30 s, and 73°C for 30 s. Melting curve analysis and gel electrophoresis were performed to confirm the exclusive amplification of the expected PCR product. Gene expression was assessed using the $2^{-\Delta\Delta CT}$ method as already described (190). The fold change in target gene relative to the endogenous control β-actin was determined by using the equation fold change=$2^{-\Delta\Delta CT}$, where $-\Delta\Delta CT=(C_{tTarget} - C_{tActin})_{treated} - (C_{tTarget} - C_{tActin})_{control}$. Primer sequences are listed in Table 1.

Table 1. Primer sequences.

GENE	PRIMER SEQUENCES
human FXII	F:5'-ACG ACC TGG CTC TGT TGC-3' R:5'-CTT GGC AGG CAC ACC GG-3'
human FXI	F:5'-TCT GGC TTG TAT TAG GGA C-3' R:5'-TCT TTG GGC CAT TCC TGG-3'
human HMWK	F:5'-AAG AGT ACA GGT GGT CGC-3' R:5'-CAA TCT AGG CTT TGG CCA AG-3'
human β-actin	F:5'-ATT GCC GAC AGG ATG CAG GAA-3' R:5'-GCT GAT CCA CAT CTG CTG GAA-3'
murine FXII	F:5'-ACA GTG CTC TGC GAG GTG G-3' R:5'-CGT TAG AGT TGG AGC AGC GAT-3'
murine FXI	F:5'-TTA CAC AGA TTT TCA GCG GCC-3' R:5'-TGT GTA CCC CCA TCC AGT CAC-3'
murine HMWK	F:5'-GGA GAA CAA AGT CGT CCC GA-3' R:5'-TGT GAC ACT CCG GAA AGG AGA-3'
murine β-actin	F:5'-AGA GGG AAA TCG TGC GTG AC-3' R:5'-CAA TAG TGA TGA CCT GGC CGT-3'

3.2.8. Protein isolation and quantification

Harvested cells and frozen lungs specimens were lysed in RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 1% Sodium deoxycholate, 0.1% SDS), which contained a protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates were incubated on ice for 30 min and then centrifuged (10000 rpm for 10 min at 4ºC). Supernatants were placed in new tubes and stored in -80˚C. Protein quantification was performed using BCA™ Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Different bovine serum albumin (BSA) concentrations were used as a standard.

3.2.9. SDS polyacrylamide gel electrophoresis

Protein samples were mixed with 5x SDS-loading buffer (0.25 mol/l Tris-HCl pH 6.8, 10% (w/v) SDS, 50% glycerol, 10% β-mercaptoethanol), boiled for 10 min, loaded onto the SDS polyacrylamide gel (stacking gel: 4% acrylamide:bisacrylamide, 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED; resolving gel (10%): 10% acrylamide:bisacrylamide, 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED) and run in a SDS-running buffer (25 mM Tris, 250 mM Glycine, 0.1% (w/v) SDS) at 100V.

3.2.10. Immunoblotting

Proteins separated on SDS polyacrylamide gel were transferred to a PVDF membrane (Amersham Biosciences, Freiburg, Germany) using the wet transfer technique in a transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 1 h at 100V. After blocking with 5 % non-fat dry milk in tris-buffered saline (TBS; 25 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 0.1 % (v/v) Tween 20 (TBS-T), the membranes were incubated at 4°C overnight with one of the following antibodies: goat anti-FXII (Zytomed Systems, Berlin, Germany), goat anti FXI (Abcam, Cambridge, UK), rabbit anti-HMWK

(Abcam), mouse anti-phospho-p44/42, rabbit anti-phospho-Akt, rabbit anti-phospho-p38, rabbit anti-phospho-Smad 3, rabbit anti-phospho-c-jun (all from Cell Signaling, Frankfurt am Main, Germany), rabbit anti-phospho-JNK (R&D Systems, Wiesbaden, Germany), mouse anti-cyclin D1 (Cell Signaling), goat anti-uPAR (Santa Cruz Biotechnology), rabbit anti-lamin B or rabbit anti-tubulin (both from Abcam). All primary antibodies were diluted 1:1000 in 1% BSA in TBS-T. After a 1 h incubation with peroxidase-labelled secondary antibody (1:3000 in 5% non-fat dry milk in TBS-T, all from Dako, Gostrup, Denmark) proteins were detected using ECL Plus Kit (Amersham Biosciences). To determine the amounts of protein loaded on the gel, blots were incubated for 1 hour in a stripping buffer (100 mM glycine, 1% HCl) and reprobed using an anti-β-actin antibody (Sigma-Aldrich) or one of the following anti-pan antibodies: anti-p44/42, rabbit anti-Akt, rabbit anti-p38, rabbit anti-Smad 3, rabbit anti-c-jun (all from Cell Signaling), rabbit anti-JNK (R&D Systems).

3.2.11. Immunocytochemistry

For immunocytochemical analysis, HLF were fixed with 4 % paraformaldehyde for 10 min, permeabilized with 0.2 % Triton X-100 in PBS for 10 min, blocked with 3 % BSA in PBS for 1 h at room temperature, and incubated overnight at 4 °C with one of the following antibodies: mouse anti-FXII, rabbit-anti-collagen IV, mouse anti-fibronectin (all from Abcam), goat anti-vimentin (Santa Cruz Biotechnology), and rabbit anti-phospho-Smad 3 (Cell Signaling). All primary antibodies were diluted 1:100 in PBS. Slides were incubated with a rhodamine-conjugated secondary antibody (Dianova, Hamburg, Germany), and mounted with Vectashield mounting medium (Vector, Burlingame, CA). Nuclei were visualized by 4′, 6-diamidino-2-phenylindole (DAPI) staining. Controls were performed by substituting the primary antibody by a non-specific antibody. Images were captured by a Leica DMR microscope (Leica, Heidelberg, Germany) with 40×/1.25-0.75 oil-objective at room temperature and photographed using MetaMorph 7.0 (Molecular Devices, Downingtown, PA). All images illustrated are representative of at least four other areas per section, seen on at least three independent sections.

3.2.12. Immunohistochemistry

Paraffin-embedded, formalin-fixed lung tissue was sectioned at 5 microns and processed for immunohistochemical staining using one of the following antibodies: rabbit anti-FXII (1:100 in PBS, kindly provided by Dr. T. Renne), goat anti-FXI (1:500 in PBS, Abcam), rabbit anti-HMWK (1:400 in PBS, Abcam), rabbit anti-fibrinogen (1:800 in PBS, Dako, Gostrup, Denmark) and ZytoChem Plus AP-Fast Red Kit according to the manufacturer's instructions (Zytomed Systems). Negative controls were obtained in all cases by omitting the primary antibody. The specificity of the staining was not proved by preabsorption of the primary antibody with the corresponding antigen.

3.2.13. Proliferation assay

The primary murine lung fibroblasts were seeded in 48-well plates, starved in serum free DMEM for 24 h, and then stimulated with various concentration of FXIIa (American Diagnostica, Stamford, CT). In some experiments cells were preincubated with anti-uPAR (5 μg/ml, R&D Systems), anti-β1-integrin or anti-α5-integrin (1:200 in DMEM, both from Millipore, Schwalbach, Germany) blocking antibodies 1-2 h before exposure to FXIIa. In addition, in some experiments 10.0 μM PD98059, 5 μM SP600125, 0.7 μM Wortmannin, 3 μM SB203580 (all from Calbiochem) were added to the cell culture medium 1-2 h prior to the addition of FXIIa. Cells were treated with 3-9 μg/ml FXIIa alone or in the presence of 12.6 μg/ml CTI (Calbiochem), or 7 μM uPAR synthetic peptides (kindly provided by Prof. A. Schmaier). After 24-36 h the cells were exposed to [^{3}H]Thymidine (0.2 μCi per well, PerkinElmer, Waltham, MA) for 6-12 h, rinsed three times with PBS and solubilized with 0.2 ml 0.5 M sodium hydroxide; 0.1 ml of the solubilized material was quantified by liquid scintillation counting (TRI-CARB® 1500, A Canbera Company, Meriden, CT). [^{3}H]Thymidine incorporation was expressed as absolute radioactivity (cpm, counts per minute per well).

3.2.14. Immunoprecipitation

Primary murine fibroblasts were either unstimulated or stimulated with 6 µg/ml FXIIa for 30 min and lysed in a buffer containing 20 mM HEPES pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF, 20 µg/ml aprotinin, 20 µg/ml leupeptin, 2 mM sodium vanadate. After 30 min incubation at 37ºC, insoluble material was pelleted by centrifugation at 10 000 rpm for 10 min at 4ºC. A hundred µl of cell lysate was further incubated overnight at 4ºC with the chicken anti-uPAR antibody (dilution 1:20, kindly provided by Dr. V. Magdolen) or with 5 µg of a nonspecific antibody. Next, 100 µl of G-Sepharose (Amersham Biosciences) was added and immune complexes were allowed to bind for 1 h at 4ºC. Next, the beads were washed four times with a lysis buffer, and adsorbed material was eluted in a 2x SDS loading buffer. After boiling, the uPAR bound proteins were analyzed by Western blotting using mouse anti-FXII antibody (1:1000 in 1% BSA in TBS-T, Abcam).

3.2.15. Generation of FXII promoter constructs and site-directed mutagenesis

The human FXII promoter fragments were amplified by PCR from human lung DNA using a Long PCR Enzyme Mix (Fermantas) according to the manufacturer's instruction. Cycling conditions were: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min. The following primers were used: human FXII-1630 forward 5'-CCG**CTCGAG**TGCTCTGTGCTTAGTAACC-3'; human FXII-907 forward 5'-CCG**CTCGAG**CAGCTACCCAGGAGGCT-3'; human FXII-577 forward 5'-CCG**CTCGAG**GCGTGGTGGTGGGCTCCT-3'; human FXII-299 forward 5'-CCG**CTCGAG**CTTAACCTCCTGATCTCC-3'; human FXII-183ΔSBE$_{-272}$ forward 5'-CCG**CTCGAG**AAACTCCCAAACTTTCC-3'; human FXII reverse 5'-CCC**AAGCTT**C-GTTGGTCCAGCTGCCTATC-3'. The PCR fragments were cloned into pGL3 Enhancer Vector (Promega, Mannheim, Germany) using XhoI and HindIII restriction sites (in bold). Point mutation was introduced into the CAGA box in pGL3-299 construct using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to

the manufacturer's instructions. The following primers were used: human FXII-299C/T forward 5'-CCACAGGACC**TAGA**GCATAAGAATG-3', human FXII-299C/T reverse: 5'-CATTCTTATGC**TCTA**GGTCCTGTGG-3'. Successful cloning and insertion of the mutation into the CAGA box were confirmed by sequencing.

3.2.16. Transient transfection and luciferase assay

NIH3T3 cells were transfected with indicated plasmids using FuGene6 (Roche) according to the manufacturer's instruction. After 48 h the cells were either unstimulated or stimulated with 10 ng/ml TGF-β1 (R&D Systems) for a further 24 h. Subsequently, the cells were harvested and assayed for luciferase reporter activity using a Promega Luciferase Assay Kit according to the manufacturer's instruction. Co-transfection with a pEGF-N1 (Clontech, Mountain View, CA) control vector was used to normalize for transfection efficiency.

3.2.17. Antisense Oligonucleotides

Pre-designed, commercially available siRNA sequences directed against human Smad 3 (Dharmacon, Chicago, IL), human JNK1 (Abnova, Heidelberg, Germany), and a universal negative-control siRNA (Ambion, Austin, TX) were employed. Cells were treated with siRNA (250 nM each) using the X-treme Gene siRNA Transfection Reagent (Roche). The siRNA-mediated downregulation of the target genes was assessed 72 h after transfection by Western blotting. At this time point the cells were either unstimulated or stimulated with 10 ng/ml TGF-ß1 for 24 h and the Western blots for FXII were prepared as described above.

3.2.18. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a Chromatin Immunoprecipitation Assay Kit from Millipore according to the manufacturer's

instruction. Briefly, NIH3T3 cells either unstimulated or stimulated with 10 ng/ml TGF-β1 (R&D Systems) were treated with 1 % formaldehyde for 10 min. The cross-linked chromatin was then prepared and sonicated to an average size of 500-800 bp. The DNA fragments were immunoprecipitated with rabbit anti-Smad 3 antibody (1:200, Cell Signaling) or IgG isotype control (5 µg/ml, Sigma Aldrich) overnight at 4°C. After reversal of cross-linking, the immunoprecipitated chromatin was amplified by PCR using the following primers: human FXII-299 bp forward: 5'- CTTAACCTCCTGATCTCC-3'; human FXII-299 bp reverse: 5'- CGTTGGTCC-AGCTGCCTATC-3'. PCR products were separated on the 2% agarose gel and visualized by ethidium bromide staining.

3.2.19. Streptavidin pull-down assay

The double-stranded biotinylated DNA fragment ($SBE_{-283/-258}$: 5'-biotin–CTTAACCTCCTGATCTCCACAGGACC**CAGA**GCATAAGAATGTCCC–3' or $SBE_{-283/-258\ C/T}$: 5'-biotin–CTTAACCTCCTGATCTCCACAGGACC**TAGA**GCATAAGAATGTCCC–3') spanning SBE was assayed for protein interaction in 100 µl binding reaction containing 20 µg of nuclear extract, 20 pmol/µl of biotinylated template, and 1 µg of poly(dI-dC). After incubation for 1 h at 30 °C, streptavidin MagneSphere paramagnetic particles (Promega) pre-equilibrated in a binding buffer (20 mM HEPES pH 7.9, 80 mM KCl, 10 mM $MgCl_2$, 10 % (v/v) glycerol, 2 mM DTT, 500 µg/ml of BSA and 0.05 % (v/v) Nonidet P-40) were added to the reaction, and incubated for another 1 h at 30°C. The DNA–protein complexes were washed three times with wash buffer (20 mM HEPES pH 7.9, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, and 8.5 % (v/v) glycerol) using a magnetic device (Dynal MPC®-E, Magnetic Particle Concentrator). After boiling, the DNA-bound proteins were analyzed by Western blot using rabbit anti-phospho Smad 3 antibody (1:1000 in 1% BSA in TBS-T, Cell Signaling). Nuclear extracts were prepared using NE-PER® Nuclear and Cytoplasmic Extraction Reagent (Pierce) according to the manufacturer's instruction.

3.2.20. Statistics

Data are presented as mean ± SD unless otherwise stated. Normal distribution was analyzed by Shapiro-Wilk test. Statistical comparisons between two populations were performed using unpaired Student *t*-tests or Mann-Whitney test. Differences between multiple groups were compared by the One-way ANOVA analyse followed by the Tukey's post test. A level of $p<0.05$ was considered statistically significant. The statistical significance between survival curves was assessed by the log-rank test.

4. Results

4.1. Expression of FXII, FXI and HMWK is altered in idiopathic pulmonary fibrosis lungs

To reveal the potential role of the intrinsic coagulation pathway in the pathogenesis of IPF, expression of FXII, FXI and HMWK in lung tissue from IPF patients and donors was analyzed using real-time PCR. As shown in Figure 4.1A, FXII and HMWK mRNA level were markedly upregulated in IPF lung specimens. The cell-specific expression of intrinsic coagulation pathway components was quantified in primary human ATII cells and fibroblasts derived from the lungs of IPF patients and donors. Lung fibroblasts isolated from IPF lung tissue expressed FXII mRNA at higher levels than those found in the cells from the donor lungs. FXII mRNA was not detected in ATII cells. FXI and HMWK expression in ATII cells and lung fibroblasts derived from the lungs of IPF patients was not changed when compared to the donor samples. Furthermore, expression of FXII, FXI and HMWK in alveolar septae microdissected from the fibrotic (F) and healthy (H) regions of IPF tissue was analyzed (Figure 4.1B). The mRNA level of FXII, FXI and HMWK in the healthy regions from IPF lungs was not changed in comparison to the levels found in the donor tissue (D) (Figure 4.1B). In contrast, there was significant upregulation of FXII, FXI and HMWK genes in the fibrotic areas of IPF lungs.

In line with the mRNA expression, protein levels of FXII and HMWK were upregulated in IPF lungs (Figure 4.2A). An activity assay using FXII chromogenic substrate demonstated increased enzymatic activity of FXII in BAL fluid from IPF patients (Figure 4.2B). The specificity of the performed assay was proved by the blockade of FXII activity by its inhibitor, CTI.

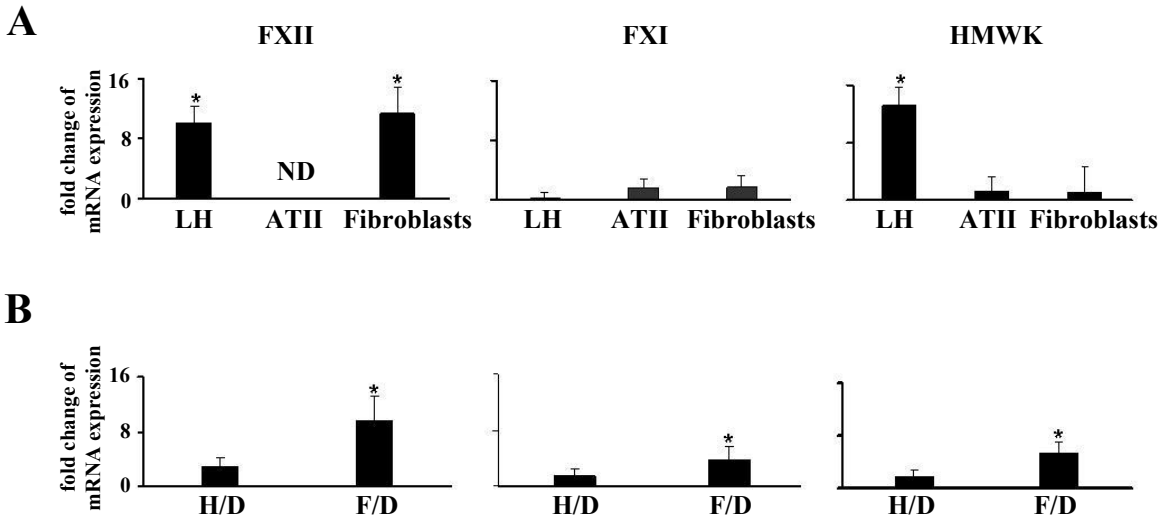


Figure 4.1. mRNA level of FXII, FXI and HMWK is elevated in the lungs of IPF patients.
(A) The expression of FXII, FXI and HMWK mRNA was assessed in donor (n=10) and IPF (n=10) lung tissue homogenates (LH) as well as in lung fibroblasts and ATII cells from donor (n=5) and IPF (n=5) lungs by real time PCR. Given is the fold-increase in mRNA expression in IPF specimens (normalized for β-actin expression) *versus* values obtained for donor samples. (B) Expression of FXII, FXI and HMWK in the healthy (H) and fibrotic (F) regions of IPF lungs. Given is the fold-increase in mRNA expression in IPF specimens (normalized for β-actin expression) *versus* values obtained for donor (D) samples; n=5 (C). Results are presented as mean ± SD, * $p < 0.05$; Student's *t*-test. ND; not detected

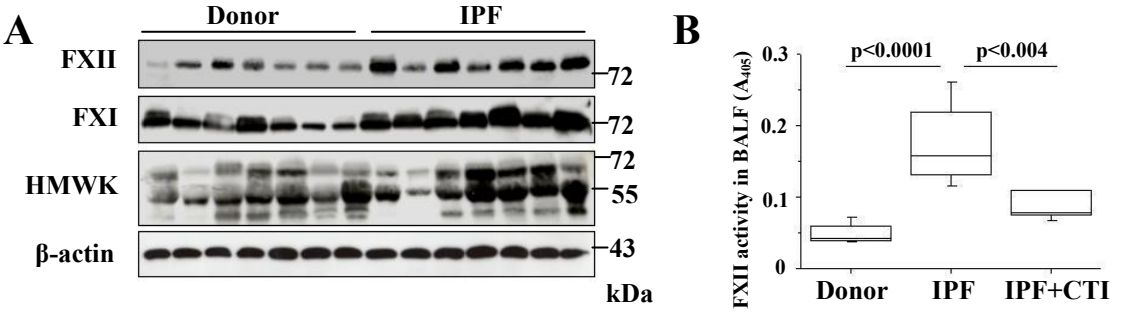


Figure 4.2. Protein level of FXII, FXI and HMWK and FXII activity are elevated in the lungs of IPF patients.
(A) FXII, FXI and HMWK expression in lung homogenates of donors and IPF patients as assessed by Western blotting. Representative donors (7 out of 20) and patients (7 out of 20) are shown. ß-actin served as a loading control. (B) FXII activity in BALF samples of donors (n=10) and IPF patients (n=10) in the absence or presence of FXII specific inhibitor CTI. Data are presented as box and whisker plots, in which the horizontal line within each box represents the median, the limits of each box represent the interquartile range and the whiskers represent the maximum and minimum values. Tested for statistical significance by Mann-Whitney test. Significance levels are indicated.

Immunohistochemistry staining of FXII, FXI and HMWK was performed to characterize localization of these factors in donor and IPF lungs. As shown in Figure 4.3 the expression of all intrinsic coagulation factors was markedly increased in IPF sections compared to donor tissue. The strongest immunoreactivity of FXII was observed in

fibroblasts and on the surface of ATII cells. FXI was mainly expressed in ATII cells and in fibroblasts, whereas HMWK was mostly present in monocytes.

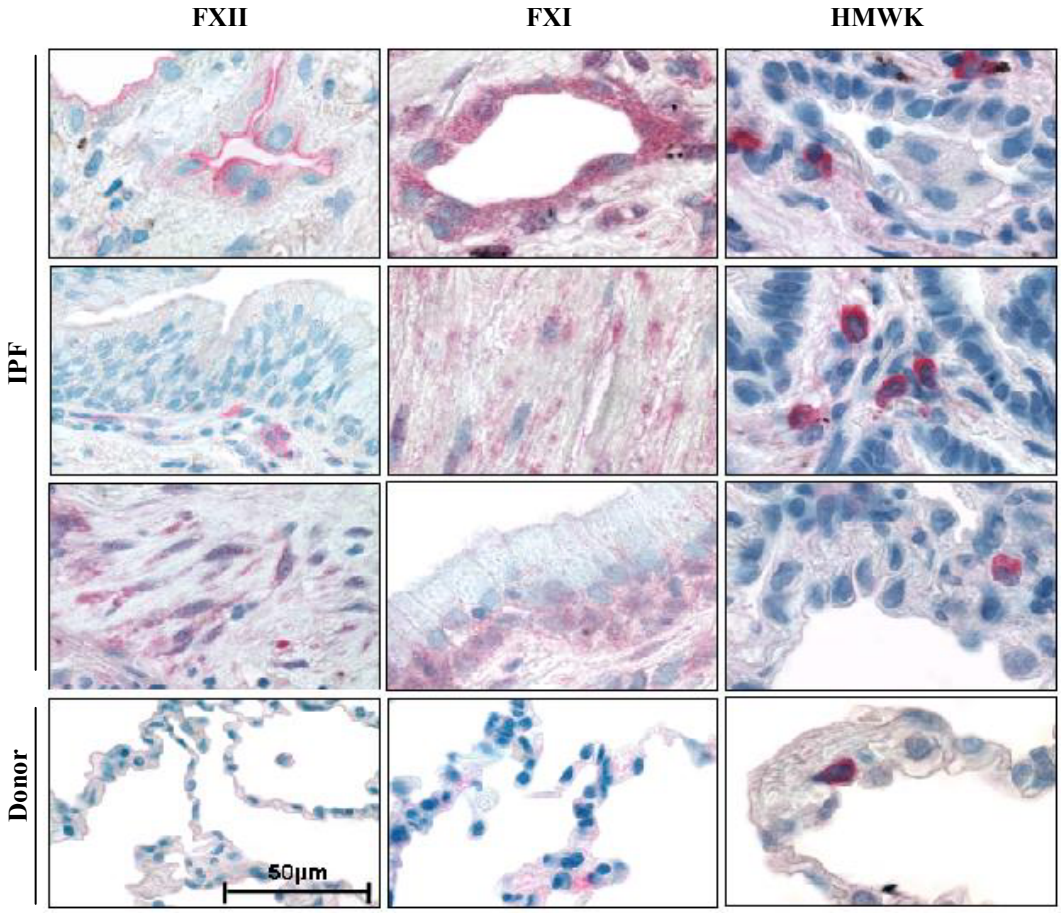


Figure 4.3. Expression and localization of FXII, FXI and HMWK in lung tissue of donor and IPF patients.
Immunohistochemical staining of FXII, FXI and HMWK in lung tissue sections obtained from donors and IPF patients. One representative IPF patient and one donor out of five per group are shown. Bar size is indicated.

4.2. Expression of FXII, FXI and HMWK is elevated in bleomycin lungs

To determine whether the findings in the IPF lungs translate to the animal model of lung fibrosis, the expression of FXII, FXI and HMWK was quantified in the lungs of bleomycin challenged mice. The real time PCR revealed upregulation of FXII, FXI and HMWK in lung homogenate from bleomycin-treated mice at day 4 and day 20 post bleomycin instillation (Figure 4.4). Interestingly, FXII mRNA was not detected in primary murine lung fibroblasts and ATII cells. FXI expression in ATII cells derived

from bleomycin lungs was not changed, while its expression in lung fibroblasts was upregulated at day 12 post bleomycin administration. There was significant upregulation of HMWK mRNA in lung fibroblasts at day 20 post bleomycin application, while no change in HMWK expression was observed in ATII cells.

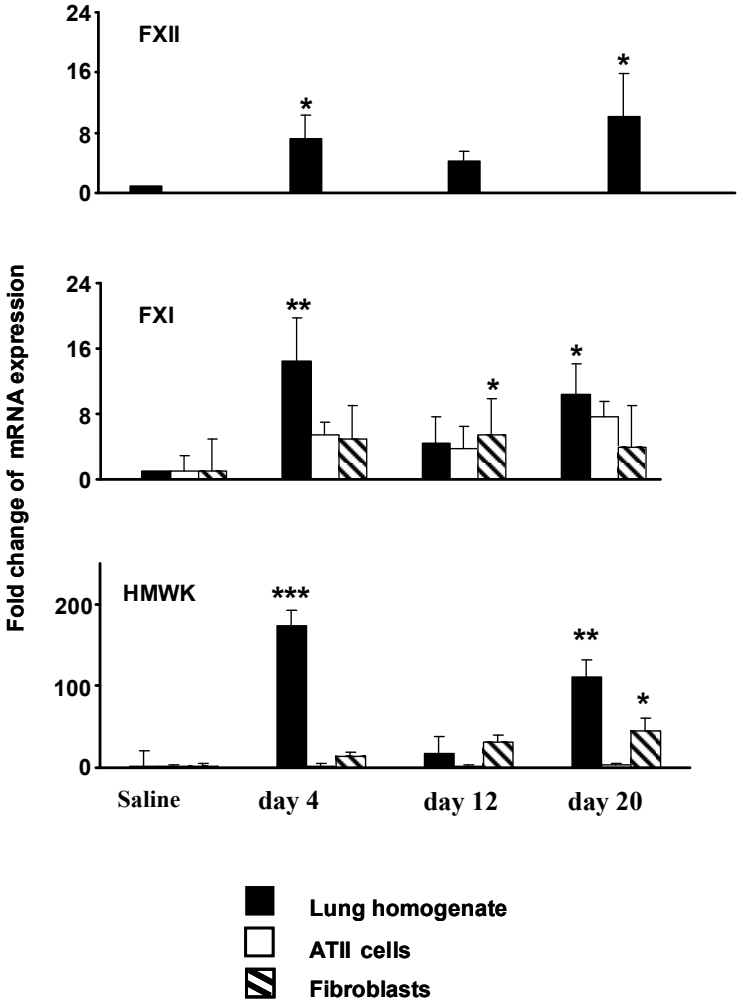


Figure 4.4. mRNA level of FXII, FXI and HMWK is elevated in the lungs of bleomycin-treated mice. Real time PCR analysis of FXII, FXI and HMWK expression in control (saline; n=10) and bleomycin (n=10) lung homogenates as well as in lung fibroblasts and ATII cells isolated from the lungs of control (n=10) and bleomycin challenged (n=10) mice. Given is the fold-increase in mRNA expression in bleomycin-treated lungs (normalized for β-actin expression) *versus* values obtained for control lungs. Results are presented as mean ± SD,*** p <0.0005, ** p <0.005,* p <0.05; Student's *t*-test.

As compared to findings from IPF tissue specimens, immunoblotting showed a similar upregulation of the FXII, FXI and HMWK protein level in lung homogenate at day 4 and 20 post bleomycin application (Figure 4.5).

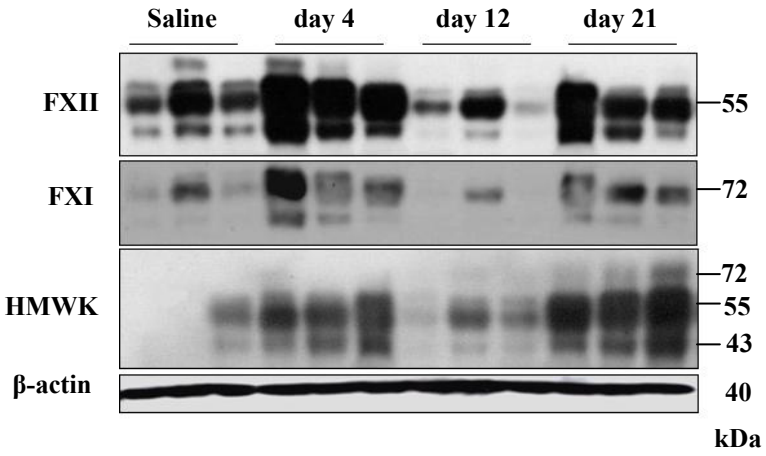


Figure 4.5. Increased protein level of FXII, FXI and HMWK in lung homogenates of control and bleomycin challenged mice.
FXII, FXI and HMWK expression in lung homogenates of control (saline) and bleomycin-treated mice. Representative saline (3 out of 10) and bleomycin challenged mice at day 4 (3 out of 10), 12 (3 out of 10) and 21 (3 out of 10) post application are shown. ß-actin served as a loading control.

Immunolocalization studies confirmed strong expression of FXII, FXI and HMWK in bleomycin-injured lungs (day 20 post application) compared to the weak signal observed in the lungs of saline treated mice (Figure 4.6). Weak FXII, FXI and HMWK staining in control lungs was mainly observed in alveolar macrophages. After bleomycin application strong FXII, FXI and HMWK immunoreactivity was visible in fibrotic regions and alveolar macrophages.

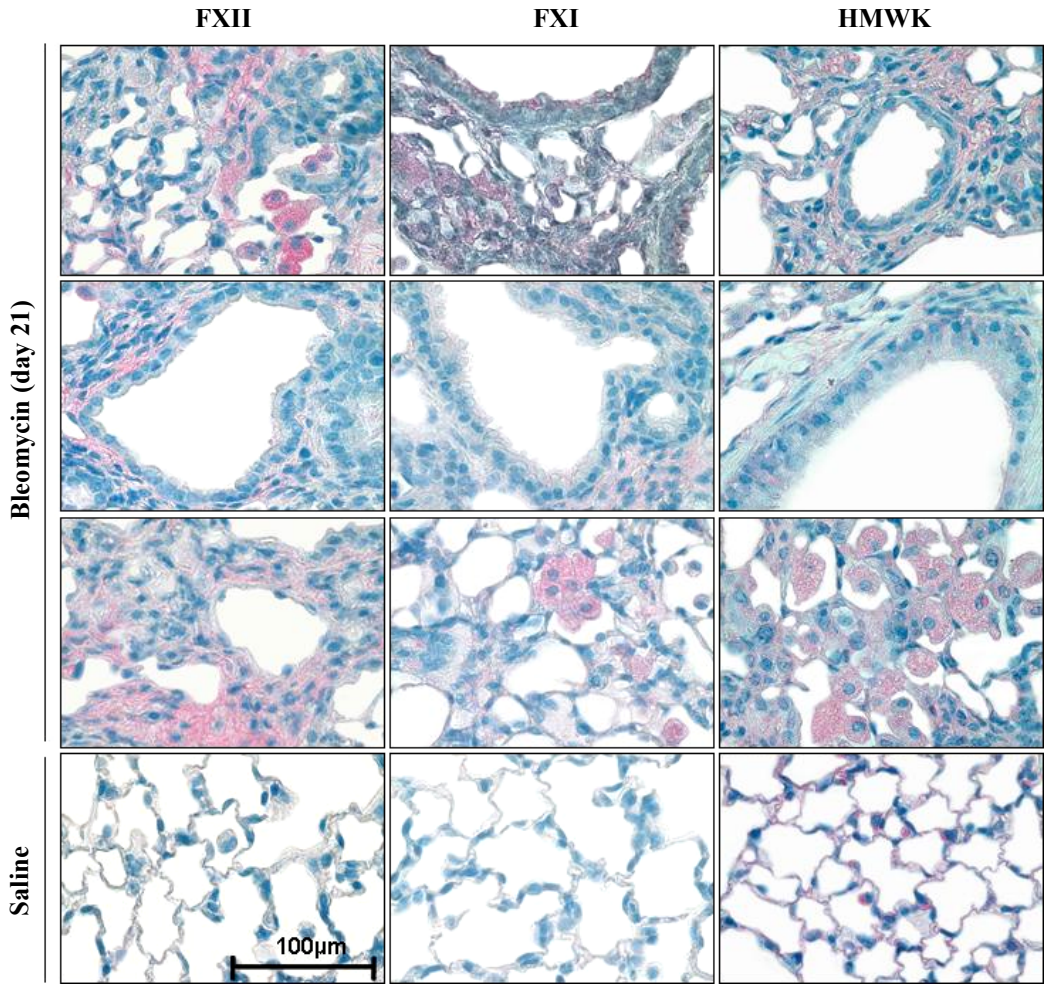


Figure 4.6. Expression and localization of FXII, FXI and HMWK in the lungs of control and bleomycin-treated mice.
Representative histological section showing FXII, FXI and HMWK localization in the lungs from saline or bleomycin-treated mice. One representative bleomycin mouse and one control out of ten per group are shown. Bar size is indicated.

4.3. FXII knockout or FXIIa inhibition protects against bleomycin-induced lung fibrosis

To determine the role of FXII in lung fibrosis, mice deficient in FXII were challenged with bleomycin. As revealed by hematoxylin and eosin staining at day 21, wild type (WT) animals treated with bleomycin showed remarkable lung fibrosis characterized by a distortion of the normal lung architecture, increased interstitial wall thickness and an increased number of fibroblasts (Figure 4.7A). In contrast FXII deficient ($FXII^{-/-}$) mice had decreased fibrotic changes in the lungs (Figure 4.7C). Trichrome staining was performed to assess abnormalities in lung collagen deposition after

bleomycin challenge. Accumulation of collagen was more remarkable in WT animals (Figure 4.7B) as compared to FXII$^{-/-}$ mice (Figure 4.7D). Finally, the mortality of FXII$^{-/-}$ mice was significantly lower in comparison to WT mice (Figure 4.7E). At day 21 post bleomycin challenge, the mortality of WT mice was 40%, whereas the mortality of FXII$^{-/-}$ was 5%.

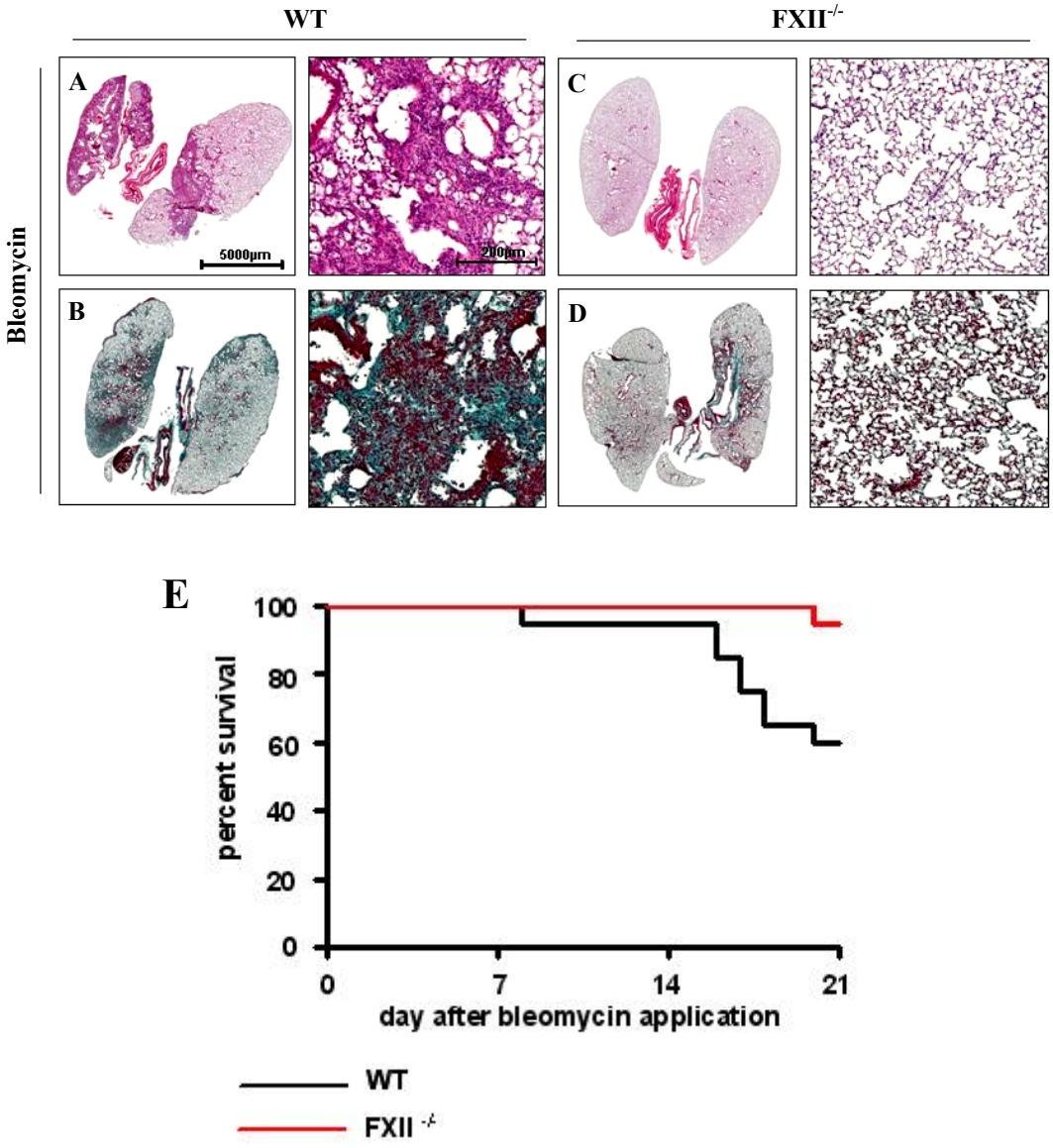


Figure 4.7. FXII$^{-/-}$ mice are protected against bleomycin-induced lung fibrosis.
Hematoxylin and eosin staining of lungs of (A) WT and (C) FXII$^{-/-}$ mice 21 days after bleomycin challenge. Trichrom staining of lungs of (B) WT and (D) FXII$^{-/-}$ mice 21 days after bleomycin administration. Green colour indicates collagen deposits. Representative lung tissue sections are demonstrated. Bar size is indicated. (E) Survival curve demonstrating mortality rate of WT and FXII$^{-/-}$ mice after bleomycin challenge, n=20 mice/group. Significant difference by log-rank test, p=0.007.

To determine whether fibrin deposition in the lungs of FXII$^{-/-}$ mice is impaired after bleomycin application, lung sections were stained with anti-fibrinogen antibody (Figure 4.8). WT animals receiving bleomycin showed extensive fibrin deposition in fibrotic areas. Fibrin staining was observed in alveolar space in close proximity and in areas of fibrosis. A similar pattern was visible in the lungs of FXII$^{-/-}$ mice challenged with bleomycin.

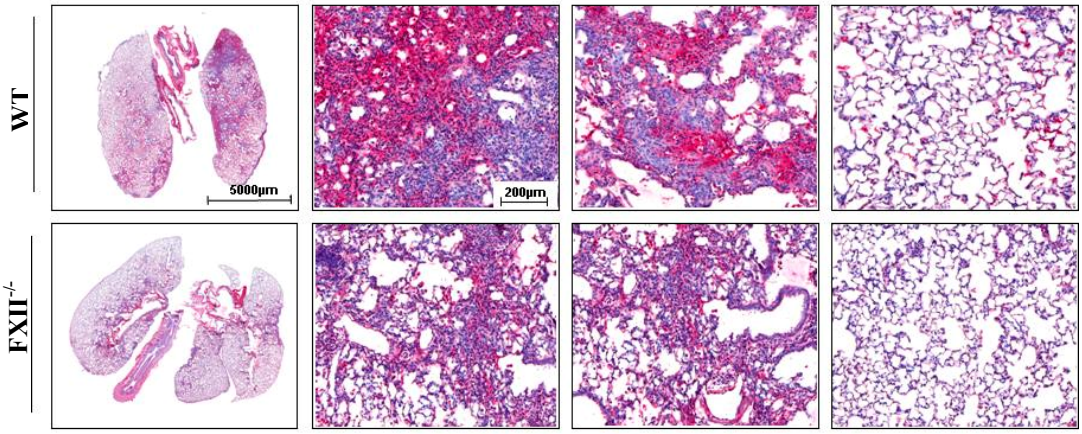


Figure 4.8. Fibrin deposition in the lungs of FXII$^{-/-}$ mice is not impaired after bleomycin application. Fibrin immunostaining in lung tissue sections of bleomycin-treated WT and FXII$^{-/-}$ mice 21 days after bleomycin instillation. Red colour indicates fibrin. The photomicrographs were selected to illustrate the pattern and extent of fibrin deposition in the abnormal and normal regions of the lung for each experimental group. Representative lung tissue sections are demonstrated. Bar size is indicated.

To evaluate the possible therapeutical effects of FXII inhibition on bleomycin-induced lung injury, the saline control mice and bleomycin challenged mice received CTI, a specific FXII activity inhibitor. Saline or CTI (at a dose 5 mg/kg body weight) were administered intratracheally at day 9, 12, 15 and 18. At day 21 the mice were sacrificed, the lung compliance was measured and lung tissue specimens were collected. Bleomycin-treated mice which received saline showed severe fibrotic changes with loss of normal architecture and extensive collagen deposition (Figure 4.9C, D). Extensive fibrosis development was reflected by a strong decrease in compliance (Figure 4.9J). In contrast, histologic findings in the lungs of bleomycin-treated mice that obtained CTI demonstrated fewer fibrotic lesions (Figure 4.9G). Collagen accumulation as assessed by Trichrom staining was markedly reduced in FXII inhibitor-treated animals (Figure 4.9H). Moreover, the compliance of CTI-treated animals was significantly improved (Figure 4.9J). At day 21 post bleomycin challenge, the mortality of saline-treated mice was 80 %,

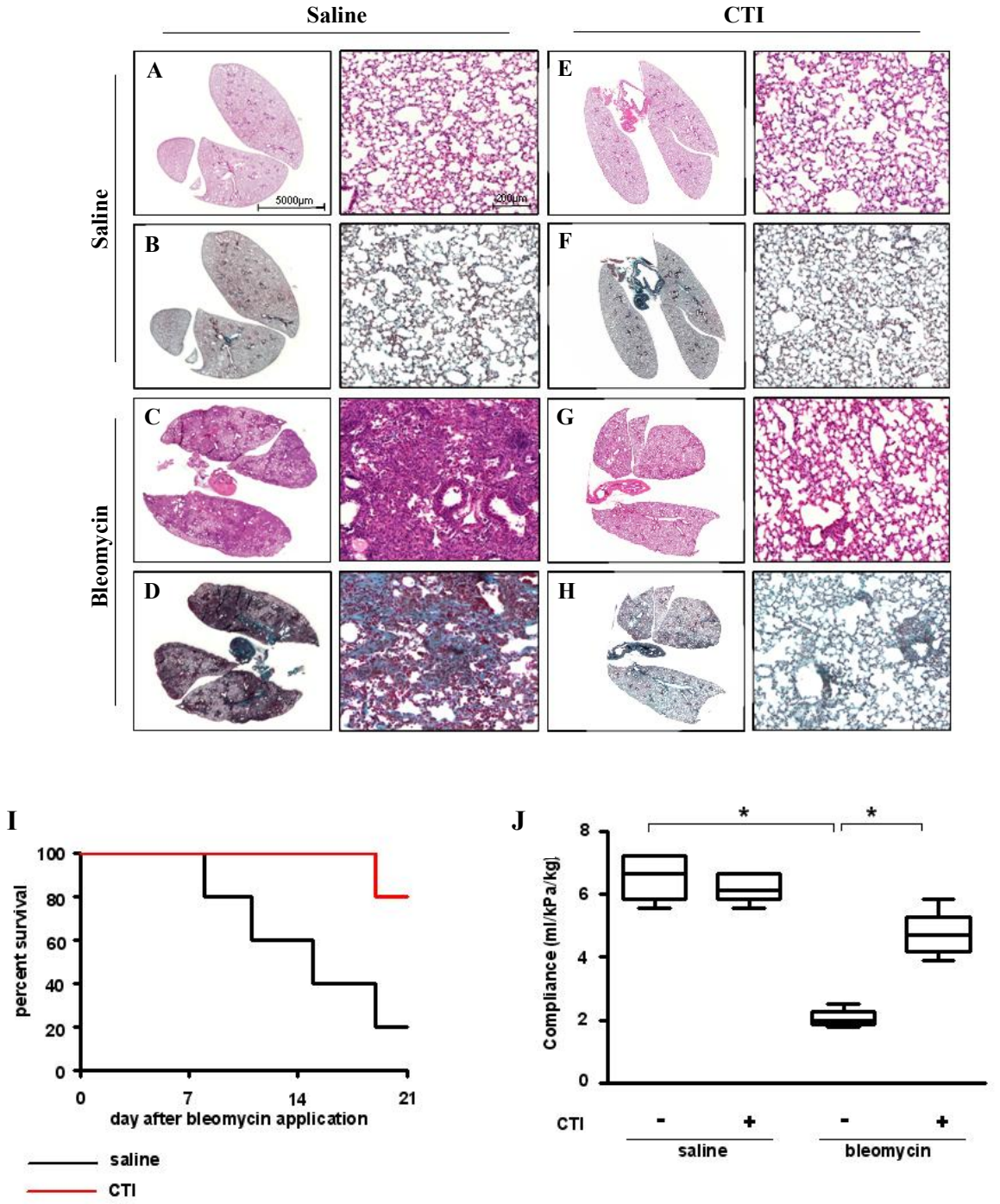


Figure 4.9. FXIIa inhibitor (CTI) attenuates bleomycin-induced lung fibrosis.
Hematoxylin and eosin staining of lung tissue sections at day 21 post (A,E) saline or (C,G) bleomycin application. Trichrom staining of lung tissue sections at day 21 post saline (B,F) or bleomycin (D,H) instillation. Green colour indicates collagen deposits. Representative lung tissue sections are demonstrated. Bar size is indicated. (I) Survival curve demonstrating mortality rate of saline- and CTI-treated mice after bleomycin challenge, n=5 mice/group. Significant difference by log-rank test, p=0.0416 (J) Lung compliance at day 21 after bleomycin or saline administration. Data are presented as box and whisker plots, in which the horizontal line within each box represents the median, the limits of each box represent the interquartile range and the whiskers represent the maximum and minimum values. n=5 mice/group; * $p < 0.05$; Mann-Whitney test.

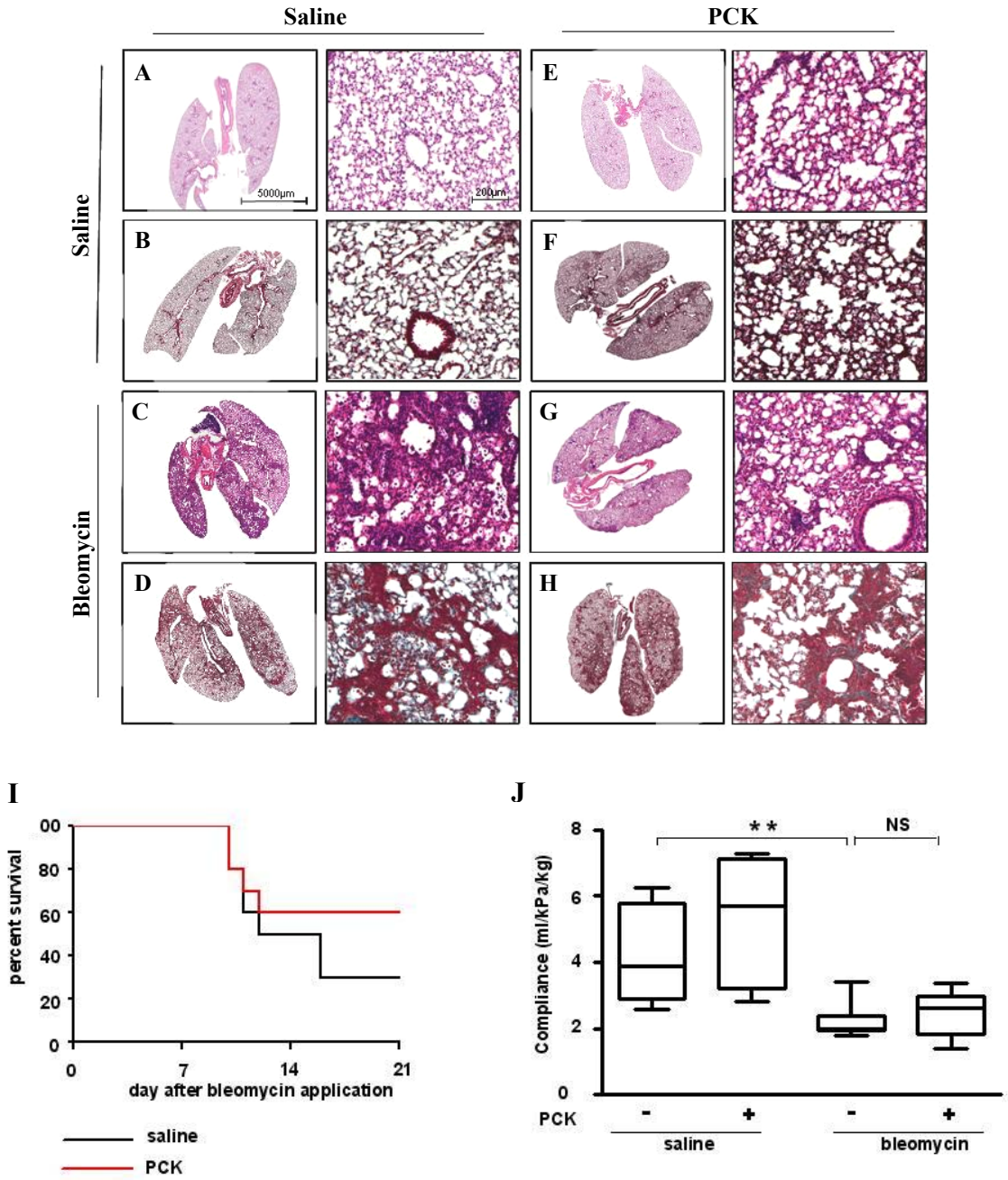


Figure 4.10. PCK administration does not improve bleomycin-induced lung fibrosis.
Hematoxylin and eosin staining of lung tissue sections at day 21 post (A,E) saline or (C,G) bleomycin application. Trichrom staining of lung tissue sections at day 21 post saline (B,F) or bleomycin (D,H) instillation. Green colour indicates collagen deposits. Representative lung tissue sections are demonstrated. Bar size is indicated. (I) Survival curve demonstrating mortality rate of saline- and PCK-treated bleomycin mice, n=10 mice/group. Statistical comparison by log-rank test, p=0.28. (J) Lung compliance at day 21 after bleomycin or saline administration. Data are presented as box and whisker plots, in which the horizontal line within each box represents the median, the limits of each box represent the interquartile range and the whiskers represent the maximum and minimum values. n=10 mice/group; ** $p < 0.005$; Mann-Whitney test. NS, not significant.

whereas the mortality of animals, which received CTI was 20% (Figure 4.9I). The control animals, which received CTI, had no histological changes in the lung but they showed a

mild degree of inflammatory cell infiltration into airspaces (Figure 4.9E). This side effect of CTI may limit its therapeutical application.

These findings led me to look for another FXII inhibitor which could be safely used in lung fibrosis treatment. PCK is the active-site directed inhibitor of activated FXII and KLK (93). Saline control mice or mice challenged with bleomycin received saline or PCK (at a dose of 8 mg/kg body weight) intratracheally at day 9, 12, 15 and 18. In contrast to previous results with CTI, after 21 days of bleomycin challenge, focal fibrotic lesions, destruction of lung architecture and collagen accumulation were seen in the lungs of both PCK- and saline-treated groups (Figure 4.10C, D, G, H) There was no significant difference in lung compliance between PCK-treated and untreated bleomycin animals (Figure 4.10J). After 21 days, when the experiment was terminated, seven of the untreated and four of the PCK-treated bleomycin mice died (Figure 4.10I). No histological changes in the control mice after PCK administration were observed (Figure 4.10E).

4.4. Bradykinin receptor 1/2 knockout mice are not protected against bleomycin-induced lung fibrosis

FXIIa is a potent activator of plasma PK. Active KLK cleaves further HMWK to HKa and BK, which acts as a vasodilator and proinflammatory peptide through two G-protein-coupled receptors: B1 and B2. To determine whether FXII-induced BK delivery contributes to the development of lung fibrosis, B1 and B2 receptors deficient mice ($B1B2^{-/-}$) were challenged with bleomycin. After 21 days of bleomycin application, WT and $B1B2^{-/-}$ animals developed strong fibrotic changes and showed collagen accumulation in the lungs as evidenced by hematoxylin and eosin staining and Trichrom staining respectively (Figure 4.11A). No improvement in lung compliance (Figure 4.11C) and survival (Figure 4.11B) was observed in $B1B2^{-/-}$ in comparison to WT mice after bleomycin administration.

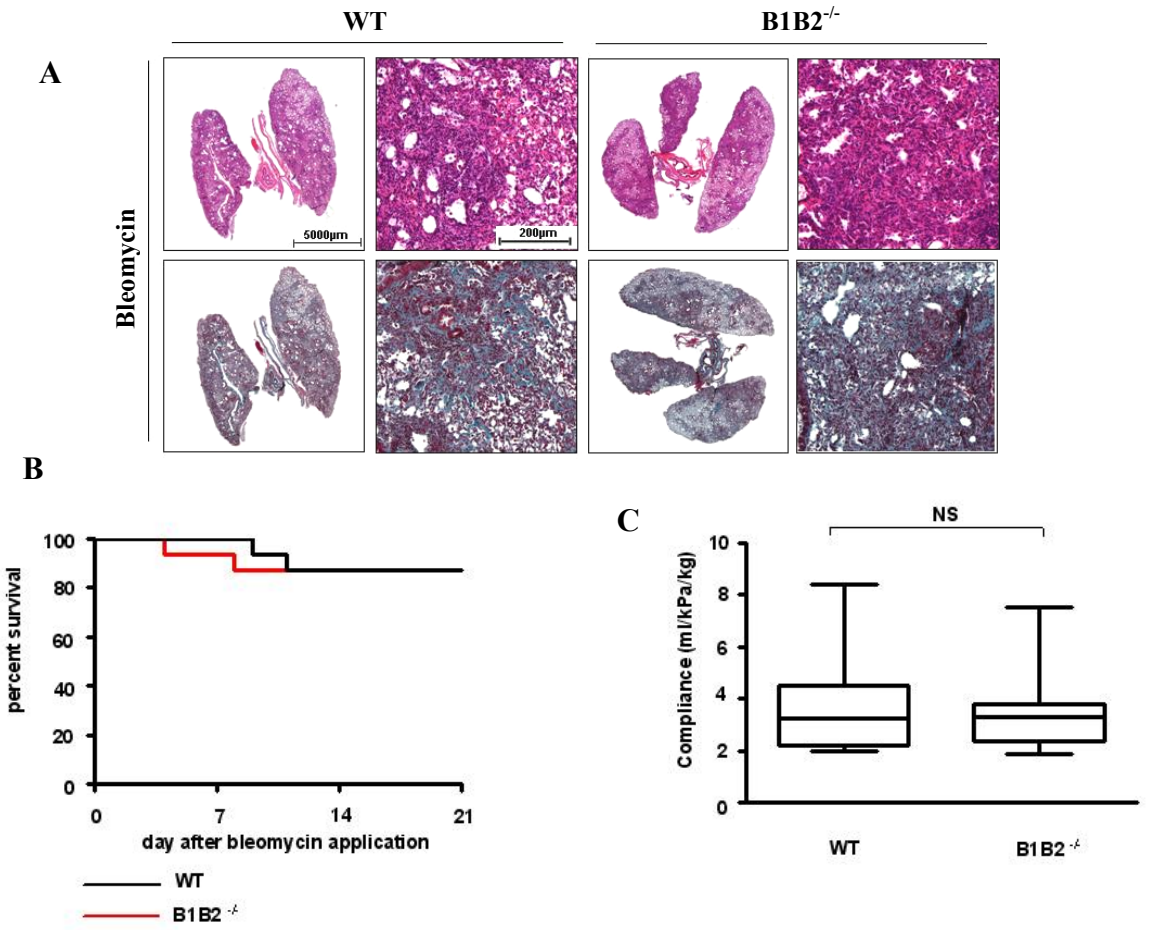


Figure 4.11. B1B2$^{-/-}$ mice are not protected against bleomycin-induced lung fibrosis.
(A) Hematoxylin and eosin (top) and Trichrom (bottom) staining of lung tissue sections of WT and B1B2$^{-/-}$ mice 21 days after bleomycin instillation. Green colour indicates collagen deposits. Representative lung tissue sections are demonstrated. Bar size is indicated. (B) Survival curve demonstrating mortality rate of WT and B1B2$^{-/-}$ mice after bleomycin challenge, n=20 mice/group. Statistical comparison by log-rank test, p=0.86. (C) Lung compliance at day 21 after bleomycin administration. Data are presented as box and whisker plots, in which the horizontal line within each box represents the median, the limits of each box represent the interquartile range and the whiskers represent the maximum and minimum values. n=20 mice/group; Mann-Whitney test. NS, not significant.

4.5. FXII stimulates proliferation of lung fibroblasts

FXII has been reported to act as mitogen for Hep2 cells, smooth muscle cells, ATII cells and endothelial cells (91). Taking into consideration that intensive fibroblast proliferation is a hallmark of lung fibrosis, it was of interest to check if FXII may control lung fibroblast proliferation as well. To answer this question murine lung fibroblasts were stimulated with an increasing concentration of FXIIa and [^{3}H]-thymidine incorporation was measured. The dose-dependent increase in DNA synthesis was observed after FXIIa

treatment (Figure 4.12A). FXII-induced proliferation was blocked by FXIIa specific inhibitor, CTI. Additionally, increased cyclin D expression after exposure to FXIIa confirmed FXIIa mitogenic activities toward murine lung fibroblasts (Figure 4.12B).

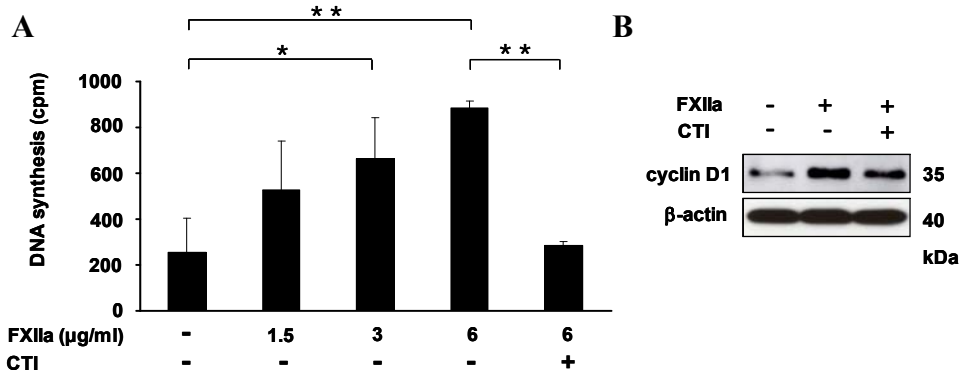


Figure 4.12. FXIIa stimulates proliferation of murine lung fibroblasts.
(A) [^{3}H]-Thymidine incorporation in murine lung fibroblasts exposed to various concentration of FXIIa in the absence or presence of CTI (12.6 µg/ml). Data represent mean values ± SD from three independent experiments, each performed in triplicate; ** $p < 0.005$; * $p < 0.05$; ANOVA, Tukey's post test. (B) Cyclin D1 expression in murine lung fibroblasts stimulated for 6 h with FXIIa (6 µg/ml) in the absence or presence of CTI (12.6 µg/ml). Data are representative of three independent experiments. ß-actin served as a loading control.

In order to investigate the contribution of different signal transduction pathways to FXIIa-induced proliferation of murine lung fibroblasts, MAPKs and Akt phosphorylation kinetics in response to FXIIa stimulation were analyzed. A marked increase in p38, p44/42 and Akt activity was visible after 15 min, whereas no phosphorylation of JNK and c-jun was observed. CTI attenuated p44/42 and Akt phosphorylation after 30 min of FXIIa exposure. After determination of the phosphorylation kinetics, it was next analyzed whether interference with these pathways would affect FXIIa-induced proliferation of murine lung fibroblasts. To assess this question, specific inhibitors of PI3K, MEK and p38 kinases (Wortmannin, PD98059, and SB203580, respectively) were used and their effect on FXIIa mitogenic activities was evaluated. As shown in Fig. 4.13 B inhibition of MEK activity by PD98059 led to reduction of murine lung fibroblast proliferation in response to FXIIa. No change in DNA synthesis was visible when cells were pretreated with inhibitors of PI3K and p38 kinases.

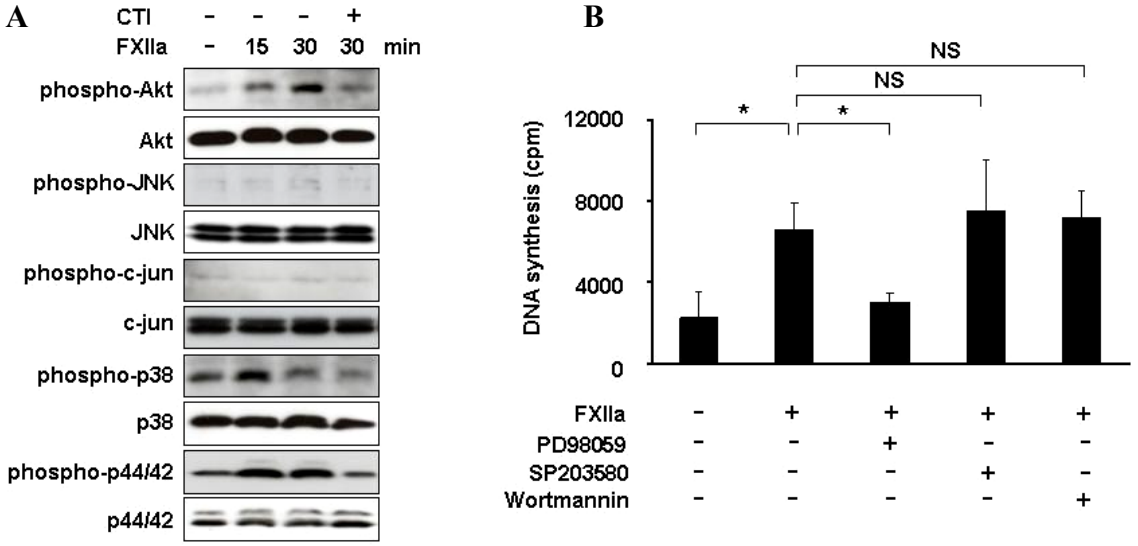
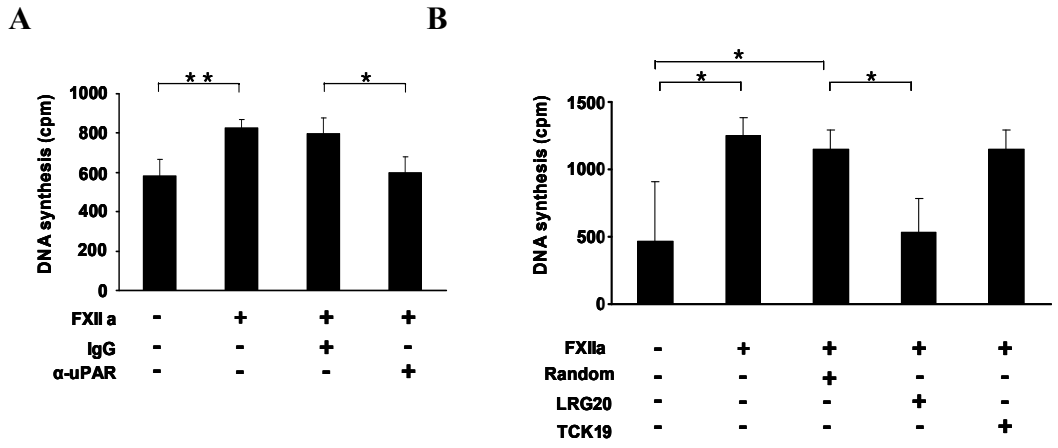


Figure 4.13. p44/42 kinase regulates FXII-induced proliferation of murine lung fibroblasts.
(A) Murine lung fibroblasts were treated for the indicated time points with FXIIa and the activity and expression of Akt, JNK, c-jun, p38 and p44/42 kinases were analyzed by Western blotting. Phosphoproteins were detected via phospho-specific antibodies as indicated. Equal loading was confirmed via pan-specific antibodies. Data are representative of three independent experiments. (B) [^{3}H]-thymidine incorporation in murine lung fibroblasts pretreated with MEK, p38, and PI3K kinases specific inhibitors (PD98059, SB203580 and Wortmannin, respectively) 1 h prior to FXIIa (6 µg/ml) stimulation. Data represent mean values ± SD from three independent experiments, each performed in triplicate;* $p < 0.05$; ANOVA, Tukey's post test. NS, not significant.

FXII has been reported to bind to the endothelial cell surface in complex with gC1qR, uPAR and CK1. Furthermore, there are studies which indicate that uPAR is involved in FXII-induced endothelial cell proliferation (191). To investigate whether uPAR mediates FXIIa mitogenic activities towards murine lung fibroblasts, cells were treated with an anti-uPAR blocking antibody prior to FXIIa stimulation. Cell proliferation, as measured by [^{3}H]-thymidine incorporation was blocked by an anti-uPAR antibody (Figure 4.14A). Moreover, experiments using peptides corresponding to uPAR's domain 2 confirmed the importance of this receptor for FXIIa mitogenic activities and revealed the potential FXII binding site on uPAR. Peptide LRG20 (position 166-185) from uPAR's domain 2 blocked an increase in DNA synthesis after FXIIa stimulation (Figure 4.14B). In comparison, peptide TCK from domain 2 (position 196-214) and random peptide had no effect on FXIIa-induced proliferation.



synthetic peptides from uPAR		
peptide	position	domain
LRG20	166-185	2
TCK19	196-214	2

Figure 4.14. uPAR mediates FXIIa-induced murine lung fibroblast proliferation.
(A) [^{3}H]-thymidine incorporation in murine lung fibroblasts pretreated with an anti-uPAR blocking antibody (5 µg/ml) or IgG isotype control antibody (5 µg/ml) prior to FXIIa (6 µg/ml) stimulation. Data represent mean values ± SD from three independent experiments, each performed in triplicate; ** $p < 0.005$; * $p < 0.05$; ANOVA, Tukey's post test. (B) [^{3}H]-thymidine incorporation in murine lung fibroblasts after exposure to FXIIa (6 µg/ml) in the presence or absence of peptides (7 µM) coresponding to domain 2 of uPAR (LRG20 or TCK19) or random peptide (7 µM). Data represent mean values ± SD from three independent experiments, each performed in triplicate; * $p < 0.05$; ANOVA, Tukey's post test.

To confirm earlier findings, murine lung fibroblasts from uPAR deficient mice ($uPAR^{-/-}$) were isolated and exposed to FXIIa. In contrast to WT cells, uPAR deficient lung fibroblasts did not respond to FXIIa (Figure 4.15A). These data indicate that uPAR is required for FXIIa mitogenic activities. This observation raised the question whether uPAR interacts with FXIIa protein. To investigate this issue, an immunoprecipitation assay was performed. For immunoprecipitation, lysates were prepared from cells either stimulated or unstimulated with FXIIa for 30 min. Using an anti-uPAR antibody FXIIa was immunoprecipitated from lysates of cells exposed to FXIIa (Figure 4.15B). uPAR did not immunoprecipitate with isotype control antibody.

Taken together, these results demonstrated that FXIIa induced murine lung fibroblast proliferation in a uPAR dependent manner. Based on the fact that uPAR has no kinase activity and does not directly interact with intracellular pathways, the question whether other proteins are involved in this mechanism was raised. It is unclear how glycosylphosphatidylinositol-anchored uPAR, which lacks a transmembrane structure, mediates signal transduction. It has been proposed that uPAR forms cis-interactions with

integrins as associated proteins in lipid rafts and thereby transduces proliferative or migratory signals to cells upon binding of its ligand, uPA (192). Studies using resonance energy transfer microscopy and co-immunoprecipitation with purified recombinant proteins indicate that uPAR forms complexes with a subset of β1- and β2-integrins (193) and modulates the signaling capacity of these molecules (194). Based on these reports and our own studies, showing increased adhesion of fibroblasts to fibronectin after FXIIa stimulation, it was investigated whether fibronectin receptor α5β1-integrin influences FXIIa mitogenic activities. Pretreatment of lung fibroblasts with anti-α5- or anti-β1-integrin blocking antibodies abolished [^{3}H]-thymidine incorporation after exposure to FXIIa, while the IgG control antibody had no effect (Figure 4.16). This observation suggests that α5β1-integrin is involved in FXIIa-induced murine lung fibroblast proliferation.

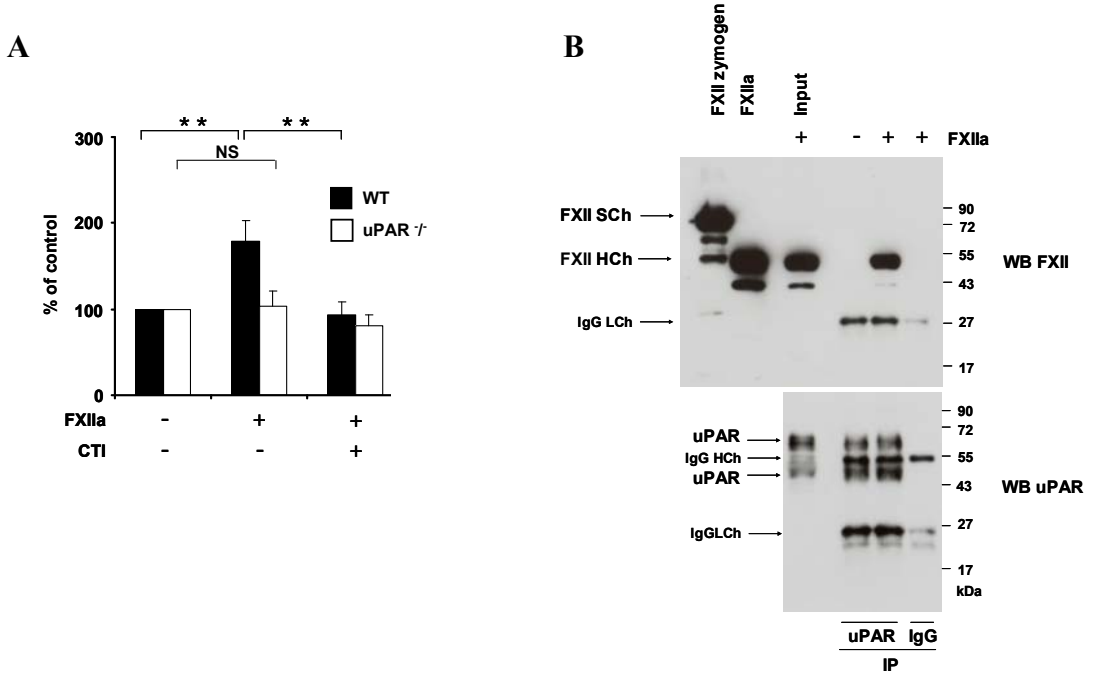


Figure 4.15. uPAR is required for FXIIa mitogenic activities.
(A) [^{3}H]-thymidine incorporation in murine lung fibroblasts isolated from WT or uPAR deficient (uPAR$^{-/-}$) mice after FXIIa (6 µg/ml) stimulation. Data represent mean values ± SD from three independent experiments, each performed in triplicate; ** $p < 0.005$; ANOVA, Tukey's post test. NS, not significant. (B) Interaction of FXII with uPAR. Antibodies specific for uPAR were incubated with lysates from cells either stimulated or unstimulated with FXIIa (6 µg/ml) for 30 min. Immunocomplexes were precipitated using protein A-agarose beads and analyzed by Western blotting using antibodies against FXII. uPAR served as a loading control. IgG LCh, IgG light chain; IgG HCh, IgG heavy chain. Data are representative of three independent experiments.

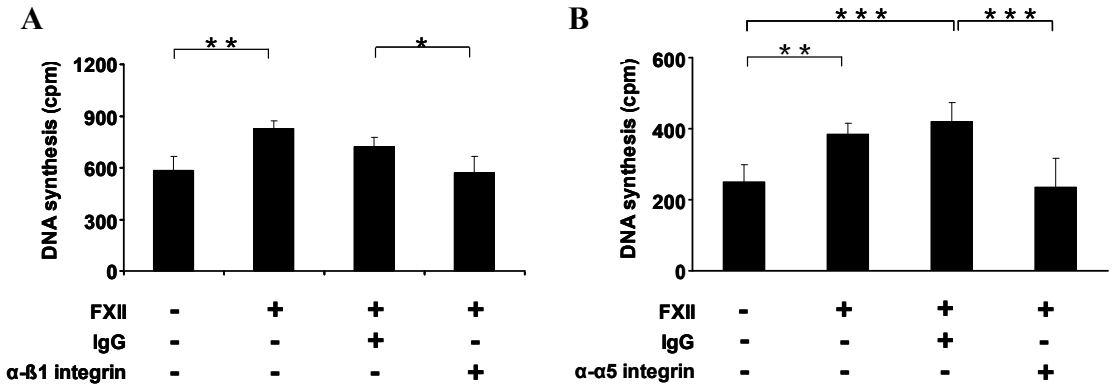


Figure 4.16. α5β1-integrin regulates FXIIa-mediated murine lung fibroblast proliferation.
(A) [^{3}H]-thymidine incorporation in murine lung fibroblasts pretreated with (A) anti-β1- or (B) anti-α5-integrin blocking antibodies (both diluted 1:200) or IgG isotype control (5 µg/ml) prior to FXIIa stimulation. Data represent mean values ± SD from three independent experiments, each performed in triplicate; *** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$; ANOVA, Tukey's post test.

4.6. TGF-β1 regulates FXII expression in human lung fibroblasts

4.6.1. TGF-β1 upregulates FXII mRNA and protein levels in HLF

Exposure of HLF to 10 ng/ml TGF-β1 stimulated the synthesis of FXII in a time dependent manner. Real time analysis demonstrated the strongest induction of FXII mRNA expression 4 h after treatment (Fig. 4.17A). The maximal FXII protein level was achieved within a 48 h stimulation period and slightly declined over 72 h (Fig. 4.17B, C). Immunofluorescence staining revealed pronounced expression of FXII in response to TGF-β1. FXII was detected on the cell surface (Fig. 4.17D, top panel), as well as in the cytoplasmic compartment of HLF (Fig. 4.17D, bottom panel). The purity of isolated HLF was verified by positive staining for fibronectin, vimentin, and collagen IV (Fig. 4.17E)

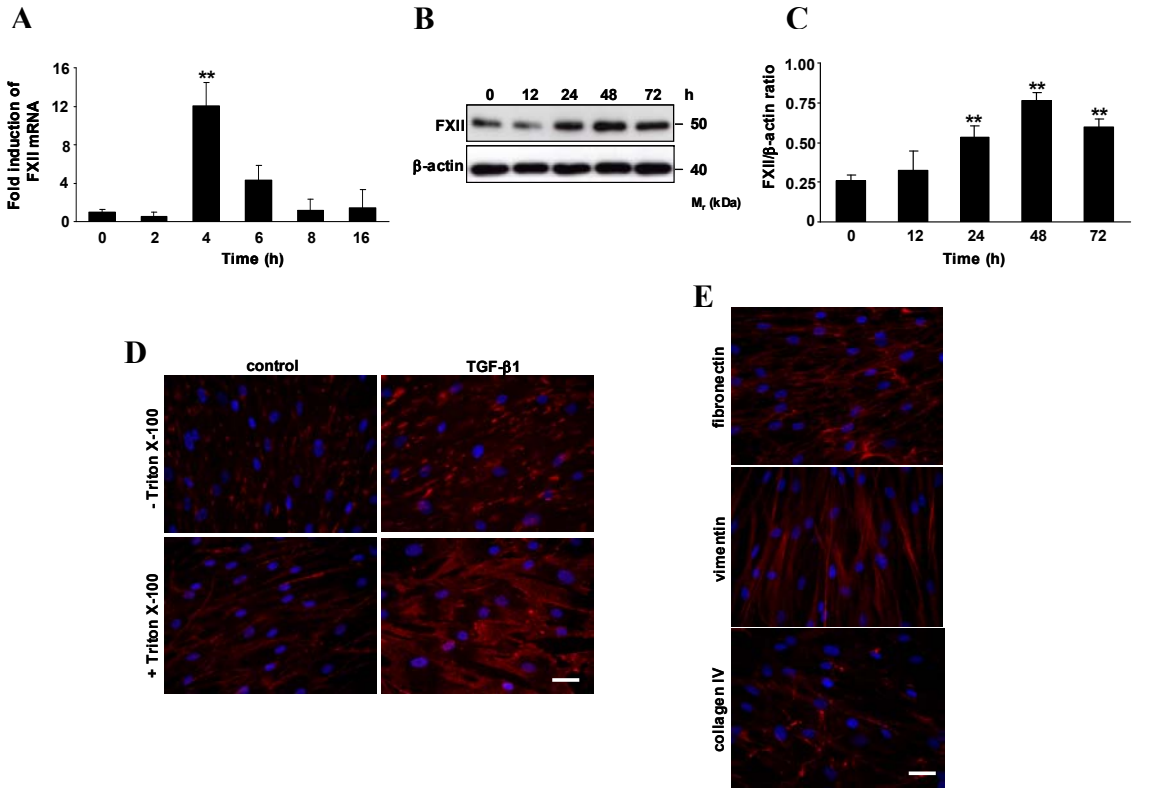


Figure 4.17. TGF-β1 upregulates FXII expression in HLF. (A, B) Time course of FXII expression in HLF following TGF-β1 stimulation as assessed by (A) real time PCR and (B) Western blotting. Real time PCR results are expressed as the fold-increase in FXII expression (normalized for β-actin expression) *versus* values obtained for unstimulated cells, and are mean ± SD; n = 3; ** $p < 0.01$; ANOVA, Tukey's post test. A representative blot out of three is illustrated. (C) Densitometric analysis of the blot presented in (B), ** $p < 0.01$; ANOVA, Tukey's post test. (D) Immunofluorescence for the detection of FXII in unstimulated or TGF-β1-treated HLF. Original magnification 40×/1.25-0.75 oil-objective. Bar size 10 μm. (E) Immunofluorescence staining for fibronectin, vimentin, and collagen IV. Original magnification 40×/1.25-0.75 oil-objective. Bar size 10 μm.

4.6.2. TGF-β1 induces phosphorylation of MAPK, Akt and Smad3

To dissect the contribution of different signal transduction pathways to TGF-β1-induced FXII production in HLF, MAPKs, Akt, and Smad phosphorylation kinetics in response to TGF-β1 stimulation were analyzed. Phosphorylation of p44/42 and p38 kinases reached a peak at 60 min and then gradually decreased. A marked increase in JNK activity was visible after 60 min, whereas enhanced phosphorylation of Akt was noted after 120 min (Fig. 4.18A). As expected, TGF-β1 induced rapid phosphorylation of Smad 3 with maximal response within 30-60 min (Fig. 4.18B). No activation of c-jun was detected (data not shown). Immunofluorescence analysis demonstrated TGF-β1 induced

translocation of phospho-Smad 3 to the nucleus (Fig. 4.18C). Accordingly increased levels of phospho-Smad 3 were observed in the nuclear extracts after TGF-β1 treatment (Fig. 4.18D). Lamin B was used as a loading control and tubulin was used to assess the purity of the nuclear fraction.

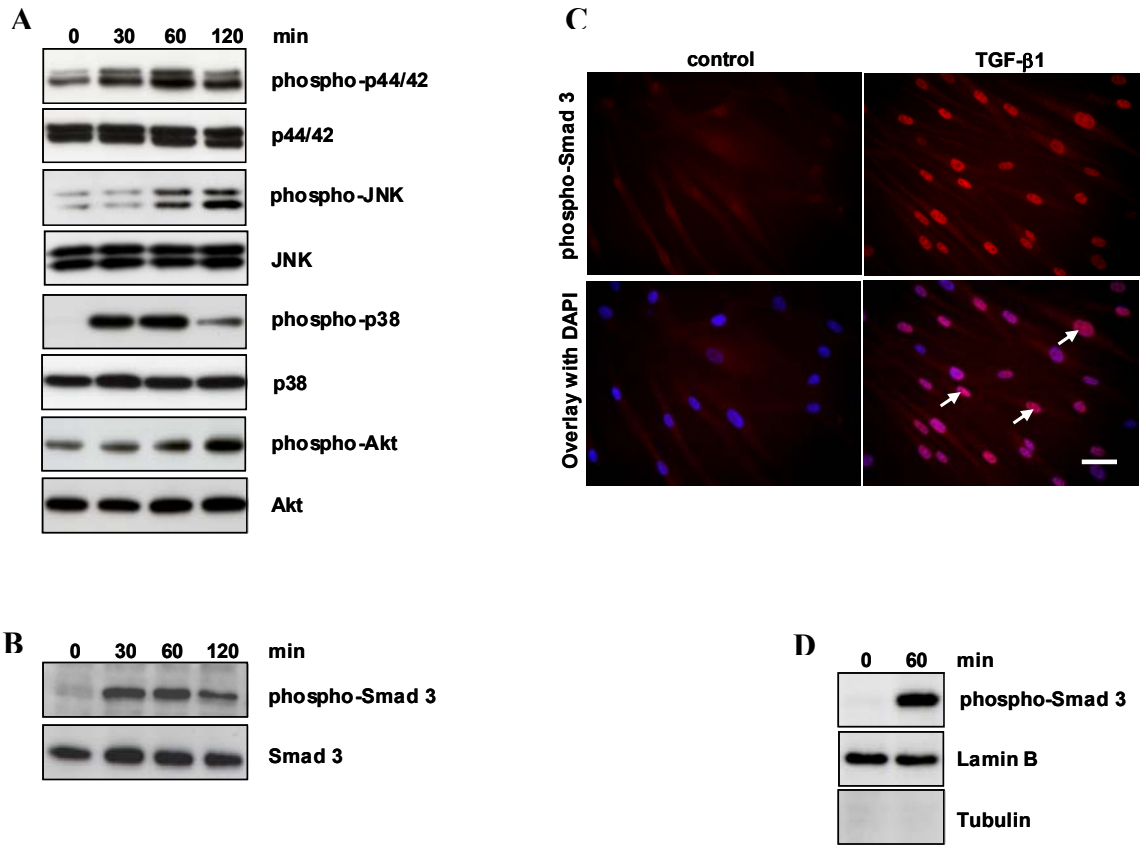


Figure 4.18. TGF-β1 induces phosphorylation of MAPK, Akt and Smad3. (A) HLF were treated for the indicated time points with TGF-β1 and the activity and expression of p44/42, JNK, p38 and Akt kinases were analyzed by Western blotting. Phosphoproteins were detected via phospho-specific antibodies as indicated. Equal loading was confirmed via pan-specific antibodies. Data are representative of four independent experiments. (B) Western blot analysis of Smad 3 phosphorylation and expression in TGF-β1 stimulated HLF. Data are representative of four independent experiments. (C) TGF-β1 dependent translocation of phospho-Smad 3 to the nucleus. HLF were incubated with TGF-β1 for 1 h then washed, fixed and stained with phospho-Smad 3 antibody. Arrows indicate nuclear localisation of Smad 3. Original magnification 40×/1.25-0.75 oil-objective. Bar size 10 µm. Data are representative of three independent experiments. (D) Western blot analysis of TGF-β1 driven translocation of phospho-Smad 3 to the nucleus. HLF were treated for 1 h with TGF-β1, nuclear extracts were prepared and immunoblotted with antibodies against phospho-Smad 3, lamin B, and tubulin. Lamin B was used as a loading control and tubulin was used to assess the purity of the nuclear fraction. Data are representative of three independent experiments.

4.6.3. Smad 3 and JNK kinase regulate TGF-β1-induced FXII expression in HLF

After determination of the phosphorylation kinetics, it was analyzed whether interference with these pathways would affect TGF-β1-induced FXII expression in HLF. To address this, specific inhibitors of TβRI, JNK, PI3K, MEK and p38 kinases (SB431542, SP600125, Wortmannin, PD98059, and SB203580, respectively) were used and their effect on TGF-β1-stimulated expression of FXII was evaluated. As depicted in Fig. 4.19A, B, inhibition of TβRI and JNK activity by SB431542 and SP600125, respectively, led to reduction of FXII expression in response to TGF-β1. No change in FXII expression was visible when HLF were pretreated with inhibitors of Akt, MEK, and p38 kinases. To further confirm these results, HLF were transfected with JNK1- or Smad 3-specific siRNAs, which caused significant knock-down of these proteins as demonstrated by Western blotting (Fig. 4.19C, D). As shown in Fig. 4.19E and G, knock-down of JNK1 resulted in inhibition of FXII expression after TGF-β1 stimulation. Similar results were obtained when Smad 3 was depleted (Fig. 4.19F, H).

4.6.4. JNK kinase does not regulate Smad3 phosphorylation and translocation to the nucleus

In next experiments the role of JNK kinase in Smad3 phosphorylation and translocation to the nucleus was investigated. Incubation of HLF with JNK inhibitor (SP600125) did not reduce Smad 3 phosphorylation, but completely abolished JNK activity (Fig. 4.20A). TβRI inhibitor (SB431542) attenuated phosphorylation of Smad 3 and JNK1 in response to TGF-β1, which indicated that phosphorylation of Smad 3 and JNK originates from TβRI, the most proximal molecule in TGF-β1 signal transduction pathway (Fig. 4.20B). Furthermore, exposure of HLF to SP600125 had no effect on TGF-β1-induced Smad 3 translocation to the nucleus, whereas SB431542 completely blocked this process (Fig. 4.20 C). Similar results were obtained by immunofluorescence analysis (Fig. 4.20D). SB431542 and SP600125 alone did not affect accumulation of Smad 3 in

the nucleus (data not shown). These results indicate that JNK kinase can regulate TGF-β1 induced FXII expression in the absence of any effects on phosphorylation and translocation of Smad 3 to the nucleus.

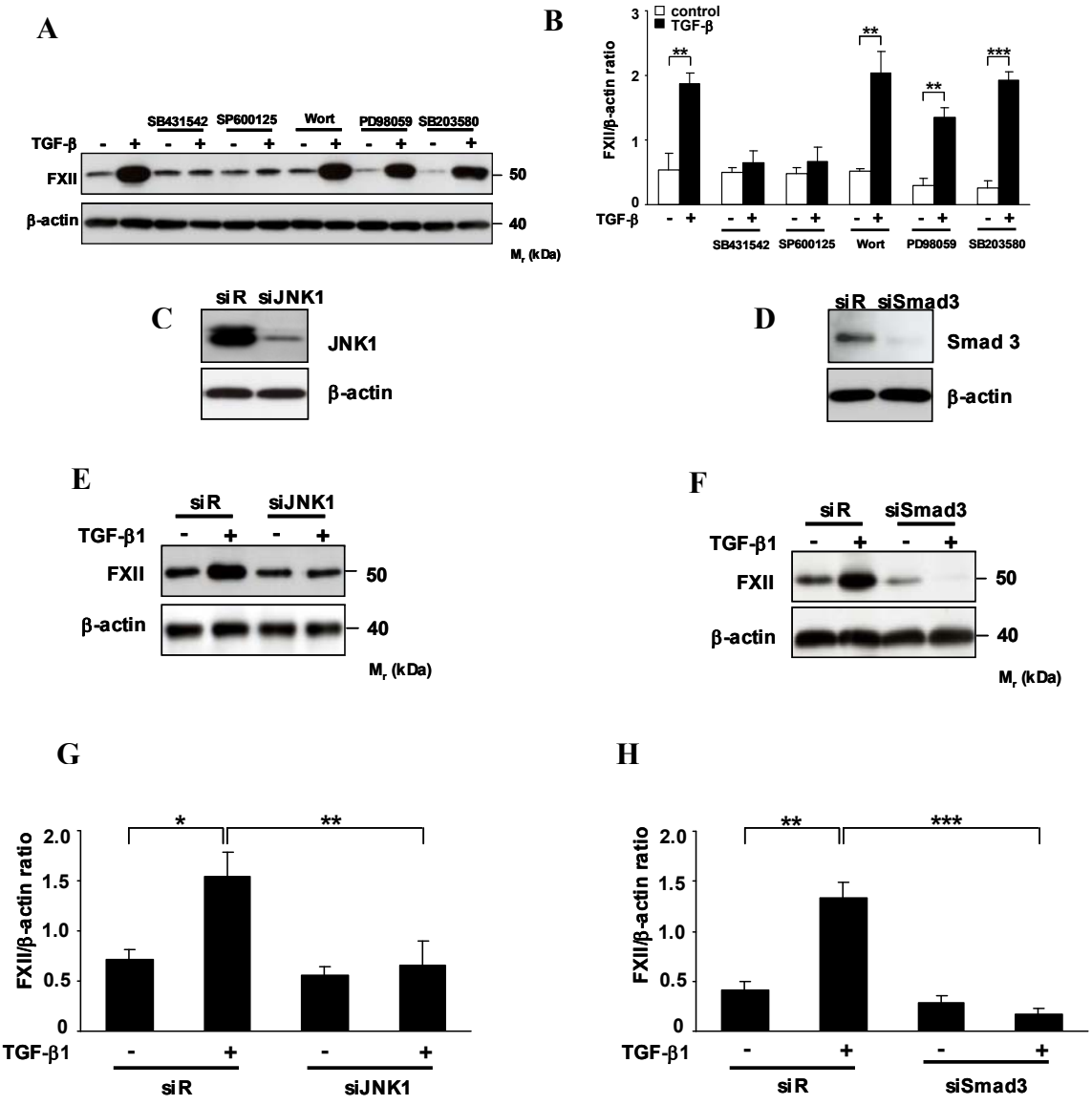


Figure 4.19. Smad 3 and JNK kinase regulate TGF-β1-induced FXII expression in HLF. (A) Western blot analysis of TGF-β1-induced FXII expression in HLF. HLF were treated with SB431542, SP600125, Wortmannin (Wort), PD98059, or SB203580 for 1 h prior to incubation with TGF-β1 for 48 h. Cell lysates were prepared and FXII expression was examined. β-actin was used as a loading control. A representative blot out of three is illustrated. (B) Densitometric analysis of the blot presented in (A); ** $p < 0.01$; *** $p < 0.001$; ANOVA, Tukey's post test. (C, D) Determination of knockdown efficiency in HLF by siRNA transfection against (C) JNK1 or (D) Smad 3 by Western blotting. Data are representative of three independent experiments. (E, F) Effect of (E) JNK1 or (F) Smad 3 knockdown on TGF-β1 induced FXII expression in HLF. Data are representative of three independent experiments. (G, H) Densytometric analysis of the blots presented in (E) and (F), respectively; * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$; ANOVA, Tukey's post test. siR, scrumble siRNA; wort, wortmannin.

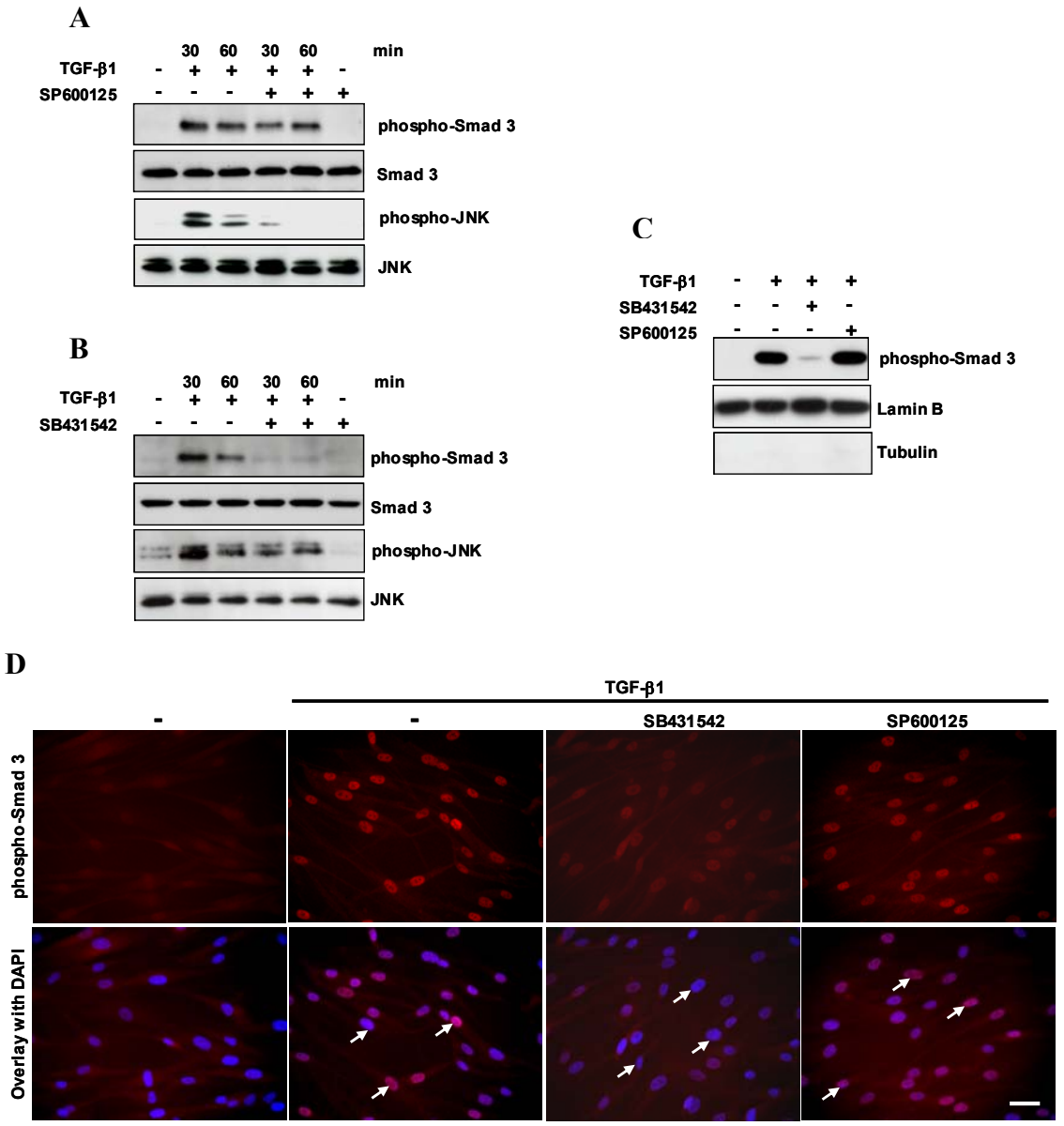


Figure 4.20. JNK1 kinase does not regulate Smad3 phosphorylation and translocation to the nucleus. (A, B) HLF were treated with TGF-β1 in the absence or presence of (A) JNK inhibitor (SP600125) or (B) TβRI inhibitor (SB431542), as indicated, and the activity and expression of Smad 3 and JNK were analyzed by Western blotting. Data are representative of four independent experiments. (C) Western blot analysis of TGF-β1 driven translocation of phospho-Smad 3 to the nucleus. HLF were pretreated with SB431542 or SP600125 and then either unstimulated or stimulated for 1 h with TGF-β1, nuclear extracts were prepared and immunoblotted with antibodies against phospho-Smad 3, lamin B, and tubulin. Lamin B was used as a loading control and tubulin was used to assess the purity of the nuclear fraction. Data are representative of three independent experiments. (D) TGF-β1 driven translocation of phospho-Smad 3 to the nucleus. HLF were preincubated with SB431542 or SP600125 1 h prior to addition of TGF-β1. After 1 h the cells were washed, fixed and stained with phospho-Smad 3 antibody. Arrows indicate nuclear localisation of Smad 3. Original magnification 40×/1.25-0.75 oil-objective. Bar size 10 µm. Data are representative of three independent experiments.

4.6.5. TGF-β1 induces FXII promoter activity via SBE located at position – 272

To identify DNA elements required for TGF-β1-induced FXII production, NIH3T3 cells were transiently transfected with a series of human FXII promoter deletion constructs (-1630 bp; -907 bp; -577 bp; -299 bp; Fig. 4.21A) and then luciferase activity in untreated and TGF-β1-treated cells was measured. NIH3T3 cells were used in these studies due to their high transfection efficiency. Cells transfected with pGL3-1630; pGL3-907; and pGL3-577 constructs displayed no increase in luciferase activity in response to TGF- β1, whereas strong induction of FXII promoter activity was observed in pGL3-299 transfected cells (Fig. 4.21B). These results indicate the presence of TGF-β1 responsive element within -299/+1 bp in the human FXII promoter. In addition, the data suggest that repressor element(s) located in the region upstream of -299 bp may dampen the stimulatory effects of TGF- β1.

To identify the TGF-β1-responsive element of the FXII promoter laying between -299/+1 bp, this DNA region was examined for consensus SBEs. This analysis identified the putative SBE at position -272 bp (SBE_{-272}, Fig. 4.21C). As expected, the point mutation of SBE_{-272} completely abolished FXII promoter activity in response to TGF-β1 (Fig. 4.21D). To confirm these results, a construct lacking SBE_{-272} was generated (Fig. 4.22A). The deletion of the sequence between -299 and -183 abrogated the ability of the FXII promoter to confer responsiveness to TGF-β1 (Fig. 4.22B).

Next, the role of JNK/Smad 3 signaling pathways in the regulation of FXII expression was tested by a gene luciferase activity assay. The cells were transfected with a pGL3-299 construct, pretreated with indicated inhibitors, and then either unstimulated or stimulated with 10 ng/ml TGF-β1. As depicted in Fig. 4.22C TGF-β1 driven luciferase activity was strongly reduced only in the presence of SB431542 and SP600125 inhibitors.

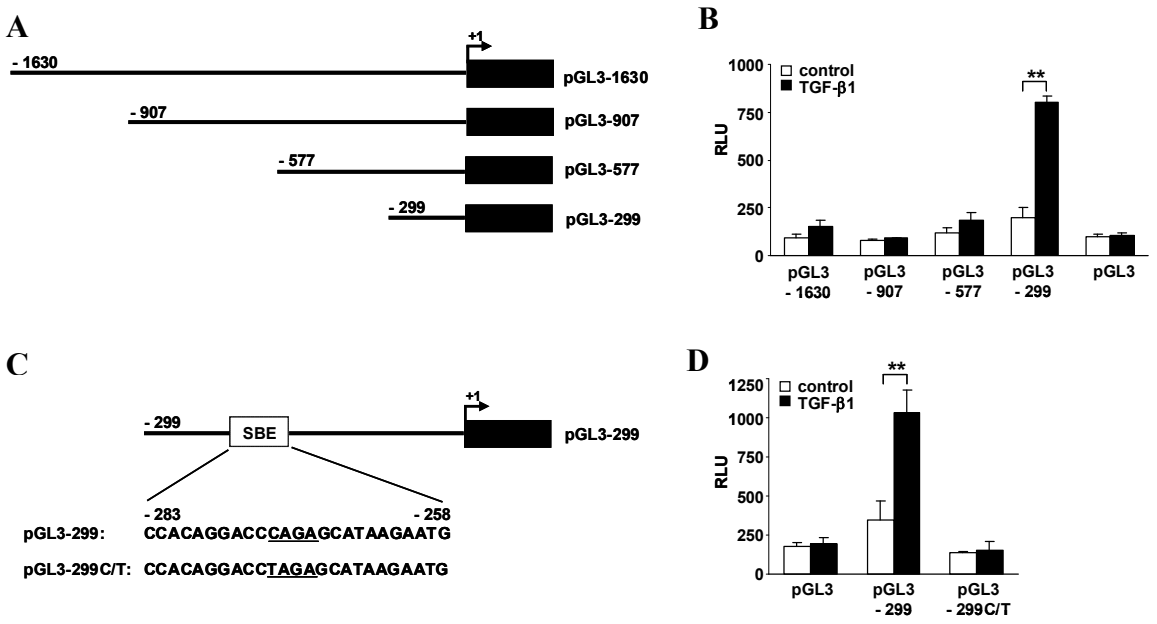


Figure 4.21. TGF-β1 induces FXII promoter activity via SBE located at position – 272. (A) Schematic representation of FXII promoter deletion luciferase reporter constructs. Angled arrow indicates the transcription start site. (B) NIH3T3 cells were transfected with the indicated FXII promoter deletion constructs and then either unstimulated (white bars) or stimulated with TGF-β1 (black bars). Luciferase activity was measured as described in "Materials and methods". Data represent mean values ± SD from four independent experiments, each performed in triplicate; ** $p < 0.01$. (C) Schematic representation of the FXII promoter region containing putative SBE at position -272. pGL3-299 C/T represents a construct in which the SBE_{-272} was mutated by the replacement of C residue at position -273 by T. (D) NIH3T3 cells were transfected with the pGL3-299 or pGL3-299 C/T constructs. Luciferase activity was determined in untreated (white bars) and TGF-β1-treated (black bars) cells. Data represent mean values ± SD from four independent experiments, each performed in triplicate; ** $p < 0.01$; ANOVA, Tukey's post test.

4.6.6. Smad 3 interacts with SBE_{-272} within the FXII promoter

To examine the interaction of Smad 3 with SBE_{-272} ChIP and streptavidin pull-down assays were performed. The ChIP assay clearly demonstrated TGF-β1-induced interaction of Smad 3 with the FXII promoter region (–299/+1 bp) flanking SBE_{-272} (Fig. 4.23A). To further analyze the binding of Smad 3 to SBE_{-272}, a streptavidin pull-down assay using biotinylated template spanning -283 and -258 bp region of FXII promoter was performed. As expected, Smad 3 was eluted from this template, whereas no interaction occurred when SBE_{-272} was mutated (Fig. 4.23B). These findings indicate that Smad 3 – SBE_{-272} form a complex after stimulation of HLF with TGF-β1.

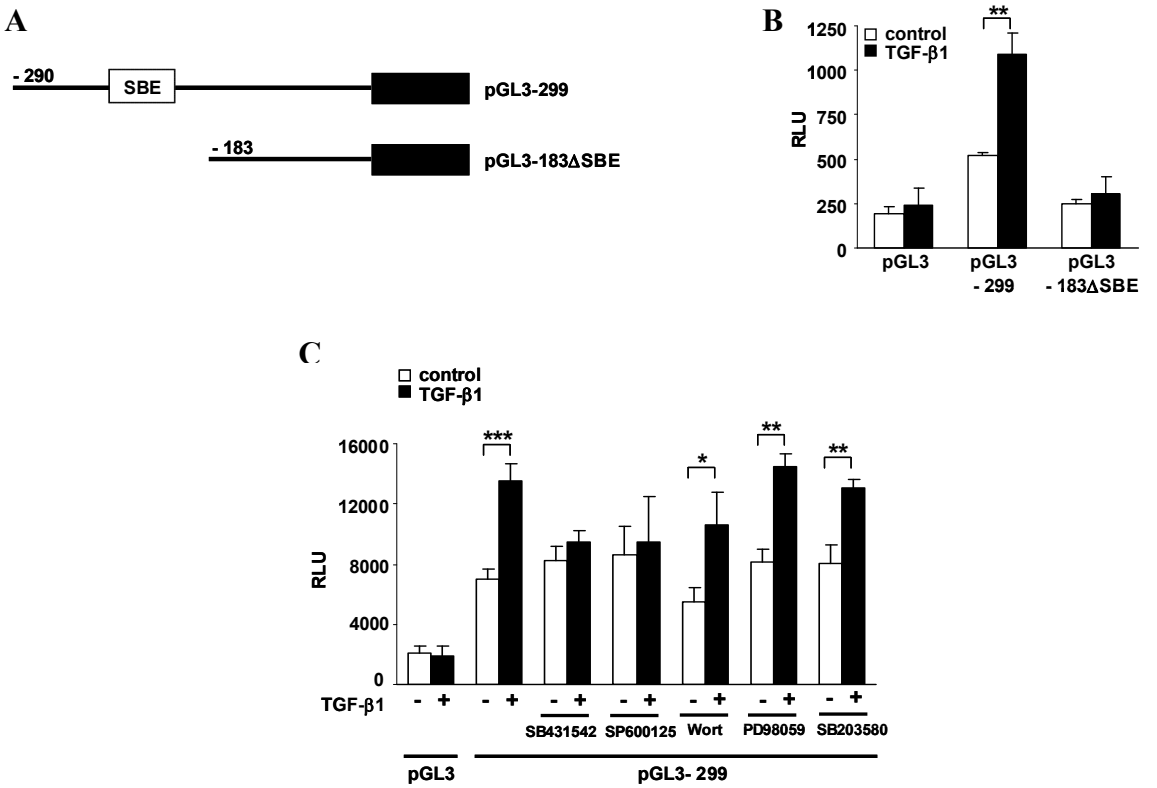


Figure 4.22. TGF-β1 induces FXII promoter activity via SBE located at position – 272.

(A) Schematic representation of pGL3-299 construct and a construct lacking SBE_{-272} (pGL3-183ΔSBE$_{-272}$). (B) NIH3T3 cells were transfected with pGL3-299 or pGL3-183ΔSBE$_{-272}$ and the luciferase activity was measured in unstimulated (white bars) and TGF-β1 stimulated (black bars) cells. Data represent mean values ± SD from three independent experiments, each performed in triplicate; ** $p < 0.01$; ANOVA, Tukey's post test. (C) NIH3T3 cells, transfected with pGL3-299 construct, were pretreated with SB431542, SP600125, Wortmannin (Wort), PD98059, or SB203580 for 1 h prior to incubation with TGF-β1. Luciferase activity was determined in untreated (white bars) and TGF-β1-treated (black bars) cells. Data represent mean values ± SD from three independent experiments, each performed in triplicate; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ANOVA, Tukey's post test.

4.6.7. JNK kinase affects binding of Smad 3 to SBE_{-272}

Since inhibition of JNK kinase did not have any impact on phosphorylation and translocation of Smad 3 to the nucleus, the involvement of this kinase in the formation of Smad 3 – SBE_{-272} complex was investigated by ChIP and streptavidin pull-down assays. HLF, pretreated with TβRI or JNK1 inhibitors (SB431542 or SP600125, respectively), were either unstimulated or stimulated with TGF-β1, lysed, and a ChIP assay was performed using an anti-Smad3 antibody and an IgG isotype control. TGF-β1 induced Smad 3-DNA complex formation. This interaction was completely abolished when SB431542 was used, and dramatically reduced in the presence of SP600125 (Fig. 4.23C).

SB431542 and SP600125 alone did not affect interaction of Smad3 with DNA (data not shown). Similar results were obtained when the streptavidin pull-down assay was performed (Fig. 4.23D).

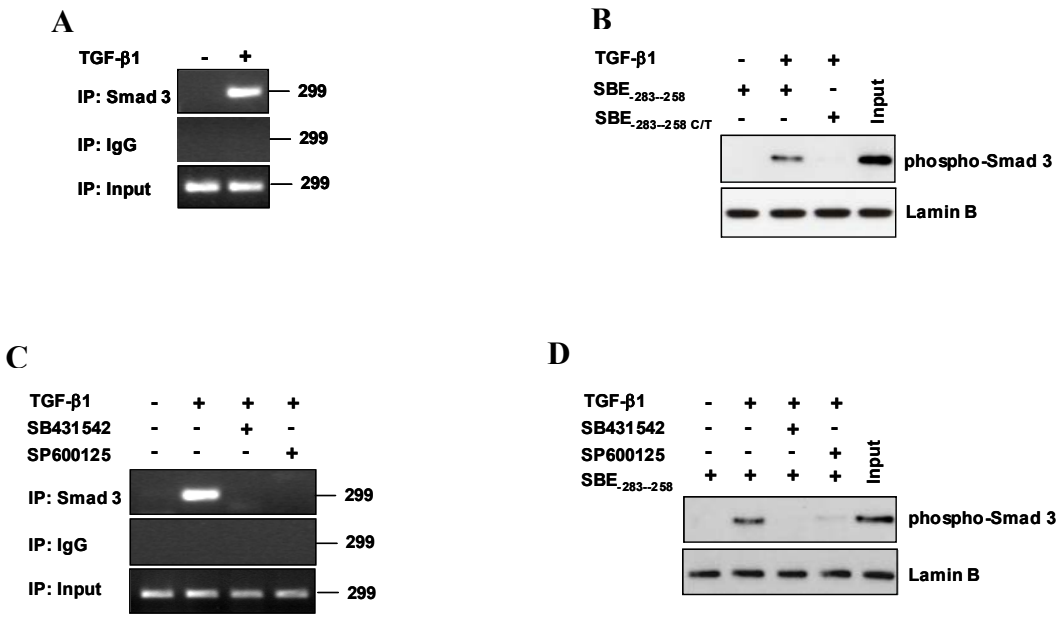


Figure 4.23. Smad 3 - SBE_{-272} interaction is suppressed in the presence of JNK inhibitor. (A) HLF were either unstimulated or stimulated with TGF-β1 and ChIP analysis was performed using a Smad 3 antibody or an isotype IgG control. PCR was performed with immunoprecipitated DNA as described in "Materials and methods". PCR products were separated by agarose gel electophoresis and detected by staining with ethidium bromide. Data are representative of three independent experiments. (B) Nuclear extracts from untreated or TGF-β1-treated cells were incubated with biotinylated templates ($SBE_{-283/-258}$ or $SBE_{-283/-258\ C/T}$) and Smad 3 was detected by Western blotting. Data are representative of three independent experiments. (C) HLF were pretreated with SB431542 or SP600125 for 1 h prior to incubation with TGF-β1. ChIP analysis was performed using Smad 3 antibody or isotype IgG control. PCR was performed with immunoprecipitated chromatin as described in "Materials and methods". Data are representative of three independent experiments. (D) HLF were preincubated with SB431542 or SP600125 for 1 h prior to addition of TGF-β1. Nuclear extracts were prepared and then incubated with a biotinylated template ($SBE_{-283/-258}$). Smad 3 was detected by Western blotting. Data are representative of three independent experiments.

5. Discussion

5.1. Expression of FXII, FXI and HMWK is elevated in lung fibrosis

It is well known that components of the coagulation and fibrinolysis pathways play important roles in the processes of tissue injury and repair (195). Coagulation mechanisms are activated at sites of inflammation, and extravascular fibrin formation commonly occurs at foci of tissue injury (196). There is accumulating evidence that increased expression and activation of the extrinsic coagulation pathway play pathophysiological roles in numerous disease settings, including acute and chronic lung injury (174).

To determine the potential role of the intrinsic coagulation pathway in human fibrotic lung disease, expression of FXII, FXI and HMWK in the lungs of patients with established IPF was examined. The expression of these factors on mRNA and the protein level was markedly elevated in IPF lung specimens, particularly from microdissected fibrotic regions. To identify the cell populations in the lung which contribute to increased content of FXII, FXI and HMWK in the alveolar compartment, the mRNA levels in ATII cells and lung fibroblasts were characterized. FXII expression in lung fibroblasts from IPF lungs was upregulated, while FXII mRNA was not detected in ATII cells. The regulation of FXII expression in lung fibroblasts will be discussed later. FXI and HMWK mRNA were expressed by lung fibroblasts and ATII cells. Neither fibroblasts nor ATII cells derived from IPF patients showed altered mRNA levels of FXI and HMWK. The lack of significant change in FXI and HMWK mRNA level in ATII and lung fibroblasts suggests that other cell populations, such as type I alveolar epithelial cells, alveolar macrophages or bronchial epithelial cells, may contribute to increased expression of FXI and HMWK in fibrotic lungs.

The presence of coagulation factors in the alveolar space in fibrotic lungs may also result from plasma spill-over through a leaky vessel barrier. Presented data give evidence that, next to the systemic circulation and the vascular bed, the resident lung cells are also capable of providing high amounts of FXII, FXI and HMWK. It has been generally accepted that the contact phase components are produced mainly by hepatocytes, and

then secreted into the blood stream. Nevertheless, FXI has been reported to be synthesized in the kidney and in the lung at a relatively high level in comparison to the liver. HMWK mRNA has been already detected in the kidney, pancreas, placenta and heart (149). The results showed that the lung is a source of intrinsic coagulation pathway factors as well. Similarly, extrinsic coagulation factors, namely TF and FX, have been found to be expressed in the lung. Different inflammatory cytokines and profibrotic growth factors are known to stimulate the expression of TF by several cell types, which leads to extrinsic coagulation pathway activation outside the blood stream (197,198). Animal studies employing inhalative bleomycin administration demonstrated that alveolar macrophages and – to a lesser extent – ATII cells are the main sources of locally produced TF in response to lung injury (199). FX was reported to be synthesized by human and murine fibroblasts in the areas of fibrotic lung tissue (200).

The enhanced procoagulant activity in BAL fluid in IPF patients has been attributed to increased expression of TF, which leads to extrinsic coagulation pathway activation and fibrin accumulation in the alveolar space (174). Similarly, this study showed the activation of the intrinsic coagulation pathway in the alveolar compartment of IPF patients due to increased FXII activity in the BAL fluid. Activation of FXII at this location may contribute to a procoagulatory state in IPF lungs through FXI activation, which results in fibrin formation in the lungs.

Elevated expression of FXII, FXI and HMWK in lung homogenate of IPF patients corresponds to their altered expression observed in the animal model of bleomycin-induced lung fibrosis. mRNA and protein levels of intrinsic coagulation pathway factors were increased at day 4 and 21 after bleomycin administration. The first week after bleomycin application is characterized by a strong inflammatory response, therefore the elevated expression of intrinsic blood coagulation factors at day 4 may result from microvascular leakage which occurs at this phase. From the second week onwards, inflammation is dramatically reduced and the fibrotic pathway takes over (171). The elevated mRNA and protein levels of FXI, FXII and HMWK at day 21 post bleomycin application suggest that resident lung cells may contribute to the synthesis of these factors. Moreover, murine fibroblasts and ATII cells were found to produce FXI and HMWK. In contrast to results in human lungs, FXII mRNA was detected neither in

murine lung fibroblasts nor in ATII cells. Therefore, it would be of interest to examine other cell types as a potential source of FXII production in murine lungs.

The activation of extrinsic coagulation pathways in fibrotic lungs has been reported to promote fibrin deposition and fibroproliferative processes. The present study demonstrates that the intrinsic coagulation pathway is activated in IPF lungs as well and may be involved in disease development. Furthermore, the evidence that resident lung cells are able to produce intrinsic coagulation pathway components was provided. However, the regulation of cell-specific expression and the contribution of the intrinsic coagulation system to pathomechanisms of lung fibrosis need further investigation.

5.2. Inhibition of FXIIa or knockout of FXII protects against lung fibrosis

Coagulation FXII is a serine protease that is capable of initiating blood coagulation under certain conditions. Upon binding to negatively charged surfaces, FXII becomes converted into a FXIIa, which exhibits serine protease-type activity in its carboxyl-terminal domain. FXIIa converts FXI to FXIa and PK into KLK. Consequently, FXI activation culminates in a series of proteolytic reactions, which results in thrombin generation and subsequent clot formation. The activation of KLK by FXII leads to BK generation and activation of fibrinolytic system. In the present study, it was hypothesized that increased expression and activation of FXII promote lung fibrosis. To prove this hypothesis, lung fibrosis in $FXII^{-/-}$ mice was induced by intratracheal administration of bleomycin. Characterization of the late fibrotic response to bleomycin injury revealed that total lung collagen accumulation was attenuated in $FXII^{-/-}$ mice compared to WT animals. Severe fibrosis after bleomycin instillation was strongly reduced in $FXII^{-/-}$ mice and the survival of $FXII^{-/-}$ mice was significantly improved.

FXIIa activates FXI, which leads to thrombin and fibrin generation. Thus, FXII deficiency might result in impaired fibrin generation due to lack of FXI activation. To investigate this possibility fibrin staining in $FXII^{-/-}$ and WT mice was performed. Fibrin accumulation in the fibrotic regions of lungs after bleomycin instillation was comparable in WT and $FXII^{-/-}$ mice. These findings indicate that FXII deficiency did not affect fibrin generation in bleomycin-injured lung tissue. These data suggest that the extrinsic

coagulation pathway accounts for fibrin deposition in lung tissue. Additionally, fibrin deposition in injured lungs of $FXII^{-/-}$ mice may result from activation of FXI by thrombin in the absence of FXII.

The strong reduction of lung fibrosis in $FXII^{-/-}$ mice suggests that the application of FXIIa inhibitor may have beneficial effects as a treatment in lung fibrosis. To reveal this hypothesis CTI, a specific FXIIa inhibitor, was administered intratracheally to bleomycin-treated mice. The application of CTI was found to improve survival, to reduce collagen content and to preserve lung structure after bleomycin challenge. The significant decrease in the grade of lung fibrosis in mice treated with CTI compared with mice treated with saline provides evidence of the possible therapeuthic outcome of CTI in experimental fibrosis.

The exact mechanism of FXIIa inhibition by CTI, a 12 kDa polypeptide derived from corn seeds, is not known. Intratracheal administration of CTI seems to have side effects as an influx of inflammatory cells to the alveolar compartment in the control animals was noticed after CTI instillation. Observed side effects may result from the activation of immune response by CTI and may restrict therapeutic application of this inhibitor in the treatment of lung fibrosis. These findings led to the administration of another FXIIa inhibitor, PCK, to bleomycin-treated mice. PCK is the active-site-directed inhibitor of FXIIa and KLK (93,94). PCK was administered intratracheally and showed no side effects in the control animals which received saline instead of bleomycin. However, PCK instillation failed to attenuate the development of lung fibrosis in bleomycin-treated animals. The discrepancy between results obtained with CTI and PCK inhibitor could be related to the ability of PCK to block KLK activity. The role of KLK in the development of lung fibrosis has never been studied. KLK is an activator of u-PA and therefore may potentiate plasmin formation. It is possible that the inhibition of KLK activity promotes fibrosis development by attenuation of fibrinolysis and by potentiation of fibrin deposition in the lungs. The dosis of PCK used in our study might be too low to inhibit FXII activity in the lung and to limit the fibrotic response. Studies with a higher PCK dose would be required to determine PCK effects on the development of bleomycin-induced lung fibrosis.

In conclusion, the findings of the present study suggest that FXIIa inhibition has a therapeutic influence on lung fibrosis development. Multitargeting anticoagulants, such

as warfarin and unfractioned heparin or inhibition of FXIa, are associated with the risk of bleeding complications. Taking into consideration that FXII deficient patients have no bleeding disorders, therapeutic approaches for lung fibrosis based on FXIIa inhibition may be beneficial and free of bleeding complications.

5.3. FXII-induced proliferation of murine lung fibroblasts may contribute to lung fibrosis development

The altered alveolar haemostatic system may contribute to the pathogenesis of fibrotic lung diseases by other mechanisms than fibrin generation. This observation is evidenced by a recent report, which showed that pulmonary fibrosis develops in fibrinogen-null mice after bleomycin administration. Besides their role in fibrin formation FXa and the TF-FVIIa complex exhibit cellular activities that contribute to the development of lung fibrosis. Thrombin and FXa stimulate fibroblast proliferation, procollagen production and the expression of profibrotic and proinflammatory cytokines (183-186).

Different mechanisms may explain FXII-associated profibrotic activities. Firstly, FXII may potentiate alveolar procoagulant activities by FXIa generation which leads to fibrin formation. Nevertheless, the physiological role of FXII in haemostasis is questioned, since FXII deficient patients have no bleeding disorders. Reports showing that FXI can be activated independently of FXII by thrombin (18) suggest that FXII promotes lung fibrosis by a mechanism independent of its procoagulant activities. Finally, activation of the extrinsic coagulation pathway has been observed in alveolar compartments of fibrotic lungs. The results presented indicate that the role of FXII in the development of lung fibrosis is not necessarily related to its procoagulant activities.

FXII displays a wide variety of important functions apart from its ability to generate FXIa. First of all, FXIIa initiates KLK activation, which leads to BK formation. BK exhibits proinflammatory and vasodilatory activities by two G-protein-coupled receptors, designated B1 and B2 (60). To evaluate the functional role of BK in the development of bleomycin-induced lung injury, mice lacking both BK receptors ($B1B2^{-/-}$) were treated with bleomycin. Similarly to WT animals, $B1B2^{-/-}$ mice developed strong

fibrosis after bleomycin instillation, as demonstrated by morphological analysis and compliance measurements. These results clearly demonstrate that BK and its receptors do not play a pivotal role in bleomycin-induced lung fibrosis and do not mediate FXII profibrotic effects.

Interestingly, besides its role in the activation of the intrinsic coagulation pathway and the KLK-kinin system, FXII exhibits mitogenic activities. FXII/FXIIa has been reported to stimulate proliferation of some cell types in a MAPK/ERK-dependent manner. The physiological significance of FXII-induced cell proliferation is unknown. Mitogenic activities of FXII may play a role in fetal lung and liver development, since fetal hepatocytes and fetal ATII cells were found to responsd to FXII/FXIIa (90). Additionally, FXII may be involved in vessel formation since it increases proliferation of smooth muscle cells and endothelial cells (90).

Intensive proliferation of fibroblasts and their differentiation into myofibroblats is a hallmark of IPF (151). These observations raise the possibility that FXII may contribute to the development of lung fibrosis by stimulating proliferation of lung fibroblasts. In line with this consideration, an increase in lung fibroblast proliferation after exposure to FXIIa was observed. The concentration of FXII required to induce cell proliferation (3-6 µg/ml) was below FXII plasma concentration. These results strongly suggest that FXIIa potentiates lung fibrosis via stimulation of lung fibroblast proliferation. Similarly, other coagulation proteases such as thrombin and FXa stimulate fibroblast proliferation in a PAR-1-dependent manner (184,185). A potential role of FX- and thrombin-induced fibroblast proliferation in lung fibrosis is further underscored by the recent finding that PAR-1 deficient mice are protected against bleomycin-induced lung fibrosis (188).

The involvement of the p44/42 signaling pathway in FXII-induced cell proliferation has already been reported. The results of the present study demonstrate that exposure of murine lung fibroblasts to FXIIa leads to rapid activation of various kinases including p44/42, Akt, and p38 in murine lung fibroblasts. The data demonstrated that p44/42 activation was essential for FXIIa mitogenic activities since PD98059 attenuated proliferation of lung fibroblasts after FXIIa stimulation. The blockade of PI3K and p38 kinases did not affect the induction of DNA synthesis by FXIIa, which indicates that activation of these kinases is not required for the enhancement of lung fibroblast proliferation by FXIIa.

The FXII receptor which would be able to transduce mitogenic activities is not known. Interestingly, FXII has been reported to assemble on the endothelial cell surface through a multiprotein complex consisting of gC1qR, uPAR and CK1 (83). uPAR has been showed to play a role in a number of biological processes including pericellular proteolysis, inflammation, angiogenesis, matrix remodelling during wound healing, tumor invasion or metastasis (201). The cellular responses activated by binding of u-PA to uPAR require transmembrane signaling. Since uPAR has no intracellular domain, uPAR responses are mediated by direct contacts of uPAR with a variety of extracellular proteins and membrane receptors, such as integrins, EGF receptor, caveolin and others. As a result of these interactions, uPAR triggers the activation of signaling pathways such as tyrosine- and serine-protein kinases, Src, focal adhesion kinase (FAK), Rac, ERK/MAPK and JAK/STAT pathways. uPAR has been reported to mediate FXII growth factor activities towards endothelial cells (191). The results indicate an essential role of uPAR in FXII-induced proliferation of primary murine lung fibroblasts as well. The increase in cell proliferation after exposure to FXIIa was blocked by an anti-uPAR blocking antibody and peptides corresponding to a sequence of uPAR in domain 2. Additionally, murine lung fibroblasts deficient in uPAR did not respond to FXIIa stimulation. Moreover, in immunoprecipitation studies, interaction between uPAR and FXIIa was demonstrated. Collectively, to the best of my knowledge for the first time, the interaction of FXIIa with uPAR on the surface of murine lung fibroblasts was evidenced.

Since uPAR has no kinase activity and does not directly interact with intracellular pathways, further studies were designed to determine the mechanism for outside-in signaling triggered by FXIIa. The presented studies showed that blocking antibodies against β1- and α5- integrins attenuated FXII–induced proliferation of murine lung fibroblasts. This finding indicates that α5β1-integrin is required for FXIIa mitogenic activities. Presumably, FXIIa-induced proliferation may occur as a consequence of FXIIa binding to uPAR, the subsequent binding of FXII/uPAR complex to α5β1-integrin and signal transduction through α5β1-integrin. Similarly, uPA binding to its receptor on the Chinese hamster ovary cell surface has been reported to trigger uPAR binding to α5β1-integrin, and subsequent cell adhesion and migration (202). Further investigations using immunoprecipitation studies are needed to define possible interactions sites between uPAR and α5β1-integrin after exposure to FXIIa.

It would be of interest to determine whether the FXII mitogenic activity depends on FXII proteolytic activity. Although CTI, the specific FXIIa inhibitor, was shown to block FXII mitogenic function, the precise mechanism of FXII inhibition by CTI is not characterized. CTI binds both FXII and FXIIa and probably blocks FXII activity by changing its conformation. The altered conformation of FXII might interfere with FXII binding to uPAR. Therefore, further studies conducting other FXII inhibitors and FXIIa blocking antibodies are needed to clarify the role of FXII protease activity in the regulation of cell proliferation**.**

The presented data strongly suggest that FXIIa-induced proliferation may play a role in the development of bleomycin-induced lung fibrosis. The findings are supported by reports which show that other coagulations factors, namely FX and TF, act as mitogens for lung fibroblasts. Moreover, inhibition of FXII by CTI and FXII-deficiency attenuated bleomycin-induced pulmonary fibrosis in mice. Thus, strategies aimed at blocking FXII mitogenic activity in fibrotic lung disease may represent new treatment opportunities. Further studies focusing on the molecular mechanism responsible for the growth factor-like function of FXII are necessary to design novel approaches to inhibit the profibrotic effects of FXII without compromising blood coagulation.

5.4. Regulation of FXII expression in human lung fibroblasts

Factor XII is believed to participate in a number of physiologial processes including inflammation, coagulation and fibrinolysis (60). The previous reports demonstrated that FXII is mainly produced by the liver and its expression is regulated by estrogen (203). The FXII promoter contains an ERE that mediates 17β estradiol-stimulated induction of FXII gene expression (143-145). Moreover, HAE type III, characterized by increased activity of FXII, appears to be correlated with high estrogen levels (104).

Since the expression of FXII was also documented in other organs such as the lung and the placenta (149), the present study elucidates FXII production in the lung and the molecular mechanism underlying the regulation of its sythesis in primary human lung fibroblasts (HLF). It was demonstrated, to the best of my knowledge for the first time, that FXII is produced by HLF and that its expression is controlled by TGF-β1. Moreover,

the requirement of JNK and Smad3 signaling pathways for TGF-β1 driven FXII synthesis in HLF was shown. The present results are supported by a recent report showing the importance of JNK kinase in TGF-β1 induced expression of connective tissue growth factor (CTGF) in HLF (204).

The involvement of different signaling components, including p44/42, Akt, p38, and JNK, in the transcriptional induction of TGF-β1 target genes has already been demonstrated. TGF-β1-mediated activation of p38 kinase was found to be essential for mammary epithelial cell apoptosis, although it was not sufficient for the epithelial to mesenchymal transdifferentiation (205). Involvement of p44/42 and Akt signaling pathways has been implicated in TGF-β1 driven induction of PAI-1 expression in endothelial and mesangial cells, respectively (206,207). The present study demonstrates that the blockade of p44/42, Akt, p38 kinases did not affect the induction of FXII expression by TGF-β1, indicating that activation of these kinases is not essential for the enhancement of FXII expression in HLF. The crucial role of JNK kinase is further confirmed by a luciferase reporter assay, where preincubation of HLF with JNK specific inhibitor (SP600125) completely abolished TGF-β1 stimulated FXII promoter activity. Surprisingly, JNK activity was not required for Smad 3 phosphorylation and translocation to the nucleus in response to TGF-β1. The presented study is in contrast to previously published data demonstrating TGF-β induced JNK-dependent phosphorylation of Smad 3 and its accumulation in the nucleus (208). The reason for this discrepancy is not clear; however, some differences in the experimental procedure, such as a choice of cell type, may be of importance. The lack of any impact of JNK kinase on Smad 3 phosphorylation and translocation to the nucleus indicates that this kinase may target other transcription factors/coactivators which, together with Smad 3, could regulate FXII transcription. To support this hypothesis, a strong reduction of Smad 3-DNA complex formation was noted when HLF were preincubated with SP600125 prior to addition of TGF-β1. Several transcription factors/coactivators, such as AP-1 (209), Sp-1 (210), IRF-7 (211) or CBP/p300 (212), were found to be able to interact with Smad molecules, therefore it is well imaginable that JNK kinase may control interaction of Smad 3 with other proteins and thus enhance TGF-β1 mediated FXII production. The role of p44/42 and Akt kinases in the modulation of DNA binding activities and transcriptional potential has already been demonstrated in other systems (207,213,214). Therefore, further efforts are needed

to clarify the composition of transcriptional machinery which is responsible for TGF- β1-induced FXII expression in HLF.

TGF-β1 induction of FXII gene transcription was further investigated by the generation of a series of FXII promoter luciferase reporter constructs. Transient transfection of NIH3T3 cells with these constructs revealed the requirement of the sequence spanning -299 and +1 bp for TGF-β1 driven FXII expression. Further analysis of this promoter region demonstrated the presence of the SBE containing the consensus sequence at position -272. This is in line with previously published reports showing the interaction of the MH1 domain of receptor associated Smad or common-mediator Smad with G/C rich sequences of DNA, termed CAGA boxes (215,216). The mutation or deletion of SBE_{-272} further underscored its importance for the TGF-β1 mediated FXII expression in HLF. In addition, using several independent approaches, direct interaction of Smad 3 with SBE_{-272} was demonstrated. Interestingly, lower TGF-β1 inducibility was observed when longer portions of FXII promoter were studied indicating the presence of repressor element(s) located upstream of -299 bp, a fact that is common to other inducible promoters as well (217). In agreement with this contention, the basal luciferase activity of the pGL3-299 construct was also elevated. Detailed studies are required to map the specific repressor binding sequence(s) within the FXII promoter.

The TGF-β1-induced expression of FXII may play a potentially important role in haemostasis and cell functions under various pathophysiological conditions. TGF-β1 is a multifunctional cytokine that is critically involved in several disease states, particularly in fibrosis of the lung (159,218), kidney (219,220) and liver (221). Recent studies suggest that induction of TF (160) and PAI-1 (222-224) by TGF-β1 may play a crucial role in the profibrotic action of this growth factor. In lung fibrosis severe alterations of the alveolar haemostatic balance favouring extravascular pulmonary fibrin accumulation have been observed. Increased alveolar procoagulant and antifibrinolytic activities under these conditions have been attributed to TF/FVII-induced activation of the extrinsic coagulation cascade and increased PAI-1 expression (162,174). Moreover, fibroblasts have been identified as an important cellular source of TGF-β1 mediated TF (160) and PAI-1 expression (225). This study adds relevant information in this regard since it demonstrates that TGF-β1 can induce expression of FXII in HLF as well. Thus, not only

proteins belonging to the extrinsic coagulation cascade but also those of the contact phase may well contribute to the shift in the alveolar haemostatic balance and subsequent fibrin accumulation in the injured lung. In line with these considerations, presented data indicate that proteins of the contact phase are highly expressed in lungs of patients with IPF. Furthermore, the presently described induction of FXII by TGF-β1 may well contribute to other pathological conditions which have been linked to TGF-β1 mediated alterations in the coagulation and fibrinolysis systems, such as obesity (198) and insulin resistance (226), neovascular ocular diseases (227) or peritoneal adhesion formation (228-230).

This study demonstrates that FXII acts as a novel mitogen for murine lung fibroblasts. Elevated expression of TGF-β1 in lungs of IPF patients may potentiate synthesis of FXII by lung fibroblasts. Consequently, FXII may stimulate proliferation of lung fibroblasts and subsequently promote the development of lung fibrosis. Thus, FXII mitogenic activities toward lung fibroblasts could mediate and enhance the fibroproliferative effects of TGF-β1. Therefore, understanding the mechanisms regulating the expression of FXII will be of importance to inhibit lung fibrosis and other fibrotic disorders. Moreover, it has been shown that FXII may exhibit growth factor activities towards HepG2 cells, aortic smooth muscle cells, epithelioid carcinoma cells (A431), bovine newborn aortic endothelial cells, fetal hepatocytes or ATII pneumocytes (90,91). Therefore, in addition to its role in the haemostatic system, FXII may act as a signaling molecule for other growth factor-sensitive cells to regulate cell growth, proliferation, and/or differentiation processes under physiological as well as pathological conditions.

In conclusion, the presented results demonstrate that TGF-β1-induced FXII production in HLF is mediated by JNK and Smad 3 signaling pathways. Moreover, SBE at position -272 bp within FXII promoter was identified and the importance of JNK activity in the Smad 3 – DNA complex formation was showed. These findings provide new insights into the molecular mechanism responsible for the regulation of FXII expression in HLF and implicate its possible role in IPF, characterized by elevated TGF-β1 levels and disregulated haemostasis.

The present study demonstrated increased expression of FXII in the lungs of IPF patients and bleomycin challenged mice. Furthermore, FXIIa was identified as a novel

mitogenic factor for murine lung fibroblasts. It was found that treatment of HLF with TGF-β increases FXII expression. Thus, elevated level of TGF-β1 in fibrotic lungs may cause the upregulation of FXII expression in IPF lungs. FXII may contribute to increased proliferation of lung fibroblasts and subsequently to the development of pulmonary fibrosis (Figure 5.1). Thus, FXII and its downstream signaling pathway in lung fibroblasts should be considered as a novel target for therapeutic interventions in pulmonary fibrosis.

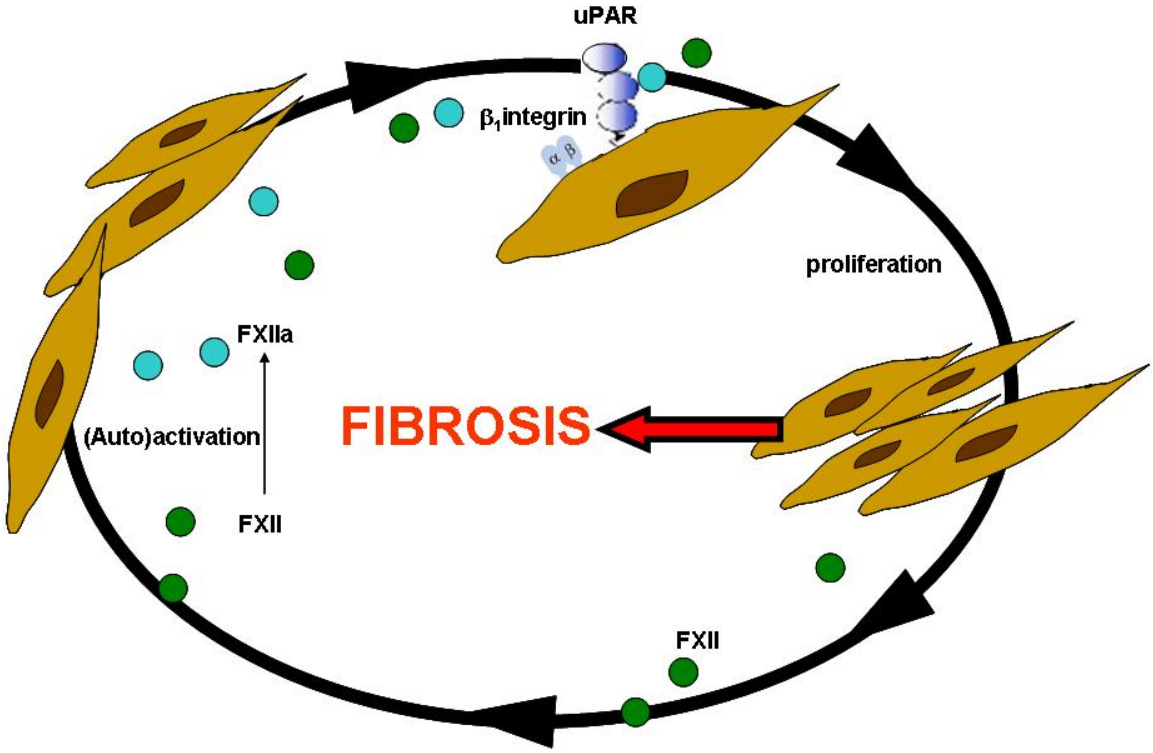


Figure 5.1. Factor XIIa may contribute to increased proliferation of fibroblasts in lung fibrosis.

6. Conclusions

Activation of the coagulation cascade is a feature of a number of lung diseases associated with inflammation and excessive deposition of extracellular matrix proteins, including lung fibrosis (174,231). IPF is a respiratory disorder caused by lung injury, increased proliferation of mesenchymal cells and excessive accumulation of extracellular matrix proteins in the lung (151). Although the primary function of the blood coagulation cascade is to preserve haemostasis and to prevent blood loss in case of tissue injury, uncontrolled activation of the blood coagulation cascade contributes to pathophysiological conditions including chronic lung injury. The extrinsic blood coagulation factors, notably TF and FVII, have been implicated in the pathogenesis of pulmonary fibrosis (174). Nevertheless, the potential role of FXII, the key intrinsic coagulation pathway factor, has never been reported in the pathomechanisms of lung fibrosis.

The aim of this study was to examine the contribution of intrinsic coagulation components to the development of pulmonary fibrosis. Increased expression of FXII, FXI and HMWK and elevated activity of FXII in the lungs of bleomycin-treated mice and IPF patients were noted. Moreover, the results provide evidence that FXII, FXI and HMWK are locally produced in the injured lungs. The study demonstrated that FXII, a central protease of the intrinsic coagulation cascade, has a capacity to drive fibrotic responses to lung injury, since knockout of FXII in mice or inhalative application of a specific FXIIa inhibitor attenuated bleomycin-induced lung fibrosis as evidenced by reduced collagen deposition and decreased mortality. The findings reported in the present study demonstrate for the first time a potential role of FXII during the fibrotic phases of experimentally induced lung injury. The evidence that targeting FXIIa may be of potentional therapeutic interest was herein provided.

Finally, it was demonstrated that FXIIa mitogenic activities may be responsible for FXII profibrotic activities. The results showed that FXIIa induces lung fibroblast proliferation via activation of the ERK 1/2. This effect required β1- and α5-integrins, uPAR, since the blockade of β1- and α5-integrin or the knockout of uPAR abolished FXIIa mitogenic activities. Moreover, FXII-dependent induction of lung fibroblast

proliferation was attenuated by a pharmacological blockade of the ERK 1/2 pathway and by a FXIIa specific inhibitor, CTI.

In addition, FXII promoter studies demonstrated that FXII expression in HLF was regulated by TGF-β1, a potent profibrotic cytokine upregulated in IPF lungs. The presented findings demonstrated that TGF-β1-induced FXII production in HLF is mediated by JNK and Smad 3 signaling pathways. Moreover, SBE at position -272 bp within the FXII promoter was identified and the importance of JNK activity in the Smad 3 – DNA complex formation was shown. The findings provide new insights into the molecular mechanism responsible for regulation of FXII expression in HLF and implicate its possible role in lung fibrosis, which is characterized by elevated TGF-β1 levels and dysregulated haemostasis.

In conclusion, this study provides evidence of a possible role of FXII in development of fibrotic lung diseases. Strategies aimed at blocking FXII in fibrotic lung disease may represent new treatment opportunities.

7. References

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9. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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