Activin receptor-like kinase 1 is a novel regulator of collagen deposition in idiopathic pulmonary fibrosis

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IV. List of abbreviations

aa	Amino acid
AEC	Alveolar epithelial cell
ActR	Activin receptor
AIP	Acute interstitial pneumonia
ALK	Activin-like kinase
AMHR	Anti-Müllerian hormone receptor
APS	Ammonium persulfate
AVM	Arteriovenous malformation
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFA	Cryptogenic fibrosing alveolitis
COL1A1	Collagen type 1α1
COP	Cryptogenic organizing pneumonia
CTGF	Connective tissue growth factor
DIP	Desquamative interstitial pneumonia
DMSO	Dimethyl sulfoxide
DPLD	Diffuse parenchymal lung disease
EC	Endothelial cell
ECM	Extracellular matrix
EDTA	Ethylendinitrilo-N,N,*N´*,*N´*,-tetra-acetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (2-amino-ethylether)-N,N,*N'*,*N',* -tetraacetic acid
EMT	Epithelial-to-mesenchymal transition
EpC	Epithelial cells
FCS	Foetal calf serum
FPF	Familial pulmonary fibrosis
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GDF	Growth and differentiation factor
HEPES	2-(4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate
hFB	Primary human fibroblast
HHT	Hereditary haemorrhagic telangiectasia
HPLF	Human periodontal ligament fibroblast
HRP	Horseradish peroxidase
HSC	Heat shock protein
IB	Immunoblotting
Id1	Inhibitor of differentiation 1
IHCH	Immunohistochemistry
IIP	Idiopathic interstitial pneumonia
IPF	Idiopathic pulmonary fibrosis
LIP	Lymphocytic interstitial pneumonia
LTBP	Latent TGF-β-binding protein
L-TGF-β	Latent TGF-β
MMP	Matrix metalloproteinase
NSIP	Nonspecific interstitial pneumonia
OD	Optical density
PAH	Pulmonary arterial hypertension
PAI1	Plasminogen activator inhibitor type 1
PBGD	Porphobilinogen deaminase
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + 0.1 % Tween 20
PCR	Polymerase chain reaction
PPH	Primary pulmonary hypertension
pSMC	Pulmonary smooth muscle cell
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time PCR
RB-ILD	Respiratory bronchiolitis-associated interstitial lung disease
Rel.	Relative
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
α-SMA	α-Smooth muscle actin
SMC	Smooth muscle cell

TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
TIMP	Tissue inhibitor of metalloproteinase
TGF-β	Transforming growth factor β
TGF-βRI	TGF-β receptor type I
TGF-βRII	TGF-β receptor type II
TNF	Tumour necrosis factor
Thy-1	Thymus cell antigen
UIP	Usual interstitial pneumonia
VBM	Vascular basement membrane

V. Summary

Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal lung disease of unknown origin, characterised by alveolar epithelial cell damage, increased deposition of extracellular matrix (ECM) in the lung interstitium, enhanced fibroblast/myofibroblast proliferation and activation, which ultimately leads to the distortion of normal lung architecture and loss of respiratory function. The interstitial fibroblast/myofibroblast represents the key effector cell responsible for the increased ECM deposition characteristic of IPF. Fibroblasts secrete large amounts of fibrillar collagens, which are the key ECM proteins, which exhibit elevated expression in this disease. The TGF-β is the primary and most potent profibrotic mediator involved in fibroblast activation and differentiation, and subsequent collagen production and deposition. Thus, it was hypothesised that the expression of TGF-β system components is altered in IPF, ultimately affecting the fibroblast activation and collagen synthesis.

In this study, the expression levels of ALK1, ALK5, TGF-βRII and endoglin, as well as Smads and TGF-β target genes, were analysed in the context of human pulmonary fibrosis. The expression of ALK1 was significantly downregulated in human lung homogenates from fibrotic lungs when compared to those from healthy subjects. Expression of other TGF-β system components was not altered in the disease. Furthermore, ALK1 and ALK5 mRNA and protein expression was localised to epithelial cells, endothelial cells, smooth muscle cells and fibroblasts, and the expression of ALK1 and ALK5 was decreased in primary fibroblasts isolated from human fibrotic lung tissue, compared to healthy controls, as assessed by quantitative RT-PCR and immunohistochemistry. The human fibroblast cell lines HFL1 and IMR-90 were selected for functional assays because these cell lines express TGF-β system components, and demonstrate active TGF-β and BMP signalling characterised by the phosphorylation of Smad2/3 and Smad1/5/8, respectively. Finally, treatment of human lung fibroblast cell lines with the siRNA specific for ALK1 attenuated collagen deposition, which was rescued by TGF-β1 stimulation. However, the impact of ALK1 on fibroblast activation and collagen deposition may not be primary, as the other signalling pathways might be involved.

These results demonstrated that ALK1 was expressed and functional in lung fibroblasts. The lack of ALK1 might be involved in the activation of fibroblasts thus leading to the collagen production, therefore being involved in the pathogenesis of pulmonary fibrosis.

VI. Zusammenfassung

Die idiopathische pulmonale Fibrose (IPF) ist eine fortschreitende und tödlich verlaufende Lungenerkrankung mit unbekanntem Ursprung, charakterisiert durch geschädigte Alveolarepithelzellen, gesteigerte Ablagerung von extrazellulärer Matrix (ECM) im Lungeninterstitium, erhöhte Fibroblasten/Myofibroblastenproliferation und -aktivierung, welche letztendlich zu einer Verformung der normalen Lungenstruktur und dem Verlust der respiratorischen Funktion führt. Der interstitielle Fibroblast/Myofibroblast repräsentiert die Schlüsseleffektorzelle, welche für die gesteigerte ECM-Ablagerung verantwortlich ist und somit charakteristisch für eine IPF. Fibroblasten sekretieren große Mengen von fibrillären Kollagenen, welche die Schlüsselproteine der ECM sind, was auch durch ihre gesteigerte Expression in dieser Erkrankung belegt wird. TGF-β ist der primäre und stärkste profibrotische Mediator, der an der Fibroblastenaktivierung und Differenzierung sowie der anschließenden Kollagenproduktion und Ablagerung beteiligt ist. Folglich war anzunehmen, dass die Expression von TGF-β Komponenten in IPF verändert ist, letztlich wirken Fibroblastenaktivierung und Kollagensynthese.

In dieser Studie wurde das Expressionsniveau von ALK1, ALK5, TGF-βRII und Endoglin, ebenso wie das der Smads und TGF-β Zielgene im Zusammenhang mit der humanen pulmonalen Fibrose untersucht. Die Expression von ALK1 war in humanen Lungenhomogenaten von fibrotischen Lungen im Vergleich zu gesunden Lungen signifikant herunterreguliert. Die Expression von anderen TGF-β Komponenten war in dieser Krankheit unverändert. Darüber hinaus war die ALK1 und ALK5 mRNA und Proteinexpression in Epithelzellen, Endothelzellen, glatten Muskelzellen und Fibroblasten lokalisiert. Die Expression von ALK1 und ALK5, die mit Hilfe quantitativer RT-PCR und Immunhistochemie ermittelt wurde, war in primären Fibroblasten, welche aus humanem fibrotischen Lungengewebe isoliert wurden, im Vergleich zu gesunden Kontrollen geringer. Für funktionelle Untersuchungen wurden die humanen Fibroblastenzelllinien HFL1 und IMR-90 ausgewählt, da diese Zelllinien Komponenten des TGF-β Signalweges exprimieren und aktive TGF-β und BMP Signaltransduktion, charakterisiert durch die jeweilige Phosphorylierung von Smad2/3 und Smad1/5/8, aufzeigen. Die Behandlung von humanen pulmonalen Fibroblastenzelllinien mit der spezifischen siRNA für ALK1 verringerte die Kollagenablagerung, welche durch eine TGF- β Stimulation hervorgerufen wurde. Dennoch dürfte der Einfluss von ALK1 auf die Fibroblastenaktivierung und Kollagenablagerung nicht der wichtigste sein, da auch andere Signalwege involviert sein könnten.

Diese Ergebnisse zeigten, dass ALK1 in Lungenfibroblasten exprimiert wird und funktionell ist. Ein Mangel von ALK1 könnte in die Fibroblastenaktivierung involviert sein und dadurch zur Kollagenproduktion führen, demzufolge kann ALK1 an der Pathogenese der pulmonalen Fibrose beteiligt sein.

1. Introduction

1.1. Idiopathic pulmonary fibrosis

1.1.1. Characteristics of idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a fatal disease of unknown cause, generally with a chronic, progressive and irreversible course and often with a fatal outcome [1]. Despite extensive research efforts over the past decades, no currently available therapy has been demonstrated to prevent or reverse the progression of this disease [2]. In principal, IPF is characterised by alveolar epithelial cell damage, increased deposition of extracellular matrix (ECM) in the lung interstitium, and enhanced fibroblast/myofibroblast proliferation and activation. These processes ultimately lead to distortion of normal lung architecture and loss of respiratory function [3].

Idiopathic pulmonary fibrosis is the most common form of idiopathic interstitial pneumonia (IIP), which constitutes a group of diffuse parenchymal lung diseases (DPLDs) also described as interstitial lung diseases. The IIPs include the entities of IPF, also referred to as cryptogenic fibrosing alveolitis (CFA), nonspecific interstitial pneumonia (NSIP), cryptogenic organising pneumonia (COP), acute interstitial pneumonia (AIP), respiratory bronchiolitis-associated interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), and lymphocytic interstitial pneumonia (LIP) [4].

Idiopathic pulmonary fibrosis is a rare disease that affects approximately five million people worldwide. Idiopathic pulmonary fibrosis does not favour particular race, ethnic group or social environment. The incidence of IPF increases with age. The mean age at presentation is 66 years. Occurrence of IPF is very rare in children [5]. The medium survival is two to five years from the time of diagnosis. Although IPF affects millions of individuals worldwide, there is still no effective therapeutic approach, and so far, lung transplantation is the only viable option for patients that are refractory to medical therapy.

Multiple lines of evidence suggest that genetic factors could impact the development of lung fibrosis [6]. Familial pulmonary fibrosis (FPF), also termed as familial interstitial pneumonia and familial idiopathic pulmonary fibrosis, is referred to those cases when two or more members of a family have an idiopathic interstitial pneumonia [7]. Clinical features of FPF are indistinguishable from those of the sporadic form, except for an earlier age of onset. Familial IPF accounts for 0.5 to 2% of all cases of IPF [5]. The largest description of FPF identified 111 families with 309 affected family members [8].

Since the pathogenesis of IPF is complex and poorly understood, identification of risk factors that may contribute to the development of the disease is essential. Although cigarette smoking [9], the presence of several viruses [10-12], and environmental factors [5, 9, 12] have been suggested to increase the risk of developing IPF, their impact remains to be fully elucidated.

The term “idiopathic” suggests that there are no known causes of IPF. Diagnostic criteria for IPF require exclusion of known causes of interstitial lung diseases [5], therefore, advances in cellular and molecular biology have extended our understanding of the biological processes involved in the initiation and progression of this disease.

1.1.2. Histopathological changes in idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis is associated with the pathologic pattern known as usual interstitial pneumonia (UIP), and therefore, is also referred to as IPF/UIP [5]. The histological hallmark is a heterogeneous appearance with alternating areas of normal lung with interstitial inflammation and fibrosis in early stages, and honeycomb change in the later stages of the disease process (Figure 1.1.). These changes are worse in the lower lobes and often seen in subpleural, peripheral and paraseptal areas [4]. Inflammatory components observed in the lungs of patients with IPF typically consist of lymphocytes and plasma cells, and to some extent eosinophils and neutrophils. Fibroblast foci, representing the sites of acute lung injury, are located within the interstitial space directly beneath alveolar epithelium and at the interphase between collogenised and normal-appearing lung. Moreover, alveolar epithelial cell (AEC) injury with hyperplasia of type II pneumocytes is an early and consistent finding in IPF. Reduced proliferative capacity, increased apoptosis, an inability to differentiate into type I AECs, and ineffective migration of type II AECs have also been observed in pulmonary fibrosis. The accumulation of ECM proteins, such as collagens, fibronectin, proteoglycans, and elastin, has been considered as hallmark of fibrosis [2, 13, 14]. The dense fibrosis causes remodelling of the lung architecture, resulting in collapse of alveolar walls followed by the loss of respiratory function [4] .

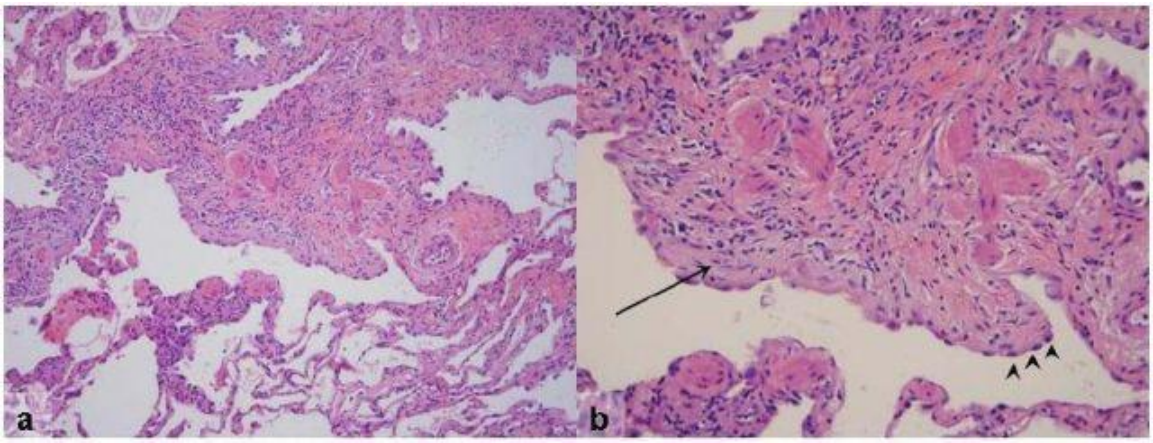


Figure 1.1. Histopathological changes in the lung in IPF.
Low-magnification photomicrograph of IPF illustrating heterogeneous involvement of the parenchyma. Zones of interstitial fibrosis are seen alternating with areas of normal lung. Original magnification is ×40 (**a**). Higher-magnification demonstrates enlarged cystic airspaces lined with hyperplastic alveolar epithelium (arrowheads). Beneath the mucosal layer is an advancing region of young fibrosis containing loose extracellular matrix (pale pink staining) and fibroblasts (arrow). Original magnification is ×200 (**b**) [5].

1.1.3. Pathogenesis of idiopathic pulmonary fibrosis

Current explanations of the pathogenesis of IPF are controversial, and ongoing research continues to investigate multiple hypotheses. From these attempts, two main hypotheses of IPF pathogenesis have arisen. The first one is the “inflammatory model of IPF pathogenesis” and the second is recognised as “epithelial/fibroblastic model”.

According to the first hypothesis, IPF has been long considered as the deleterious consequence of an unresolved chronic inflammatory process that follows an unrecognised insult [3], and injures the lung and modulates lung fibrogenesis, leading to the end-stage fibrotic scar. This hypothesis is based on the idea that injury/inflammation of the alveolar-capillary constituents and basement membrane leads to the loss of type I epithelial and endothelial cells, the proliferation of type II pneumocytes, the loss of alveolar space integrity, the recruitment and proliferation of stromal cells, and the deposition of the ECM [15]. However, there is little evidence that inflammation is prominent in early disease, and it is unclear whether inflammation is relevant to the development of the fibrotic process. Evidence suggests that inflammation does not play a pivotal role as most patients with IPF do not respond to anti-inflammatory drugs [4]. Thus, the chronic process of fibrosis may be separated from the acute process of inflammation, and inflammation appears necessary but not sufficient to explain the pathophysiology of fibrosis [16].

It has been suggested that IPF is characterised by a sequence of events that start with alveolar epithelial micro-injuries followed by the formation of fibroblastic foci and result in an exaggerated deposition of ECM, which drives the destruction of the lung parenchyma architecture [3]. The primary sites of ongoing injury and repair are the regions of fibroblastic proliferation, so-called fibroblastic foci. These small aggregates of actively proliferating and secreting fibroblasts/myofibroblasts constitute multiple sites of alveolar epithelial injury with exuberant deposition of ECM. The alveolar epithelium exhibits a marked loss of, or damage to, type I cells, and hyperplasia of type II cells. Alveolar epithelial cells express several enzymes, cytokines and growth factors, like for instance, that may promote fibroblast migration and proliferation, their differentiation to myofibroblasts. Subsequently, myofibroblasts may provoke basement membrane disruption and alveolar epithelial cell apoptosis, leading to the inappropriate reepithelialisation. The result is the excessive deposition of ECM with the destruction of alveolar-capillary units and progression to dense fibrosis with loss of lung function [3, 4, 17-19].

1.1.4. Fibroblasts - key effector cells in IPF

Fibroblasts are the most versatile of the connective-tissue cell family and possess a remarkable capacity to undergo various phenotypic conversations between distinct but related cell types. Fibroblasts participate in repair and regenerative processes in almost every human tissue and organ. Their primary function is to secrete ECM proteins that provide a tissue scaffold for normal repair events such as epithelial cell migration. Eventual dissolution of this scaffold and apoptosis of fibroblasts/myofibroblasts are critical for restoration of normal tissue architecture [20].

In the normal adult lung, fibroblasts are present in the adventitia of vascular structures and airways. They are commonly cultured as adherent cells exhibiting spindle-shape morphology and expressing interstitial collagens (type I and III), but they do not express markers of other differentiated cell types, therefore, there is the relative lack of specific marker to indicate the purity of isolated population [21]. The interstitial fibroblasts comprise 30-40% of the cells in the normal adult human lung.

Although fibroblasts in the normal lung synthesise very little matrix, activated myofibroblasts are major contributors to fibrotic lung disease (Figure 1.2.). These mesenchymal cells represent foci of organising acute lung injury and actively ongoing fibrogenic process, and they are considered as the key player, that is able to transform a

potentially reversible disorder to a progressive and irreversible one [18]. Myofibroblasts possess ultrastructural features intermediate between fibroblasts and smooth muscle cells. They are identified by the expression of α-smooth muscle actin (α-SMA).

The origin of pathological fibroblast foci within the IPF lesion remains puzzling. Possibilities include differentiation of resident fibroblasts [21], recruitment of circulating fibroblast precursors (fibrocytes) [22-24] and transdifferentiation of epithelial cells into pathological fibroblast phenotypes during the process called epithelial-to-mesenchymal transition (EMT) [25, 25-28].

The factors regulating activation and differentiation of myofibroblasts are poorly understood, although the importance of transforming growth factor (TGF)-β in this process has been widely appreciated [29]. Moreover, myofibroblasts are characterised by the production and secretion of collagen and a variety of cytokines, including the profibrotic TGF-β1 [17]. Thus, the well-known effect of TGF-β on α-SMA expression and myofibroblasts differentiation suggest the importance of the canonical TGF-β-associated Smad pathway. *In vitro* evidence indicates the importance of Smad3 in α-SMA expression in lung fibroblasts, and Smad3 deficiency *in vivo* results in a significant reduction in pulmonary fibrosis [30, 31].

Fibroblasts from the lungs of IPF patients produce a number of ECM proteins and integrin molecules. This is accompanied by an imbalance in the production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). The expression of the four TIMPs is demonstrated to be higher in the interstitium, where ECM accumulates, suggesting that a nondegrading fibrillar collagen microenvironment is present in pulmonary fibrosis. For instance, notable expression of TIMP-2 in the fibroblastic foci may be related to longer survival of myofibroblasts since in addition to its MMP inhibitory function it is also able to induce proliferation [20, 32-34].

Regarding the contribution of fibroblasts/myofibroblasts to the abnormal alveolar reepithelialisation, it has been demonstrated that fibroblasts from the lungs of IPF patients produce angiotensin peptides able to induce epithelial cell death *in vitro*, which also probably occurs *in vivo.* In addition, myofibroblasts from the lungs of IPF patients synthesise gelatinases A (MMP-2) and B (MMP-9), two matrix MMPs that degrade basement membrane molecules, contributing to the failure of the repair of alveolar type I epithelial cells and enhancing the migration of fibroblasts/myofibroblasts into the alveolar space [18, 20, 33].

Myofibroblasts at various states of development and activation express high or low levels of the cell surface marker thymus cell antigen (Thy)-1. Rat fibroblasts with high

levels of Thy-1 show a less contractile phenotype and α-SMA expression than Thy-1-low fibroblasts [35]. Similar phenotypic differences are found in humans, with the fibroblastic foci characteristic of IPF containing a fibroblast population whose Thy-1 expression level is far lower that the rest of the lung [36]. Whether Thy-1 expression causes or results from fibroblastic foci formation, as well as the relevance of this finding to the pathogenesis of IPF, are questions that have yet to be answered [29].

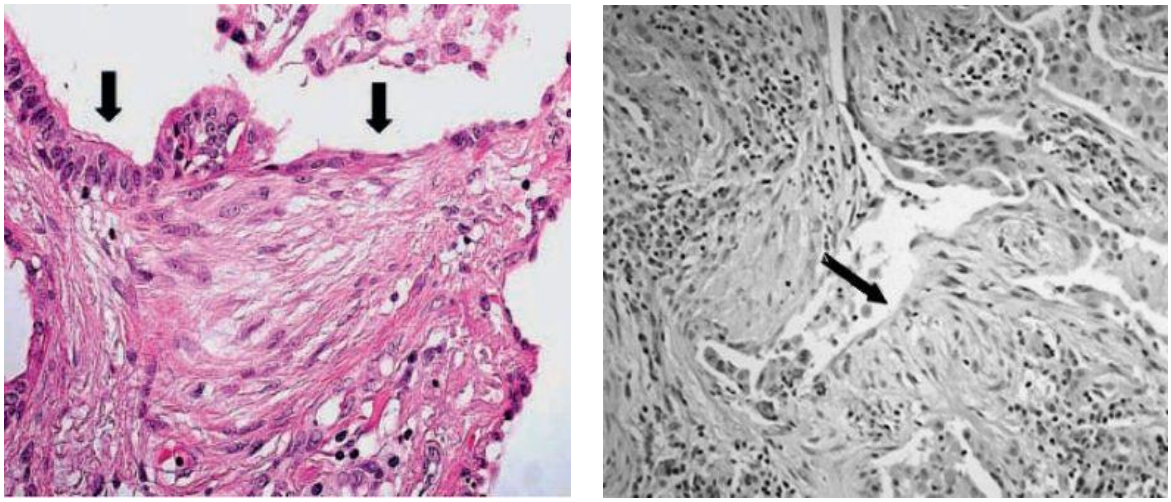


Figure 1.2. Subepithelial fibroblastic foci in lungs of patients with IPF.
Fibroblasts foci are indicated by arrows. Hematoxylin and eosin staining; original magnification is ×200. [17, 18]

1.1.5. Collagen - a key component of the extracellular matrix

Extracellular matrix remodelling is a dynamic process involved in development, fibrosis, tissue repair, tumor progression and metastasis. Under physiological conditions this process is tightly controlled. Disturbances either in the synthesis or in the degradation of the ECM result in an accumulation of ECM, primarily of fibril-forming collagens, which has been linked to the aberrant remodelling characteristics of lung fibrosis. The fibrotic lung contains approximately two to three times more ECM than normal lung, including collagens I, III, V, VI and VII, fibronectin, elastin, and proteoglycans. Proteases (MMPs) and their inhibitors play an important role in both, the degradation of ECM proteins as well in the activation and regulation of the processes that underlie their deposition [37, 38]. Two subtypes of type I collagen, COL1A1 and COL1A2, are the major collagens synthesised during abnormal wound repair, and their expression is regulated by TGF-β1 [39].

Collagens are abundant proteins and typically represent 25% of the total protein content of mammals. Currently, there are 28 collagen molecules, which are grouped in subfamilies depending on their structure and function. Collagen fibrils are the key ECM proteins that display significantly increased levels in IPF. The fibrils are synthesised and secreted by fibroblasts, but how this process is controlled during regeneration and tissue repair remains poorly understood. Collagens are trimeric molecules in which each chain consists of repeating Gly-X-Y triplets, where X and Y are usually proline and hydroxyproline, respectively. This triplet motif results in a left-handed helix that, together with two other helices, can form a right-handed triple-helical structure that (dependent on collagen type) can be homotrimeric or heterotrimeric.

Biosynthesis of collagen is a complex process that requires the formation of procollagen, which undergoes extensive post-translational modification. These modifications occur prior to triple helix formation, and consist of hydroxylation of proline and lysine. Hydroxylation of L-proline occurs in an ascorbic acid-dependent manner and is essential for collagen stability [38, 40].

1.2. Transforming growth factor (TGF)-β signalling

In the late 1970s and early 1980s, it was discovered that polypeptides secreted by Moloney sarcoma virus-infected mouse 3T3 cells exhibited the ability to confer a “transformed” phenotype to non-neoplastic cells, such as rat NRK fibroblasts. In 1983, this transforming activity could be assigned to a combination of two entirely different polypeptides, termed transforming growth factor (TGF)-α and TGF-β. Transforming growth factor α was identified to be an analogue of epidermal growth factor (EGF), while TGF-β represented a novel growth factor completely unrelated to any known polypeptide at the time. The name “transforming growth factor” was thus designed in the context of this observation. This original nomenclature, however, is misleading, as TGF-β elicits very potent tumor suppressor and antiproliferative activities, especially on epithelial cells. Today, TGF-β represents the prototypic member of a large and still growing family of secreted polypeptide growth factors that exerts pleiotropic effects on many cell types. Transforming growth factor β plays essential roles in embryonic development and cellular differentiation, regulate cellular proliferation and cell death, induce ECM synthesis, and modulate the immune response [41, 42]. Human genome contains 28 genes that encode

members of this family. The ligands, receptors and their intracellular effectors, the Smads, are conserved in eukaryotes from *Caenorhabditis elegans* and *Drosophila* to mammals [43].

1.2.1. The TGF-β ligands

More than 60 TGF-β family members have been identified in multicellular organisms (Figure 1.3.). Among these, there are many multifunctional cytokines including TGF-βs, activins, inhibins, anti-Müllerian hormone (AMH), bone morphogenetic proteins (BMPs), myostatin, Müllerin inhibiting substance (MIS), growth and differentiation factors (GDFs), Nodal, Vg1 and others [44, 45]. Three isoforms of TGF-β, termed TGF-β1, -β2 and -β3, are present in mammals.

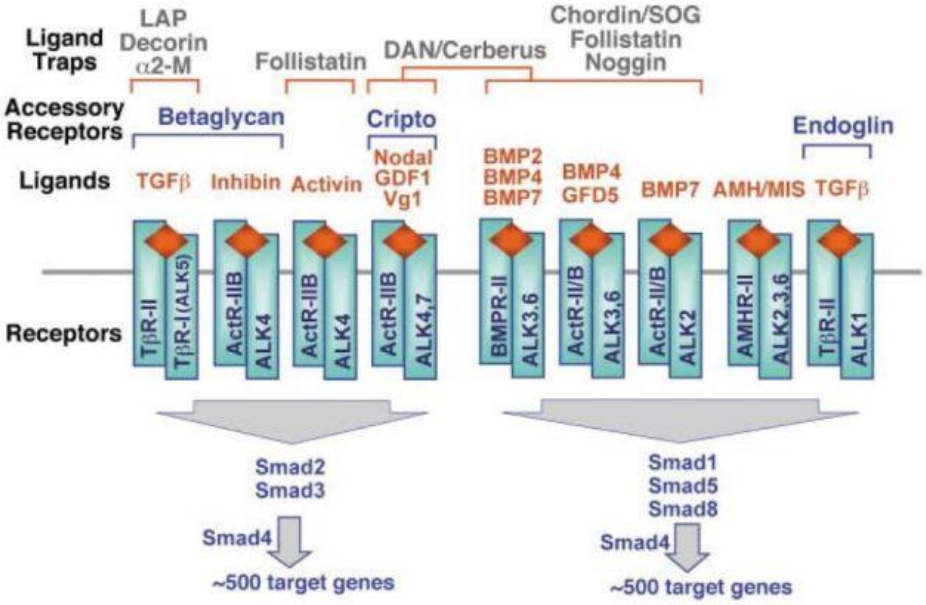


Figure 1.3. Schematic relationship between TGF-β superfamily members in vertebrates. The downstream R-Smads 1, 2, 3, 5 and 8 are grouped based on their signalling specificity. Commonly used alternative names are: ALK2/ActRI, ALK3/BMPRIA, ALK4/ActRIB, ALK5/TβRI, and ALK6/BMPRIB [45].

Transforming growth factor-β is secreted predominantly as a latent complex that must be activated before being capable of eliciting biological effects. The three isoforms of TGF-β are secreted as latent precursor molecules (L-TGF-β) that contain an amino-terminal hydrophobic signal peptide region, the latency associated peptide (LAP) region

and the carboxyl-terminal potentially bioactive region. The L-TGF-β is complexed with latent TGF-β-binding protein (LTBP), requiring activation into a mature form for receptor binding and activation of signal transduction pathways. The LTBP is removed extracellularly either by proteolytic cleavage by proteases such as plasmin, or through the action of binding proteins, such as thrombospondin [46, 47]. After proteolytic cleavage of the mature carboxyl-terminal part, biologically active TGF-β proteins are generated.

1.2.2. The TGF-β receptors

Transforming growth factor β superfamily members bind to three types of TGF-β receptors, which are classified as the type I (53 kDa), type II (73-95 kDa) and type III (110 kDa) receptors, depending on their molecular masses. The type I and type II receptors contain serine/threonine kinase domains, whereas the type III receptors lack a cytoplasmic kinase domain. This suggests that the type III receptors may serve as accessory receptors promoting ligand access to the signalling receptors. All members of these subgroups share structural and functional similarities within their own subgroups [48]. Generally, type I and II receptors are glycoproteins with core polypeptides of 500 to 570 amino acids including the signal sequence. They contain a cystein-rich extracellular domain, a short transmembrane helix and a cytoplasmic serine/threonine kinase domain (Figure 1.4.).

The extracellular region is relatively short (approximately 150 aa), N-glycosylated, and contains 10 or more cysteine residues that may determine the general fold of this region. The transmembrane region and cytoplasmic juxtamembrane region of type I and II receptors have no singular structural features. However, the GS domain, the highly conserved 30-amino acid region immediately preceding the kinase domain, is a unique feature of type I receptors. This region is called GS domain because of a characteristic SGSGSG sequence it contains. Ligand-induced phosphorylation of the serines and threonines in the TTSGSGSG sequence of TGF-βRI by the type II receptor is required for activation of signalling. The GS domain is a key regulatory part of that may control the catalytic activity of type I receptor kinase. The kinase domain consists of the canonical sequence of a serine/threonine protein kinase domain. Transforming growth factor β receptor type I have been shown to phosphorylate their substrates – Smad proteins – on serine residues, whereas TGF-β type II receptors autophosphorylate themselves and transphosphorylate type I receptors on serine and threonine residues [49].

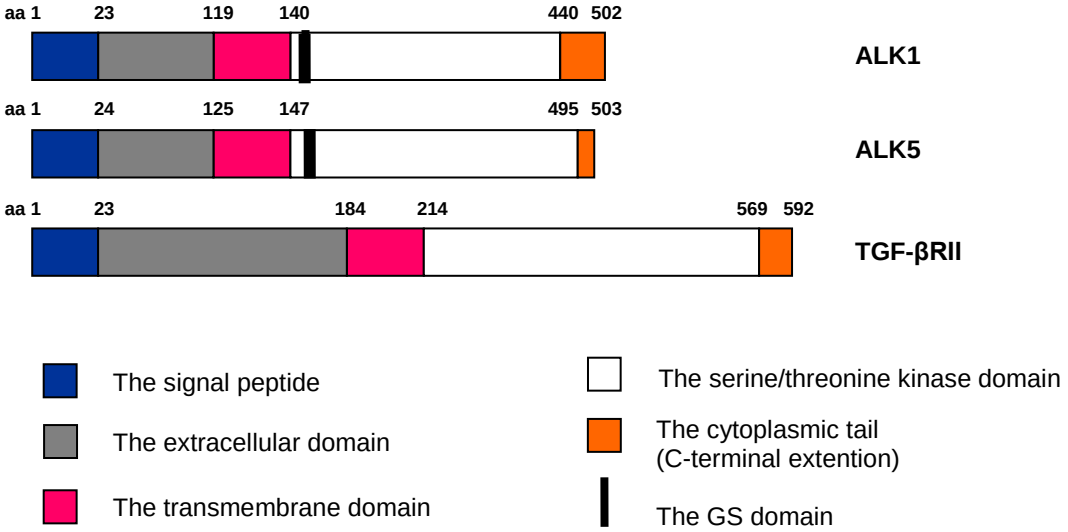


Figure 1.4. The structure of the TGF-β type I (ALK1 and ALK5) and type II (TGF-βRII) receptors.
There is homology between TGF-β types I receptors, which possess the specific GS domain, not present in type II receptors. The constitutively active type II receptor phosphorylates and activates type I receptors in their GS domain.

The receptor serine/threonine kinase family in the human genome comprises 12 members – seven type I and five type II receptors – all dedicated to TGF-β signalling. The following receptors are ranked amongst the TGF-β type I receptors:

- ALK1 (ACVRL1) for TGF-β ligands,
- ALK2 (ActRI) for activins, BMP2 and BMP4 ligands,
- ALK3 (BMPRIA) for BMP ligands,
- ALK4 (ActRIB) for activins and TGF-β ligands,
- ALK5 (TGF-βRI) for activins and TGF-β ligands,
- ALK6 (BMPRIB) for BMP ligands,
- ALK7 for Nodal, GDFs and Vg1 ligands.

The following receptors are ranked amongst type II receptors:

- TGF-βRII for TGF-β ligands,
- ActRII for activins, BMP2, 4, 7,
- ActRIIB for activins, BMP2, 4, 7,
- BMPRII for BMPs,
- AMHRII for anti-Müllerian hormone.

There are two TGF-β type III receptors known as betaglycan (TGF-βRIII) and endoglin (CD105). The evidence suggests that accessory receptors do not have any

intrinsic signalling function, some of these molecules act as supplementary receptors hat assist the type I and II subgroups for ligand binding, and potentiate the signalling cascade, other function as inhibitors of the signalling pathway [49-51].

1.2.3. The Smad proteins

Smad proteins function as signal transducers of TGF-β family members and they are the first identified substrates of type I receptor kinases. The name Smad originates from a fusion between *Drosophila mothers against dpp (Mad)* and *C. elegans Sma* [52, 53].

Based on structural and functional considerations, eight members of the Smad family have been identified which can be further classified into three distinct subfamilies:

- Smads that are direct substrates of TGF-β family receptor kinases, the receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, Smad8),
- Smads that participate in signalling by associating with these receptor-regulated Smads, the common-mediator Smads (Co-Smads: Smad4 and Smad4β),
- Antagonistic Smads that inhibit the signalling function of the other two groups, the inhibitory Smads (I-Smads: Smad6 and Smad7) (Fig. 1.5.).

The subfamily of R-Smads can be further divided into 2 groups:

- BMP-Smads, being activated in the BMP signalling pathway and phosphorylated by BMP type I receptors (Smad1, Smad5, and Smad8),
- TGF-β/activin-Smads, activated and phosphorylated by TGF-β type I receptors and activin type I receptors (Smad2 and Smad3).

The overall structure of R-Smads and Co-Smads comprises the highly conserved N-terminal Mad homology 1 (MH1) and the C-terminal Mad homology 2 (MH2) domains which form globular structures and are linked by a divergent proline-rich region of variable length. The I-Smads likewise contain the conserved MH2 domain but show very little similarity to other Smads in their N-terminal part [54]. The R-Smads have a unique SSXS motif at the C-terminus, which is directly phosphorylated by activated TGF-β type I receptor on at least two serine residues [55]. Co-Smads have both types of MH domains but TGF-β type I receptor is unable to phosphorylate this class of proteins since they do not have SSXS phosphorylation motif at the C-terminus. The MH1 domain of R-Smads and Co-Smads, except for Smad2, can bind to the specific DNA sequences. The MH2 domains of R-Smads and Co-Smads are indispensable for homomeric and heteromeric complex formation. The L3 loop in the MH2 domain of R-Smads determine the specificity of the interaction with type I receptors [49, 52, 54, 56].

Smad proteins do not contain any intrinsic enzymatic activity but rather exert their function through protein-DNA and protein-protein interactions via their MH1 and MH2 domains. These domains of R-Smads have intrinsic affinity for each other and inhibit each other's functions. The R-Smads are present predominantly as monomers in the steady state. Ligand stimulation promotes R-Smads to form homo-oligomers or hetero-oligomers that are composed of R-Smads alone or together with Co-Smads. The phosphorylation of R-Smads allows them to interact with DNA and other proteins in the nucleus. The binding of Smads to DNA occurs with rather low affinity and sequence specificity. Therefore, Smads need to cooperate with each other and/or with other DNA-binding proteins to regulate TGF-β target gene transcription [52, 57-60].

The I-Smads interact efficiently with the activated type I receptor, thereby preventing access of R-Smads to the activated type I receptor. Whereas Smad6 appears to preferentially inhibit BMP signalling, Smad7 acts as a general inhibitor of TGF-β signalling [52].

1.2.4. The TGF-β signalling pathway

In the current model of TGF-β signal transduction, biological effects of TGF-β are induced after binding of active TGF-β ligand to the ligand binding serine/threonine kinase receptor type II. The TGF-βRII has intrinsic kinase activity. The TGF-βRI is then recruited into a heterotetrameric receptor complex and phosphorylated in its GS domain by TGF -βRII, leading to activation of its kinase activity and subsequent intracellular signalling into the nucleus. This occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad proteins family. Type I receptors specifically recognise and phosphorylate the ligand-specific receptor-activated Smads (R-Smads). Upon phosphorylation, R-Smads form heteromeric complexes with common Smad, such as Smad4. These complexes are translocated into the nucleus, where they function as transcription factors, binding DNA either directly or in association with other DNA binding proteins (Figure 1.5.) [61, 62].

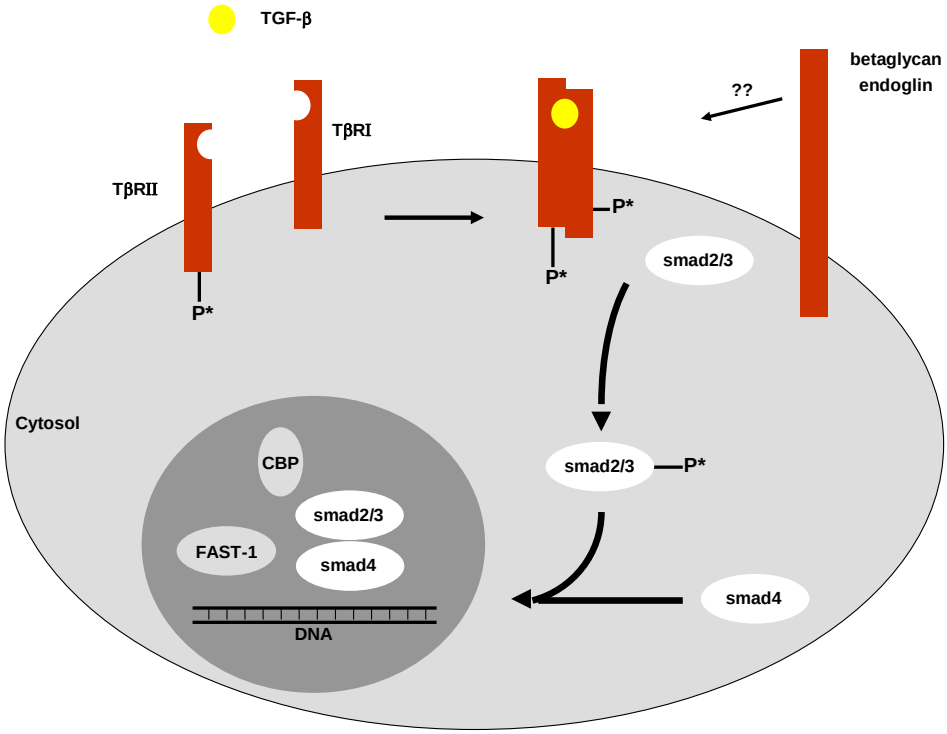


Figure 1.5. TGF-β signal transduction.
Transforming growth factor β signal transduction is initiated by binding of TGF-β ligand to TGF -βRII. The TGF-βRI is then recruited into the receptor complex, in which the type I receptor, after having being phosphorylated, recruits and phosphorylates Smad2/3 molecules. These molecules form dimers with Smad4 and the heterodimer complex is translocated into the nucleus, where it activates or represses target gene transcription together with other cofactors [62].

1.2.5. The role of TGF-β in idiopathic pulmonary fibrosis

Transforming growth factor β is a multifaceted cytokine produced by several cell types, such endothelial cells, vascular smooth muscle cells, myofibroblasts, macrophages and other haematopoietic cells. Transforming growth factor β is involved in the modulation of a wide array of biological processes including cell growth and differentiation, cell adhesion, cell migration, cell apoptosis, ECM production, immune response, embryonic development, and wound healing [63]. Transforming growth factor β plays a pivotal role in the fibroproliferative changes that follow tissue damage in many vital organs and tissues, including liver [64, 65], lung [5], kidney [66], skin [47], heart, and arterial wall [67].

Transforming growth factor β1, β2 and β3 play a pivotal role in the regulation of lung fibrosis. Transforming growth factor β modulates lung fibrosis through recruitment and activation of monocytes and fibroblasts, induction of ECM, and stimulation of

angiogenesis. Fibroblasts are induced by TGF-β to differentiate into myofibroblasts, which represents the main source of ECM during pulmonary fibrosis. Transforming growth factor β signalling modulates ECM production by promoting ECM gene transcription, including collagens I, III, IV, and V, fibronectin, and proteoglycans, and by suppressing the activity of MMPs, plasminogen activators, and elastases, which results in the inhibition of collagen degradation [68-72]. Moreover, the intracellular factor Smad3 has been demonstrated to be downstream of TGF-β1 in studies on the targeted repression of this pathway in mice, which fail to develop pulmonary fibrosis when challenged with TGF-β1 [73-75]. Additionally, TGF-β1 levels are elevated in fibrotic organs, and are often specifically localised to fibrotic areas. This is correlated with increased level of Smad2, Smad3 and Smad4, but reduced level of Smad7, which was investigated in cardiac fibrosis [76].

The potent profibrotic cytokine TGF-β induces matrix synthesis in fibroblasts and fibrotic responses *in vivo* and *in vitro.* Genetic and pharmacological studies have suggested the broad targeting of general TGF-β signalling pathways might be optional for treating fibrotic diseases, but on the other hand could be problematic due to the pleiotropic nature of TGF-β. However, further clarification of the differential contribution of TGF-β to the pathogenesis of IPF may lead to the discovery of novel therapeutic options for the treatment of fibrotic diseases.

1.3. Two distinct TGF-β type I receptors: ALK1 and ALK5

The actions of TGF-β are highly dependent on cellular context. In TGF-β signalling, one TGF-β type II receptor and two distinct TGF-β type I receptors, the endothelium restricted activin receptor-like kinase (ALK)1 and the broadly expressed ALK5, have been implicated. Recent studies now challenge the previous dogma concerning receptor complexes and signal transduction schemes and demonstrate that, in endothelial cells (ECs), TGF-β signals through a heteromeric receptor complex consisting of TGF-βRII, ALK5 and ALK1, resulting in activation of both classes of Smad proteins, which mediate both selective and antagonistic effects on the transcriptional output.

1.3.1. The ALK1/ALK5 balance in endothelial cells

In the vascular system, TGF-β regulates the process of angiogenesis, which involves the activation, remodelling, and expansion of pre-existing networks of vessels. Vessels are formed by two main cell types – ECs and perimural cells – that enshroud the endothelium. Angiogenesis can be divided into an activation phase and a resolution phase. Under baseline conditions, the endothelium is quiescent due to the stabilisation of the vessels by mural cells. During the activation phase, smooth muscle cells detach, vascular basement membranes depredate, and EC proliferate and migrate, to form a new tube. During the resolution phase, basement membrane is reformatted, smooth muscle cells are recruited to cover the new tube and to inhibit the proliferation and migration of the endothelial cells [77]. These two phases are self-limiting processes in the human body. The existence of a balance between activation and resolution phase of angiogenesis is pivotal for homeostasis.

One of the aspects that have puzzled researchers for years is that TGF-β exerts bifunctional effects on EC proliferation: TGF-β can both stimulate and inhibit proliferation of ECs. Low doses of TGF-β stimulate EC proliferation and migration, while high doses of TGF-β inhibit these processes [78]. Recent results have reported that TGF-β regulates the activation state of the endothelium via a fine balance between ALK5 and ALK1 signalling (Figure 1.6.) [79].

Although ALK5 is a predominant receptor that mediates TGF-β signalling, ALK1 can also form complexes with the type II receptor. In ECs, both ALK1 and ALK5 are expressed and bind TGF-β. Activin receptor-like kinase 1 expression is restricted to the ECs and during embryogenesis at active sites of angiogenesis.

Transforming growth factor β/activin receptor-like kinase 5 signalling induces Smad2/3 phosphorylation and blocks angiogenesis by inhibiting EC proliferation, tube formation and migration [79, 80]. Activin receptor-like kinase 5 induces the expression of fibronectin and plasminogen activator inhibitor type 1 (PAI1), a negative regulator of EC migration. Activin receptor-like kinase 5 has been reported to increase TGF-β-induced EC permeability and actin cytoskeleton remodelling [81]. By enhancing TGF-βRII/ALK5 assembly, clustered VE-cadherin promotes persistent and elevated TGF-β-induced Smad2/3 activation, indicating a positive role for VE-cadherin in TGF-β/ALK5-induced vessel stabilisation [82]. Taken together, TGF-β/ALK5 signalling plays an important role in keeping the endothelium quiescent.

In contrast to TGF-β/ALK5, TGF-β/ALK1 signalling induces Smad1/5 activation and has been shown to stimulate EC migration, proliferation and tube formation [80].

Caveolin1 was shown to associate with ALK1 and to promote TGF-β/ALK1-induced responses [83]. An important intracellular effector of ALK1 is inhibitor of differentiation 1, an inhibitor of basic helix-loop-helix proteins that promotes angiogenesis. Its upregulation was shown to be required for TGF-β/ALK1-induced EC migration and tube formation [79]. However, an inhibitory effect of ALK1 signalling on EC proliferation, migration and sprouting has also been reported [84-87]. Bone morphogenetic protein 9, identified as a ligand for ALK1 and BMPRII complex in ECs, was shown to inhibit EC migration and VEGF-induced angiogenesis [88, 89]. These observations suggest that the effect of ALK1 signalling on angiogenesis is dependent on the context and specific ligand by which it is activated [90].

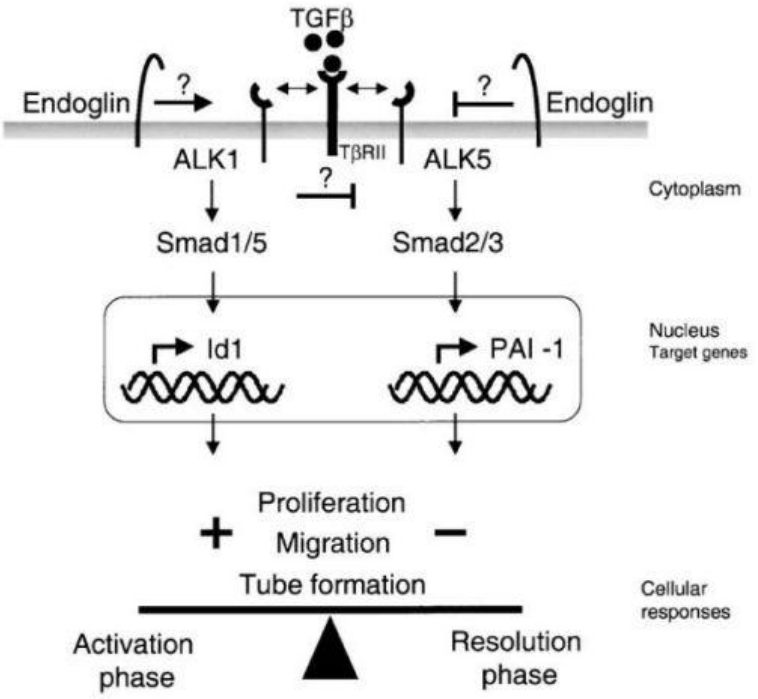


Figure 1.6. A model of TGF-β control of the angiogenic switch.
Transforming growth factor β regulates the state of the endothelium via a balance between ALK1 and ALK5 signalling. Activation of ALK5, phosphorylates Smad2/3, which induces PAI1 and fibronectin expression and inhibits migration, proliferation, and tube formation, resulting in the resolution phase of angiogenesis. Transforming growth factor β binding to ALK1 leads to the phosphorylation of Smad1/5, which induces Id1 expression and stimulates migration and proliferation, processes involved in the activation phase of angiogenesis [91].

Activin receptor-like kinase 1 and ALK5 signalling not only elicit opposite responses, but also physically interact with each other in ECs. Activin receptor-like kinase 5-deficient ECs are not only defective in TGF-β/ALK5 signalling but also exhibit impaired

TGF-β/ALK1 responses; ALK5 was found to be necessary for recruitment of ALK1 into a TGF-β receptor complex, and the kinase activity of ALK5 is essential for maximal ALK1 activation [80]. Furthermore, ALK1 can directly antagonise ALK5/Smad2/3 signalling at the level of Smads [86]. The cross-talk between ALK1 and ALK5 signalling provides ECs with a TGF-β-dependent switch to fine-tune EC function.

Interestingly, there are endothelial cells that express betaglycan and those that express endoglin. Endothelial cells expressing betaglycan respond to all three isoformes of TGF-β, whereas ECs that express endoglin respond to TGF-β1 and –β3, but not –β2 [92]. The co-receptor endoglin is predominantly expressed in highly proliferating vascular ECs. Endoglin regulates the fine-tuning between the ALK1 and ALK5 signalling pathways. Endoglin is required for TGF-β/ALK1 signalling and indirectly inhibits TGF-β/ALK5 signalling [93, 94], thus promoting the activation phase of angiogenesis. Moreover, in the absence of endoglin, ECs do not grow and ALK1 signalling is abrogated whereas ALK5 signalling is stimulated. Endoglin may thus function as a modulator of the balance between the TGF-β/ALK1 and the TGF-β/ALK5 signalling pathways [95].

1.3.2. Fibrosis and angiogenesis

In recent years, several new discoveries have been made in the fields of fibrosis and angiogenesis [47, 67, 96, 97]. Although most of these discoveries were made in the context of either fibrosis or angiogenesis, a new appreciation for a connection between these two fields is emerging. The cytokines and ECM molecules involved in fibrosis are also pivotal for the formation of new capillaries [98].

Extracellular matrix proteins exist outside the cells to provide structural and functional support for cells. Extracellular matrix also exists as thin layer called the basement membrane, which provides a supporting structure on which epithelial and endothelial cells can grow. Vascular basement membrane (VBM) constitutes the rigid structural wall of newly-established capillaries, and is speculated to play an important role in regulating pro- and anti-angiogenic events. In this regard, several endogenous inhibitors of angiogenesis have been discovered, which are fragments of ECM molecules. These fragments of large collagen proteins, are released by the action of matrix-degrading enzymes such as MMPs and elastase during the activation phase of angiogenesis, and during the resolution phase the expression of these proteases is downregulated, and the expression of matrix proteins is upregulated in association with the formation of new VBM, pericyte proliferation and attachment [99]. In future, it will be

interesting to study the role of these inhibitors of angiogenesis in organ fibrosis and organ development. Similarly, during fibrosis, the expression of these enzymes is significantly downregulated and the expression of inhibitors of these enzymes, such as TIMPs, is upregulated. These observations suggest that fibrosis and angiogenesis share some common features. One example of he coordinated interplay of molecular mechanisms associated with fibrosis and angiogenesis is the process of wound healing [98].

Transforming growth factor β is produced by many different cell types, including epithelial cells, endothelial cells and cells of mesenchymal origin, and is typically a negative regulator of cell proliferation in epithelial and endothelial cells but a positive regulator in mesenchymal cells [49, 59]. Many different cell types including fibroblasts, endothelial and epithelial cells respond to TGF-β by upregulating the expression of matrix and matrix-associated proteins such as fibronectin, collagens, proteoglycans, laminin and thrombospondin. Transforming growth factor β is associated with the increasing level of PAI1, TIMPs and integrins, and with decreasing level of MMPs and plasminogen activators [70, 99]. Interestingly, in the context of fibrosis and angiogenesis all these molecules exhibit the same pattern of expression [100].

TGF-β can induce the formation of new blood vessels *in vivo*, essentially in concert with basic fibroblast growth factor (bFGF) [100, 101]. Transforming growth factor β may have growth inhibitory effects on endothelial monolayer, and play a role in pericyte maturation, indicating a role for this cytokine in the resolution phase of angiogenesis. Additionally, as described in the chapter 1.2.5., TGF-β plays a pivotal role in the pathogenesis of idiopathic pulmonary fibrosis. The question remains, however, as to whether the two processes are mediated by the same or different activities of TGF-β, or by specific switch between the TGF-β receptors and intracellular mediators.

2. Aim of the study

It is well established that an imbalance between ALK1/ALK5 may contribute to the development of several diseases, including HHT, pulmonary hypertension and fibrosis. These TGF-β type I receptors may significantly affect collagen synthesis due to their opposite effect on the different cell proliferation, migration and differentiation, and the subsequent collagen deposition. As these processes are reported to be involved in the pathogenesis of the fibrosis of many organs, this project aims to functionally characterise the action of ALK1 in idiopathic pulmonary fibrosis. In this context, the research focus was:

1. The expression analysis of ALK1/ALK5 receptors in the human lungs exhibiting the pathological features of IPF and the human lung fibroblasts
2. The elucidation of the role of ALK1 receptor and its impact on fibroblasts activation and collagen deposition.

3. Materials and Methods

3.1. Materials

3.1.1. Equipment

ABI PRISM 7500 Sequence Detection System	Applied Biosystems, USA
Cell Culture Incubator; Cytoperm2	Heraeus, Germany
Developing machine; X Omat 2000	Kodak, USA
Electrophoresis chambers	Bio-Rad, USA
Film cassette	Sigma-Aldrich, Germany
Filter Tip FT: 10, 20, 100, 200, 1000	Greiner Bio-One, Germany
Filter units 0.22 µm syringe-driven	Millipore, USA
Freezer -20 °C	Bosch, Germany
Freezer -40 °C	Kryotec, Germany
Freezer -80 °C	Heraeus, Germany
Fridge +4 °C	Bosch, Germany
Fusion A153601 Reader	Packard Bioscience, Germany
Gel blotting paper 70 × 100 mm	Bioscience, Germany
Glass bottles: 250, 500, 1000 ml	Fischer, Germany
GS-800TM Calibrated Densitometer	Bio-Rad, USA
Light microscope Olympus BX51	Olympus, Germany
Mini spin centrifuge	Eppendorf, Germany
Multifuge centrifuge, 3 s-R	Heraeus, Germany
Multipette® plus	Eppendorf, Germany
Nanodrop®	Peqlab, Germany
PCR-thermocycler	MJ Research, USA
Pipetboy	Eppendorf, Germany
Pipetman: P10, P20, P100, P200, P1000	Gilson, France
Power Supply; Power PAC 300	Bio-Rad, USA
Petri dish	Greiner Bio-One, Germany
Pipette tip: 200, 1000 µl,	Sarstedt, Germany
Pipette tip: 10 µl, 20 µl, 100 µl	Gilson, USA

Radiographic film X-Omat LS	Sigma-Aldrich, Germany
Serological pipette: 5, 10, 25, 50 ml	Falcon, USA
Test tubes: 15, 50 ml	Greiner Bio-One, Germany
Thermo-Fast® 96 PCR Plate	Thermo Scientific, USA
Tissue culture dish 100 mm	Greiner Bio-One, Germany
Tissue culture flask 250 ml	Greiner Bio-One, Germany
Tissue culture plates: 6, 48 well	Greiner Bio-One, Germany
Western Blot Chambers: Mini Trans-Blot	Bio-Rad, USA
Vortex machine	Eppendorf, Germany
Vacuum centrifuge	Eppendorf, Germany

3.1.2. Reagents

Acetone pure	Merck, Germany
Acrylamide solution, Rotiphorese Gel 30	Roth, Germany
Agarose	Invitrogen, UK
Albumine, bovine serum	Sigma-Aldrich, Germany
Ammonium persulfate	Promega, Germany
β-glycerophosphate	Sigma-Aldrich, Germany
β-mercaptoethanol	Sigma-Aldrich, Germany
Bone morphogenetic protein 2 (BMP2)	R&D Systems, USA
Bromophenol blue	Sigma-Aldrich, Germany
Complete™ Protease inhibitor	Roche, Germany
D-(+)-Glucose	Sigma-Aldrich, Germany
D-MEM + GlutaMAX™-I (1×) medium	Gibco BRL, Germany
D-MEM/F12 + GlutaMAX™-I (1×) medium	Gibco BRL, Germany
D-MEM medium	Sigma-Aldrich, Germany
Dimethyl sulfoxide	Sigma-Aldrich, Germany
DNA Ladder (100 bp, 1 kb)	Promega, USA
Ethylendinitrilo-N, N, *N´, N´,* -tetra-acetic-acid (EDTA)	Promega, USA
Ethylene glycol-bis (2-amino-ethylether)-N,N, *N´, N´* -tetraacetic-acid (EGTA)	Sigma-Aldrich, Germany
Dulbecco's phosphate buffered saline 10×	PAA Laboratories, Austria
Dulbecco's phosphate buffered saline 1×	PAA Laboratories, Austria
Ethanol absolute	Riedel-de Haën, Germany
ECL Plus Western Blotting Detection System	Amersham Biosciences, UK
Ethidium bromide	Roth, Germany
Foetal calf serum (FCS)	PAA Laboratories, Austria
Glycine	Roth, Germany
GoTaq® Flexi DNA polymerase	Promega, USA
Hydrochloric acid	Sigma-Aldrich, Germany
2-(4-2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES)	Sigma-Aldrich, Germany
Igepal CA-630	Sigma-Aldrich, Germany
MuLV Reverse Transcriptase	Applied Biosystems, USA

L-Glutamine 200 mM (100×)	PAA Laboratories, Austria
Lipofectamine™ 2000	Invitrogene, UK
Luciferase Assay Reagent 10-Pack	Promega, USA
Luciferase Cell Culture Lysis 5× Reagent	Promega, USA
Magnesium chloride	Sigma-Aldrich, Germany
Methanol	Fluka, Germany
N,*N*,*N′*,*N′*-tetramethyl-ethane-1,2-diamine (TEMED)	Bio-Rad, USA
Non-essential amino acids	PAA Laboratories, Austria
Opti-MEM medium	Gibco BRL, Germany
PCR Nucleotide Mix	Promega, USA
Penicillin-streptomycin	PAA Laboratories, Austria
Potassium acetate	Sigma-Aldrich, Germany
Potassium borate	Grom-chromatography, Germany
Potassium chloride	Merck, Germany
Potassium phosphate	Sigma-Aldrich, Germany
Precision Plus Protein™ Standards	Bio-Rad, USA
2-Propanol	Merck, Germany
Pure Yield Plasmid Midiprep System	Promega, Germany
QIAprep Spin Miniprep Kit	Qiagen, Germany
Quick Start™ Bradford Dye Reagent	Bio-Rad, USA
Random Hexamers (50 µM)	Applied Biosystems, USA
RNase inhibitor	Applied Biosystems, USA
RNaseZAP®	Sigma-Aldrich, Germany
PeqGOLD Total RNA Kit	Peqlab, Germany
Roti®-Quick-Kit	Roth, Germany
Silencer® Negative siRNA control #1 (50 µM)	Ambion, Germany
Sircol™, Soluble Collagen Assay	Biocolor, UK
Sodium acetate	Sigma-Aldrich, Germany
Sodium chloride	Merck, Germany
Sodium dodecyl sulfate (SDS)	Promega, USA
Sodium ortho vanadate	Sigma-Aldrich, Germany
Sodium phosphate	Sigma-Aldrich, Germany
SuperSignal® West Pico Chemiluminescent Substrate	Thermo Scientific, USA

SYBER® Green PCR Kit	Invitrogene, UK
Transforming growth factor β1 (TGF-β1)	R&D Systems, USA
Tween 20	Sigma-Aldrich, Germany
Tris	Roth, Germany
Triton X-100	Promega, USA
Trypsin-EDTA	Gibco BRL, Germany

3.1.3. Mammalian cells

3.1.3.1. Cell lines

HFL1 (human foetal lung fibroblasts), ATCC, USA
IMR-90 (human lung fibroblasts), ATCC, USA

3.1.3.2. Primary cells

Human primary lung fibroblasts were isolated from tissues obtained from healthy transplant donors and fibrotic patients, as described in section 3.2.11.2.

3.1.4. Human tissues

Lung tissue samples were obtained from twelve patients with IPF (mean age 51 ± 11 years) and twelve control subjects (mean age 48 ± 14 years). The study protocol was approved by the Ethics Committee of the University of Giessen School of Medicine (AZ 31/93). Informed consent was obtained from each subject for the study protocol.

3.2. Methods

3.2.1. RNA isolation

Isolation of RNA from lung tissue and cultured cells material was performed according to the manufacturer's instructions provided with Roti®-Quick-Kit and peqGOLD Total RNA Kit, respectively.

3.2.2. Determining RNA concentration

The concentration of isolated RNA was determined according to a protocol from Peqlab by applying 1.5 µl of the sample to a Nanodrop® spectrophotometer.

3.2.3. Reverse transcription reaction

Reverse transcription polymerase chain reaction (RT-PCR) is an enzymatic process performed by an enzyme called reverse transcriptase. This enzyme synthesises complementary DNA (cDNA) using RNA as a template.

In order to perform RT-PCR, 500 ng of human total RNA was added to the autoclaved water up to 10 µl of total volume. The reaction mixture was heated to 70 °C for 15 min, chilled on ice, and the following RT reagents were added:

RT reaction component	**Volume**	**Final concentration**
10× RT Buffer II ($MgCl_2$ free)	2 µl	1×
25 mM $MgCl_2$	4 µl	5 mM
10 mM dNTP mix	1 µl	0.5 mM
Random hexamers (50 µM)	1.5 µl	3.75 µM
RNAse inhibitor (20 U/µl)	0.5 µl	10 U
Reverse transcriptase (50 U/µl)	1 µl	50 U

To synthesise cDNA, the reaction mixture was incubated at 20 °C for 10 min, then at 43 °C for 75 min and at 99 °C for 5 min. Synthesised cDNA was stored either at -20 °C or used for other experiments immediately.

3.2.4. Polymerase chain reaction

The polymerase chain reaction (PCR) is an enzymatic technique that facilitates the production of millions of copies of specific DNA. An enzyme called DNA polymerase carries out the amplification of cDNA, previously reverse-transcribed from RNA. Principally, each PCR cycle consists of three steps:

Denaturation - separation of double-stranded DNA into single strands,
Annealing - primer binding to the appropriate sequence of single DNA strands,
Elongation - synthesis of a new DNA strand by DNA polymerase.

3.2.4.1. Semi-quantitative polymerase chain reaction

A semi-quantitative PCR reaction proceeds, in principal, as described in section 3.2.3. Amplification of cDNA was performed according to the manufacturer's instructions provided with the GoTaq® Flexi DNA Polymerase. The PCR reaction mix was prepared as follows:

PCR reaction component	**Volume**	**Final concentration**
5× PCR Buffer (free $MgCl_2$)	10 µl	1×
25 mM $MgCl_2$	5 µl	2.5 mM
10 mM dNTP mix	1 µl	0.2 mM
10 µM forward primer*	1 µl	0.2 µM
10 µM reverse primer*	1 µl	0.2 µM
Taq DNA Polymerase (5 U/µl)	0.25 µl	1.25 U
cDNA template	1 µl	not applicable
H_2O (autoclaved)	up to 50 µl	not applicable

* All primer sequences are listed in Table 6.1.2.

The PCR reaction components were mixed on ice and transferred to a PCR machine. To perform effective amplification of cDNA, the following program was performed:

Step	**Time**	**Temperature**
First denaturation	5 min	95 °C
Second denaturation	1 min	95 °C
Annealing	0.5-1 min	57-60 °C*
Elongation	1 min	72 °C
Final extension	10 min	72 °C

* Annealing temperatures varied depending on the primers used in the experiment.

The steps were repeated for 22-28 cycles depending on the amplified sequence. Amplicons were immediately separated by the agarose gel electrophoresis and visualised by staining with ethidium bromide.

3.2.4.2. Real-time polymerase chain reaction

Quantitative real-time PCR is used to simultaneously quantify and amplify specific sequence of DNA. The procedure follows the PCR strategy but after each amplification round, the DNA is quantified. Quantification is performed by means of fluorescent dye – SYBR® Green I – that directly binds to double-stranded DNA. The bound dye generates a signal that is proportional to the DNA concentration.

Reactions were performed according to the manufacturer's instructions provided with a SYBER® Green PCR Kit. PCR reaction mix was prepared as follows:

PCR reaction component	Volume	Final concentration
Platinum® Syber® Green qPCR SuperMix-UDG	13 µl	1×
50 mM $MgCl_2$	1 µl	2 mM
10 µM forward primer*	0.5 µl	0.2 µM
10 µM reverse primer*	0.5 µl	0.2 µM
cDNA template	2 µl	not applicable
H_2O (autoclaved)	up to 25 µl	not applicable

* All primer sequences are listed in Table 6.1.1.

The amplification and quantification of cDNA was carried out by means of following program:

Step	Time	Temperature
Activation of polymerase enzyme	2 min	50 °C
First denaturation	5 min	95 °C
Second denaturation	5 s	95 °C
Annealing	5 s	59-60 °C*

Elongation	30 s	72 °C
Dissociation step 1	15 s	95 °C
Dissociation step 2	1 min	60 °C
Dissociation step 3	15 s	95 °C
Dissociation step 4	15 s	60 °C

*Annealing temperatures varied depending on the primers used in the experiment.

The steps were repeated for 45 cycles.

A ubiquitously and equally-expressed gene that is free of pseudogenes was used as the reference gene in all quantitative real-time PCR reactions. The relative transcript abundance of a gene was presented as ΔCt values ($\Delta Ct = Ct_{reference} - Ct_{target}$). Relative changes in transcript levels compared to controls were displayed as ΔΔCt values ($\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$). All ΔΔCt values corresponded approximately to the binary logarithm of the fold change.

3.2.5. DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed in order to separate and analyse DNA products obtained by PCR. Percentage of the gels varied 1–2.5 % depending on the size of the DNA amplicon. Agarose was mixed with 1× Tris-acetate-EDTA (TAE) buffer and 0.5 µg/ml ethidium bormide, a DNA intercalating dye, that enables visualisation of the DNA fragments under ultraviolet light. The DNA samples were mixed with 6× DNA loading buffer and loaded onto the gel. Electrophoresis was performed at 100 V/cm in 1× TAE buffer.

<u>1× TAE:</u>
40 mM Tris-acetate, pH = 8.0
1 mM EDTA, pH = 8.0

<u>6× DNA loading buffer:</u>
0.025 % (w/v) bromophenol blue
40 % (w/v) sucrose

3.2.6. Protein isolation

Proteins were extracted from either total lung tissue or mammalian cells. Depending on the biological material, two different extraction procedures were performed.

3.2.6.1. Protein isolation from tissues

Lung tissue preserved in liquid nitrogen was homogenised by addition of ice-cold tissue lysis buffer. Tissue lysate was then passed 5–8 times through a 0.9 mm gauge needle fitted to an RNAse-free syringe. Homogenised tissue was then incubated for 30 min on ice and centrifuged 15000 × g for 15 min at 4 °C. Resulting supernatant was used as tissue extracts and stored at -20 °C for further experiments.

Tissue lysis buffer:

20 mM Tris-HCl, pH = 7.5

150 mM NaCl

1 mM EDTA

1 mM EGTA

1 % Trition X-100

2.5 mM Na_3PO_4 phosphatase inhibitor

1 mM β-glycerophosphate inhibitor

1 mM Na_3VO_4, phosphatese inhibitor – added immediately prior to homogenisation

Complete™, protease inhibitor mix – added immediately prior to homogenisation

3.2.6.2. Protein isolation from cells

Cells were harvested at indicated time points by scraping in the cell lysis buffer with a rubber policeman. Collected cells were passed 5–8 times through a 0.9 mm gauge needle until a homogenous lysate was obtained. Lysates were then incubated for 30 min on ice and centrifuged 15000 × g for 15 min at 4 °C. Resulting supernatants were used as cell extracts and stored at -20 °C for further experiments.

Cell lysis buffer:

20 mM Tris-HCl, pH = 7.5

150 mM NaCl

1 mM EDTA

1 mM EGTA

0.5 % Igepal CA-630

1 mM Na_3VO_4, phosphatese inhibitor – added immediately prior to homogenisation

Complete™, protease inhibitor mix – added immediately prior to homogenisation

3.2.6.3. Protein precipitation from cell culture media

Three ml of cell culture media was collected in 15-ml Falcon tubes after the experiments were performed. Twelve ml of acetone was added and the mixture was incubated for over night at -20 °C. The following day, the samples were centrifuged 13000 × g for 20 min at 4 °C. The acetone was removed completely by evaporation at room temperature. Collected pellets were resuspended in cell lysis buffer (the same used for protein isolation from cells) and passed 5–8 times through a 0.9 mm gauge needle until a homogenous lysate was obtained. Lysates were then incubated for 30 min on ice and centrifuged 15000 × g for 15 min at 4 °C. The resulting supernatants were stored at -20 °C for further experiments.

3.2.6.4. Protein quantification

Protein concentrations in tissue and cell extracts were spectrophotometrically determined using Quick Start™ Bradford Dye Reagent and a Fusion A153601 Reader according to the manufacturer's instructions. The protein assay is based on the colour change of Coomassie Brilliant Blue G-250 dye after binding proteins. The dye binds primarily to basic and aromatic amino acids residues. The 10 µl of sample was mixed with 200 µl of Bradford Dye Reagent and transferred to a 96-well plate. Six dilutions of protein standard, bovine serum albumin, 0.05–0.5 µg/µl, were prepared and mixed with Bradford Dye Reagent in the same ratio as the sample of unknown concentration. Reaction mixtures were incubated for 15 min at room temperature. The absorbance of the samples was measured at 570 nm. The unknown amount of protein in the sample

was determined by interpolation, reading the concentration of protein on the standard curve that corresponded to its absorbance.

3.2.7. SDS polyarcrylamide gel electrophoresis

Protein extracts were separated by means of SDS polyacrylamide gel electrophoresis (SDS-PAGE). Before loading onto the gel, 30 µg of protein was combined with 10 × SDS-loading buffer and denaturated by heating for 10 min at 95 °C. Separation of proteins was performed in the gel consisting of 5 % stacking gel and 10 % resolving gel. Electrophoresis was carried out in SDS-running buffer at 120 V.

10× SDS-loading buffer:
625 mM Tris-HCl, pH = 6.8
50 % (v/v) glycerol
20 % (w/v) SDS
9 % (v/v) β-mercaptoethanol
0.3 % (w/v) bromophenol blue

Stacking gel:
5 % 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 % acrylamide:bisacrylamide
375 mM Tris-HCl, pH = 8.8
0.1 % (w/v) SDS
0.1 % (w/v) APS
0.1 % (v/v) TEMED

SDS-running buffer:

25 mM Tris

250 mM glycine

0.1 % (w/v) SDS

3.2.8. Immunoblotting

Immunoblotting was performed in order to visualise and detect specific proteins separated by SDS-PAGE.

3.2.8.1. Protein blotting

Proteins separated by SDS-PAGE were transferred to 0.25 µm pure nitrocellulose membrane. Transfer was performed in transfer buffer at 120 V for 1 h.

Transfer buffer:

25 mM Tris

192 mM glycine

20 % (v/v) methanol

3.2.8.2. Protein detection

Membranes were blocked in blocking solution for 1 h at room temperature. After blocking, membranes were incubated with the appropriate primary antibodies for overnight at 4 °C *. Membranes were washed three times for 10 min with 1× Phosphate-buffered saline + 0.1 % Tween 20 (PBST) buffer, incubated with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature, and then washed five times for 10 min with 1× PBST. Specific bands were visualised by chemiluminescence using an Enhanced Chemiluminescence Immunoblotting system. In order to re-probe membranes with another antibody, membranes were stripped with stripping buffer for 15 min at 52 C and subsequent protein detection was performed as described above.

Blocking solution:
5 % (w/v) non-fat dry milk
1× PBS
0.1 % (v/v) Tween-20

1× PBST:
1× PBS
0.1 % (v/v) Tween-20

Stripping buffer:
62.5 mM Tris-HCl, pH = 6.8
2 % (w/v) SDS
100 mM β-mercaptoethanol

* Primary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in Tables 6.2.1. and 6.2.2.

3.2.9. Immunohistochemistry

In order to localise and detect the expression of proteins in lung tissue, immunochistochemical analysis was performed using a Histostain-SP Kit. Whole lung sections were deparaffinised in xylene, three times for 10 min, and rehydrated in 100 % ethanol twice for 5 min, 95 % ethanol twice for 5 min, 70 % ethanol twice for 5 min, and 1× PBS twice for 5 min. Antigen retrieval was performed by incubation of the slides in 1× citrate buffer for 20 min at 100 °C, followed by cooling the slides for 10 min. Endogenous peroxidase activity was quenched by incubating sections with 3 % (v/v) H_2O_2 for 10 min. Sections were blocked with blocking solution provided in the kit for 10 min at room temperature, followed by incubation with the primary antibody overnight at 4 °C *. Normal mouse IgG was used as a negative control. Sections were washed twice with 1× PBS for 5 min, and incubated with biotinylated secondary antibodies for 10 min, followed by the Streptavidin-conjugated enzyme for 10 min, and chromogenic substrate for 10 min. Slides were developed for 5 min with diaminobenzidine (DAB) and counterstained with Mayers hematoxylin. Sections were mounted using mounting medium and examined for staining using an Olympus BX51 microscope.

* Primary antibody concentrations varied depending on the antibodies used in the experiment, and are presented in Tables 6.2.1. and 6.2.2.

3.2.10. Culture of mammalian cells

3.2.10.1. Cell culture condition

The human fibroblast cell lines, HFL1 and IMR-90, and primary human lung fibroblasts were grown in plate culture dishes in appropriate medium containing 10 % (v/v) foetal calf serum (FCS) at 37 °C, 5 % CO_2 and 95–100 % humidity. Human foetal lung fibroblasts 1 were grown in D-MEM/F12 + GlutaMAX^{TM} -I (1×), IMR-90 in D-MEM + GlutaMAX^{TM} -I (1×) containing additionally non-essential amino acids and primary human lung fibroblast in D-MEM containing additionally 10.4 mM HEPES and 4 mM (1×) L-glutamine. Cells were passaged after achieving 80-90 % confluence. Cells were washed with 1× PBS and then incubated with 3 ml trypsin/EDTA solution for 2–3 min in order to detach the cells. The 7 ml of D-MEM containing FCS was added to cells to further block the enzymatic reaction. The cell suspension was transferred to the new culture dishes in dilution 1:5 or 1:10.

PBS (phosphate-buffered saline):
0.08 % (w/v) NaCl
0.02 % (w/v) KCl
0.115 % (w/v) $Na_2HPO4\ 2H_2O$
0.02 % (w/v) $KH_2PO4\ 2H_2O$
pH = 7.4 adjusted with NaOH; sterilised for 20 min at 121 °C, 15 psi

Trypsin/EDTA solution:
0.25 % (w/v) trypsin
1.23 g/l EDTA

3.2.10.2. Isolation of primary lung fibroblasts

Human lung primary fibroblasts were derived from healthy transplant donors and patients with IPF. In order to isolate primary lung fibroblasts, lungs were perfused free of

blood, and individual lung lobes were removed to a petridish. Using sterile technique, lung lobes were finely minced with scissors, and the minced lungs were cultured for 10–14 days in D-MEM containing 10 % FCS, 10.4 mM HEPES, 4 mM (1×) L-glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). After 10 days, fibroblasts had grown out from the tissue and were passaged by standard trypsinisation. To insure the purity of the culture, cells were morphologically characterised by light microscopy, additionally specific staining for α-SMA was performed. For experiments described here, the cells were used between the second and fifth subpassages. Cells were plated at 30000 cells/cm^2 either in 6-well plates or in 100-mm plates, depending on the experiment.

3.2.10.3. Transfection with small interfering RNA

The ON-TARGETplus SMARTpool siRNA oligonucleotides specific for human ALK1 and ALK5 mRNA (Table 6.3.) were obtained from Dharmacon Inc. (Lafayette, USA). Human foetal lung fibroblasts 1 cells were transiently transfected with 100 nM ALK1 or ALK5 siRNA or Silencer® Negative siRNA control #1 using DharmaFECT 1 transfection reagent. Briefly, DharmaFECT 1 was added to OptiMEM and incubated for 5 min. Short interference ALK1, siALK5 or non-specific siRNA was added to OptiMEM, and this mix was transferred into DharmaFECT 1 and OptiMEM mix after 5 min. The mix was incubated for another 20 min at room temperature with constant shaking. Afterwards the siRNA mix was added to the cells, after 24 h cells were lysed, and efficiency of gene knockdown was monitored by real-time PCR.

3.2.10.4. Calculations for siRNA data

The remaining gene expression calculated as a percent and percent knockdown of gene expression was determined to asses the effect of siRNA on the silencing the target gene with real-time PCR. The C_T method ($\Delta\Delta C_T$) for relative quantitation is a commonly used method for measuring siRNA-induced knockdown of a particular gene. In this method, data are normalised using a control transcript (PBGD), and the normalised expression value (ΔC_T) for the gene of interest in the experimental sample is compared to the equivalent ΔC_T for the negative control siRNA-treated sample by calculating $\Delta\Delta C_T$ ($\Delta C_{T\text{-Sample}} - \Delta C_{T\text{-Negative control}}$). The $\Delta\Delta C_T$ is used to calculate then the percent remaining

gene expression (% Remaining = $2^{-\Delta\Delta CT}$) and the percent knockdown (% Knockdown = 100-% Remaining) [102] .

3.2.11. Sircol collagen assay

Total collagen content in cell lysates was determined using the Sircol Collagen Assay Kit according to the manufacturer's instructions. This is a quantitative dye–binding method designed to analyse the amount of collagen produced in mammalian tissues or cells during *in vitro* culture. The dye reagent contains the Sirius Red anion with sulfonic acid side chain groups, which reacts with the side chain groups of the basic amino acids present in collagen.

Equal amounts of cell lysates containing 100 µg of protein were added to 1 ml of Sircol dye reagent followed by 30 min of mixing at room temperature. After centrifugation at 10000 × g for 10 min, the supernatant was carefully removed and 1 ml of alkali reagent was added, and the pellet was dissolved by vortexing. Samples and collagen standards were analysed at 540 nm in a Fusion A153601 Reader. Collagen concentrations were calculated using standard curve with acid–soluble type I collagen.

3.2.12. Statistical analysis of data

Values are presented as mean ± SEM. The mean of indicated groups were compared using two-tailed Student´s *t*-test. A level of $p<0.01$, $p<0.02$, $p<0.05$, $p<0.005$, or $p<0.0005$ was considered statistically significant, depending on the experiment. All experiments were performed at least three times.

4. Results

4.1. Analysis of the expression of TGF-β system components in human lungs

4.1.1. Expression analysis of TGF-β receptors by semi-quantitative PCR

To investigate potential changes in the levels of the TGF-β system components in human lungs, mRNA expression of TGF-β receptors in human lung tissue was analysed. The RNA was isolated from human lung homogenates of transplant donors and IPF patients. The mRNA levels of receptors were determined by semi-quantitative RT-PCR (Figure 4.1.). No significant changes in the expression were observed in most of the receptors. Interestingly, only TGF-β type I receptor, ALK1 mRNA expression level was decreased in the fibrotic samples compare to healthy subjects.

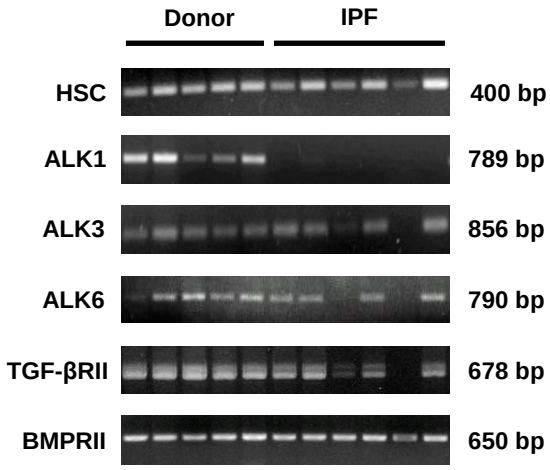


Figure 4.1. Expression analysis of TGF-β receptors in the lungs of transplant donors and IPF patients.
Transforming growth factor β receptor mRNA expression was analysed by semi-quantitative RT-PCR in homogenates from healthy lungs (donor; n=5) and lungs from IPF (n=6) patients. The RNA was isolated from the lungs directly frozen after explantation. Heat shock protein 70 (HSC) was used as the loading control.

4.1.2. Expression analysis of Smads by semi-quantitative PCR

To further investigate potential changes in the levels of the TGF-β system components in human lungs, mRNA expression of intracellular mediators, Smads, in human lung tissue was analysed. The RNA was isolated from homogenates of transplant donors and IPF patients, previously used to assess TGF receptor expression. The mRNA levels of Smads were determined by semi-quantitative RT-PCR (Figure 4.2.). No changes in the expression were observed for Smads, except for Smad3 and Smad4 mRNA expression levels, which were decreased in the fibrotic samples compared to healthy donors.

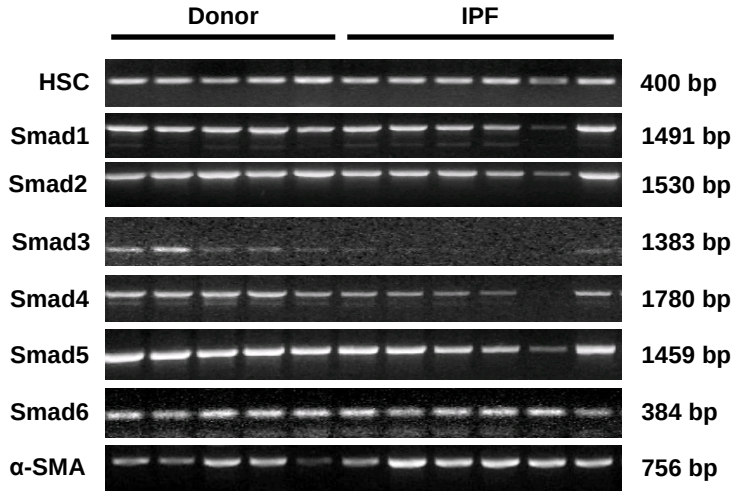


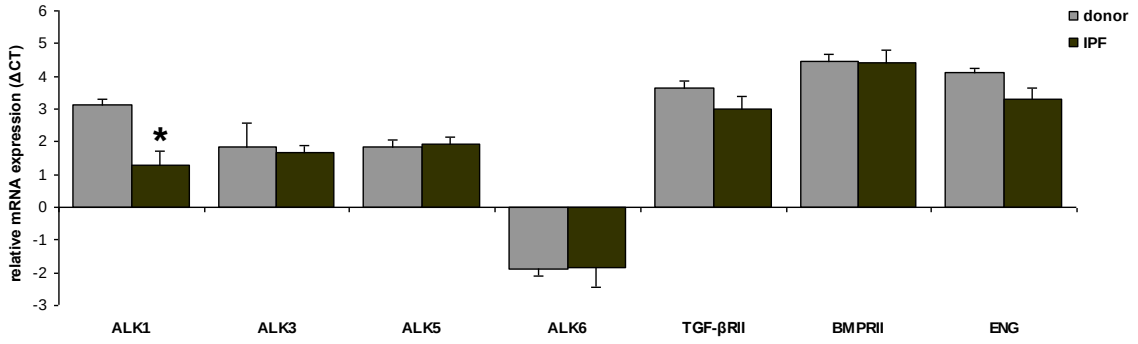
Figure 4.2. Expression analysis of Smads in lungs of transplant donors and IPF patients.
Smads mRNA expression was analysed by semi-quantitative RT-PCR in homogenates from healthy lungs (donor; n=5) and lungs from IPF (n=6) patients. RNA was isolated from the lungs directly frozen after explantation. Heat shock protein 70 (HSC) was used as the loading control, α-SMA was used as the positive control for fibrotic samples.

4.1.3. Expression analysis of TGF-β receptors and Smads by quantitative PCR

To confirm the semi-quantitative RT-PCR results, the mRNA expression level of TGF-β receptors and intracellular mediators, Smads, in human lung tissues were analysed. The RNA was isolated from human lung homogenates of transplant donors and IPF patients. Quantitative real-time PCR (Figure 4.3.) was used to determine the mRNA levels of receptors and Smads. Of all genes investigated, the type I TGF-β

receptor ALK1 (ΔCt of 1.28 and 3.11 in IPF and controls, respectively) exhibited significantly lower expression levels in IPF lungs. No evidence of altered Smad3 mRNA expression was detected by quantitative PCR in fibrotic samples, as detected by semi-quantitative PCR (Figure 4.2.)

A.



B.

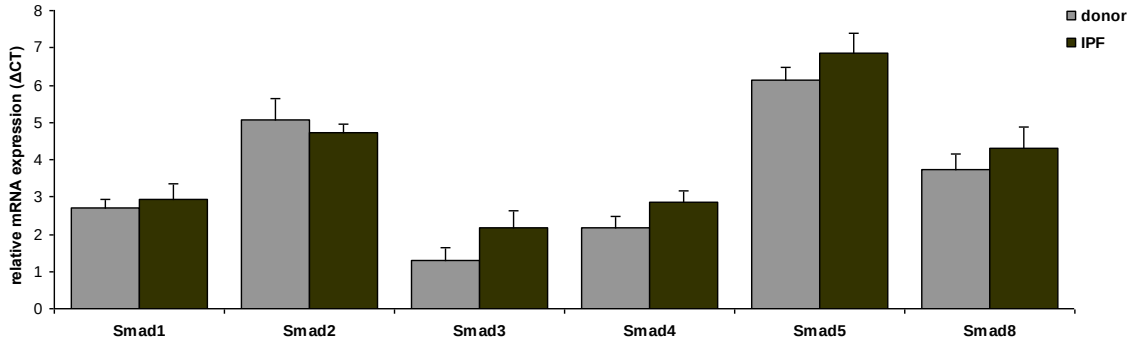


Figure 4.3. Expression analysis of TGF-β receptors and Smads in lungs of transplant donors and IPF patients.
Transforming growth factor β receptors (**A**) and Smads (**B**) mRNA expression was analysed by quantitative real-time PCR in homogenates from healthy lungs (donor) and lungs from IPF patients. Values are represented as mean ± S.E.M. (n=12). Statistically significant differences of $p \leq 0.01$ (by Student's t-test) are marked by *.

4.1.4. Expression analysis of TGF-β target genes

There are many target genes regulated by TGF-β. Among them are genes encoding proteins involved in the activation of fibroblasts, driving the production of ECM, and are, therefore, crucial for the pathogenesis of IPF. α-Smooth muscle actin and collagen type I (COL1A1) have been identified as the main participants in this disease,

and the expression levels of these proteins in human lung tissue were assessed. Additionally, the expression pattern of the target genes of TGF-β/ALK1 and TGF-β/ALK5 pathways, Id1 and PAI1, respectively, were analysed. The mRNA levels of target genes were determined by quantitative PCR (Figure 4.4.).

As expected, the mRNA levels of α-SMA (ΔCt of 1.7 and -0.2 in IPF and controls, respectively) and COL1A1 (ΔCt of 5.5 and 1.9 in IPF and controls, respectively) were significantly elevated in fibrotic samples. The mRNA expression levels of Id1 (ΔCt of 4.3 and 5.1 in IPF and controls, respectively) and PAI1 (ΔCt of 2.0 and 1.1 in IPF and controls, respectively) revealed similar patterns of expression as ALK1 and ALK5, respectively in human lung tissue.

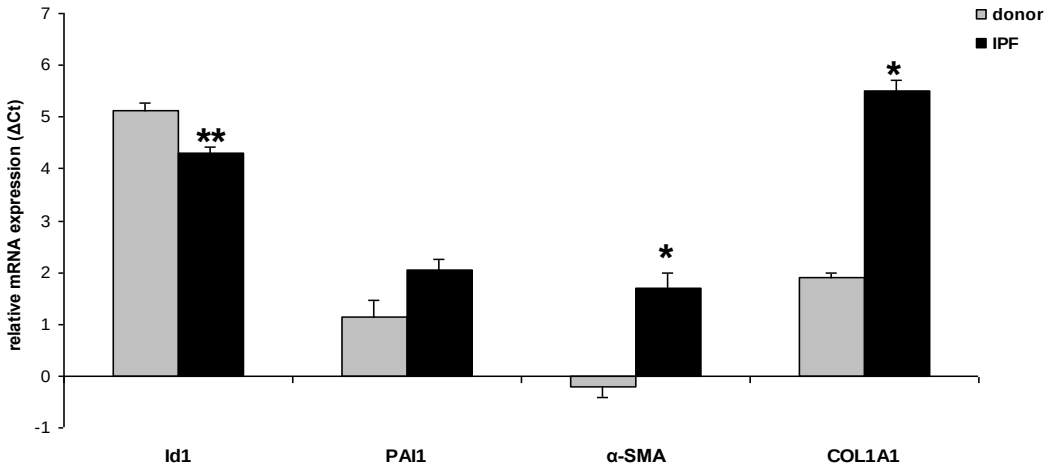


Figure 4.4. Expression analysis of TGF-β target genes in lungs of transplant donors and IPF patients.
Transforming growth factor β target genes mRNA expression was analysed by quantitative real-time PCR in lung homogenate from healthy lungs (donor) and lungs from IPF patients. Values are represented as mean ± S.E.M. (n=6). Statistically significant differences of $p \leq 0.01$ and $p \leq 0.02$ (by Student's t-test) are marked by * and **, respectively.

4.2. Localisation of ALK1 and ALK5 in human lungs

Previous results suggested a decrease in ALK1 mRNA expression in total lung homogenates from transplant donors and IPF patients. In order to verify ALK1 expression patterns in lung tissue, immunohistochemistry was performed on the sections obtained from healthy and fibrotic patients (Figure 4.5.). Moreover, it was examined whether the expression was specifically localised to particular cell types in the human lung.

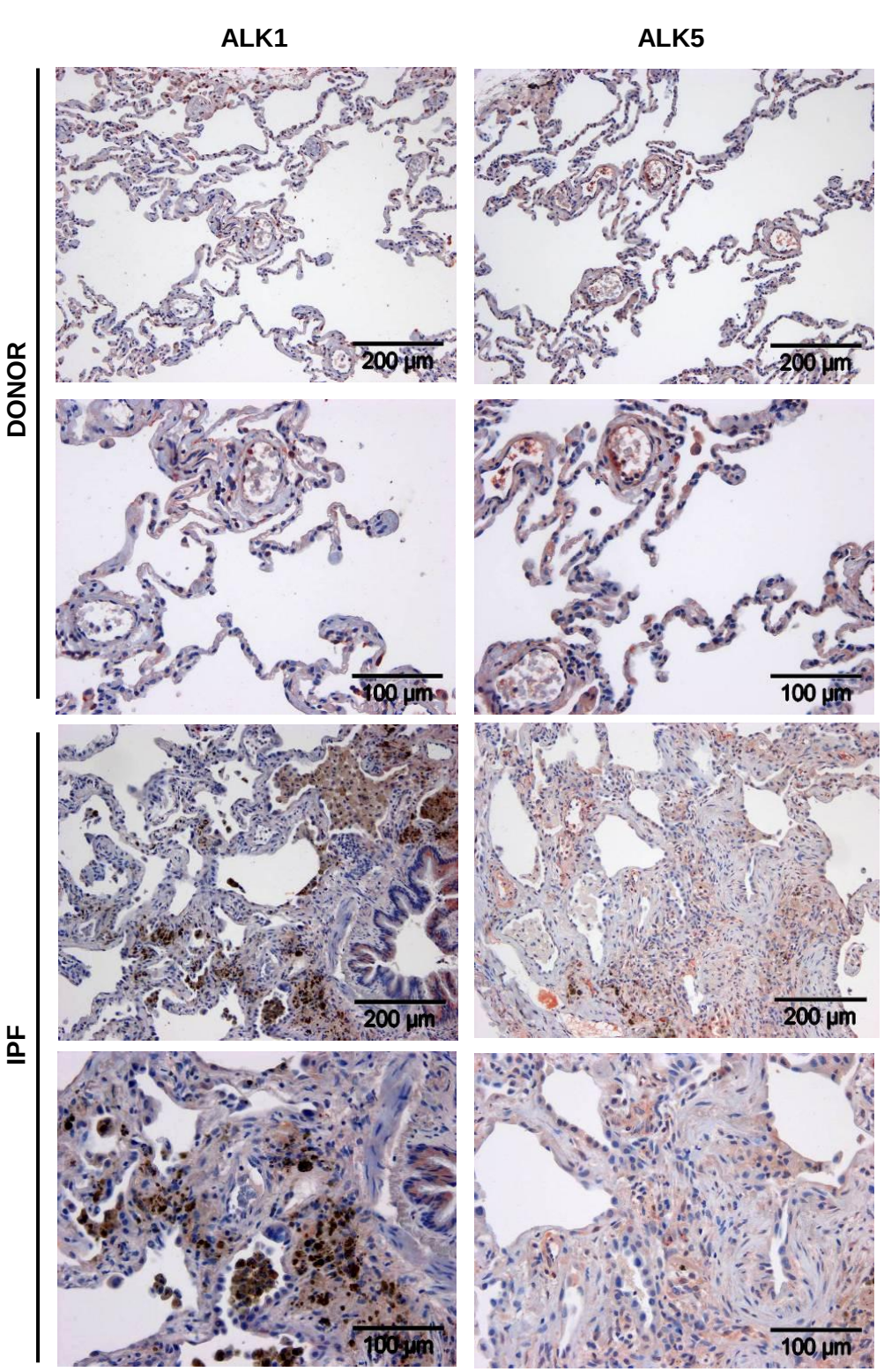


Figure 4.5. Localisation of ALK1 and ALK5 in tissue sections from the lungs of transplant donors and IPF patients.

Paraffin-embedded lung sections from healthy (donor) and IPF patients were stained for ALK1, ALK5 (**A**) and α-SMA (**B**), as indicated. The IgG control (**B**) was performed as the primary antibody control. The pictures are representative of three independent experiments.

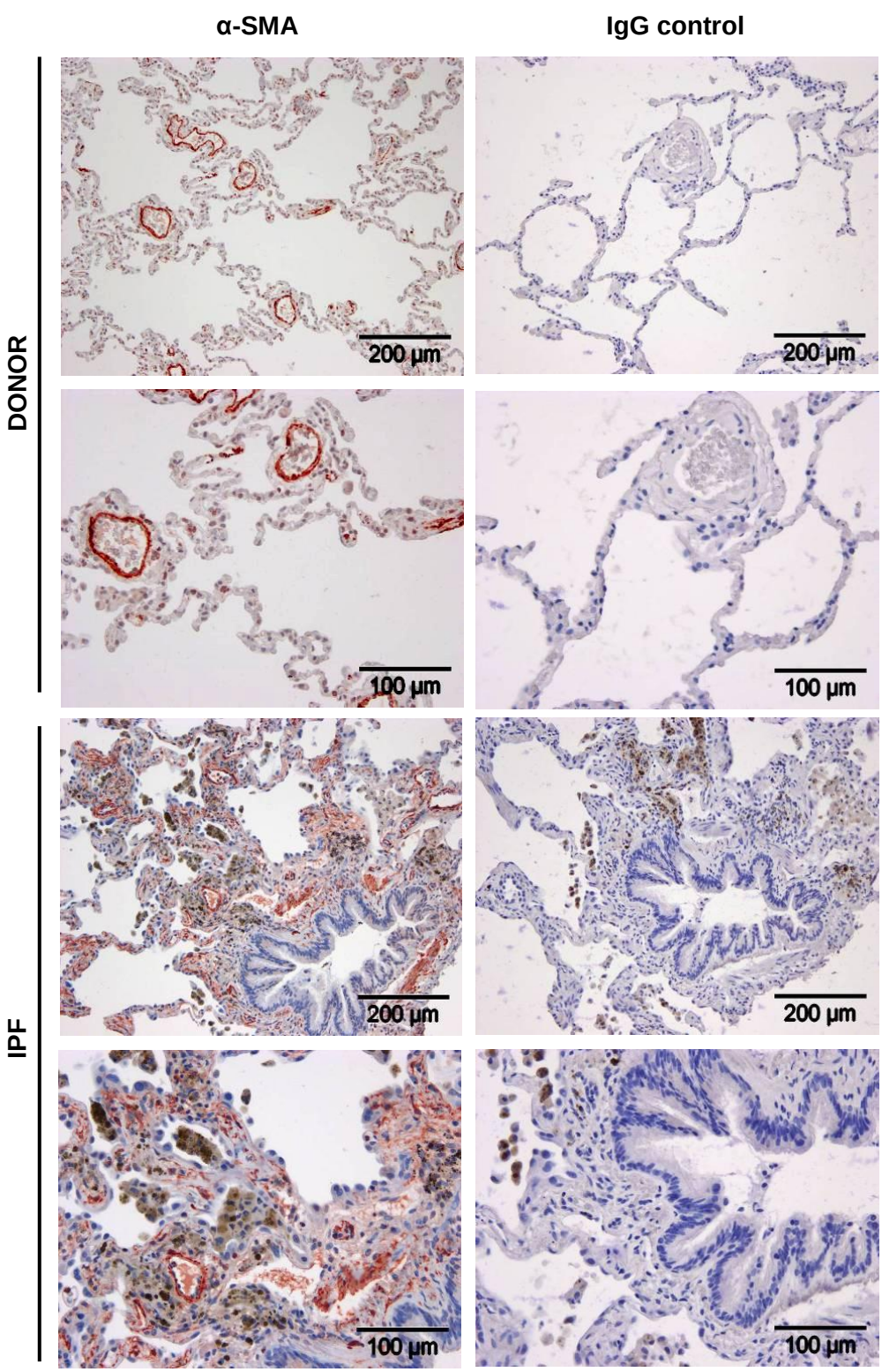


Figure 4.5. Localisation of ALK1 and ALK5 in tissue sections from the lungs of transplant donors and IPF patients.

Paraffin-embedded lung sections from healthy (donor) and IPF patients were stained for ALK1, ALK5 (**A**) and α-SMA (**B**), as indicated. The IgG control (**B**) was performed as the primary antibody control. The pictures are representative of three independent experiments.

Activin receptor-like kinase 5 was localised widely to endothelial cells, bronchial and alveolar epithelial cells, smooth muscle cells, as well interstitial fibroblasts, whereas ALK1 was localised mainly to endothelial cells, and to a certain extent, to epithelial cells, and to the interstitial regions which were α-SMA positive. Furthermore, ALK1 localised to cells that also showed localisation of α-SMA which stains interstial regions representing activated myofibroblasts. On comparing donors with fibrotic sections, ALK1 staining appears to be highly expressed in IPF than in donors, but this is not the case since immunohistochemistry is not a quantitative method and is widely applicable for determining cell specific localisation of proteins of interest.

4.3. Analysis of the expression of TGF-β system components in primary human cells and cell lines

4.3.1. Expression analysis of TGF-β system components in primary human cells

4.3.1.1. Expression analysis of TGF-β system components and target genes in primary human fibroblasts

As immunohistochemical results indicated the localisation of ALK1 in lung fibrotic foci, further investigations were performed in order to examine the expression of this receptor and other TGF-β system components as well as the target genes in human primary lung fibroblasts - the key cell type responsible for increased collagen deposition during lung fibrosis. Therefore, isolation of mRNA was carried out using primary fibroblasts derived from the lungs of healthy and IPF patients. The mRNA expression analysis was performed by quantitative real-time PCR (Figure 4.6.).

As expected, expression levels of ALK1 (∆Ct of -3.2 and -1.5 in IPF hFB and donor hFB, respectively) was significantly downregulated in the fibrotic primary human fibroblasts. The other TGF-β receptors and Smads did not show any significant changes, except for Smad1 (∆Ct of -1.2 and 0.2 in IPF hFB and donor hFB, respectively) revealing downregulation in the fibrotic fibroblasts, but these differences were not significant. The mRNA expression level of target genes, α-SMA and COL1A1, was increased, but not significantly, in the fibrotic fibroblasts. The expression level of Id1 and PAI1 exhibit the

similar pattern of expression as in the lung homogenates, although the mRNA relative expression units (ΔCt) were different in both of these experiments.

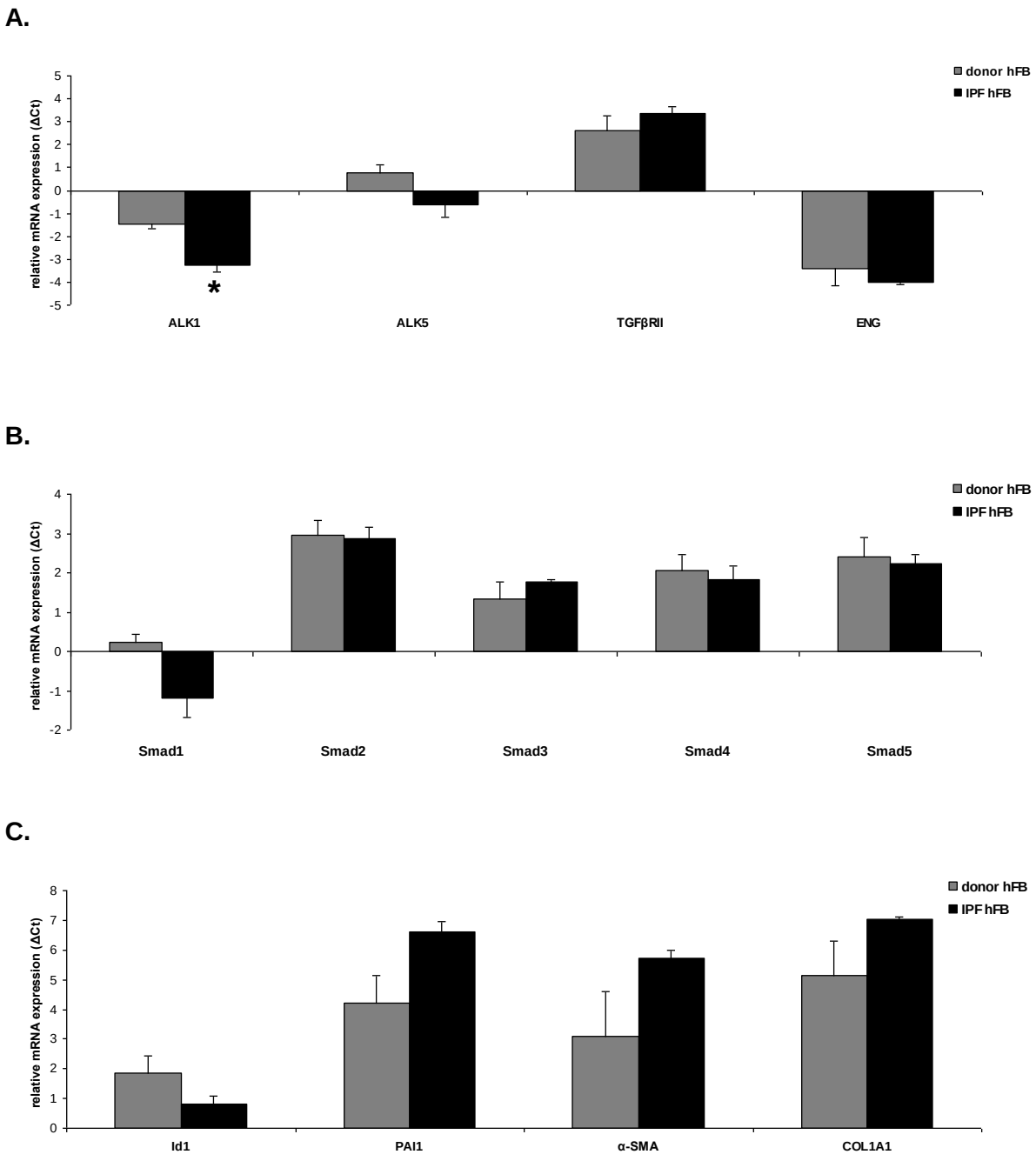


Figure 4.6. Expression analysis of TGF-β system components in primary fibroblasts from the lungs of transplant donors and IPF patients.

Transforming growth factor β receptors (**A**), Smads (**B**) and the TGF-β target genes (**C**) mRNA expression was analysed by quantitative real-time PCR in the fibroblasts isolated from healthy (donor) and fibrotic (IPF) human lungs. Values are represented as mean ± S.E.M. (n=3). Statistically significant differences of $p \leq 0.05$ (by Student's t-test) are marked by *. hFB – human fibroblasts.

4.3.1.2. Expression analysis of TGF-β system components in pSMC, EpC and HUVEC

The mRNA expression of ALK1 in the primary fibroblasts was found to be attenuated in lung fibrosis. To further verify the expression of ALK1 in other human primary cells, the endogenous expression level of TGF-β receptors was examined in human primary pulmonary smooth muscle cells (pSMC), primary epithelial cells (EpC) derived from donor and fibrotic human lungs, and human umbilical vein endothelial cells (HUVEC). The endogenous level of TGF-β target genes was verified in pSMC and HUVEC. The mRNA expression analysis was performed by quantitative PCR (Figure 4.7.).

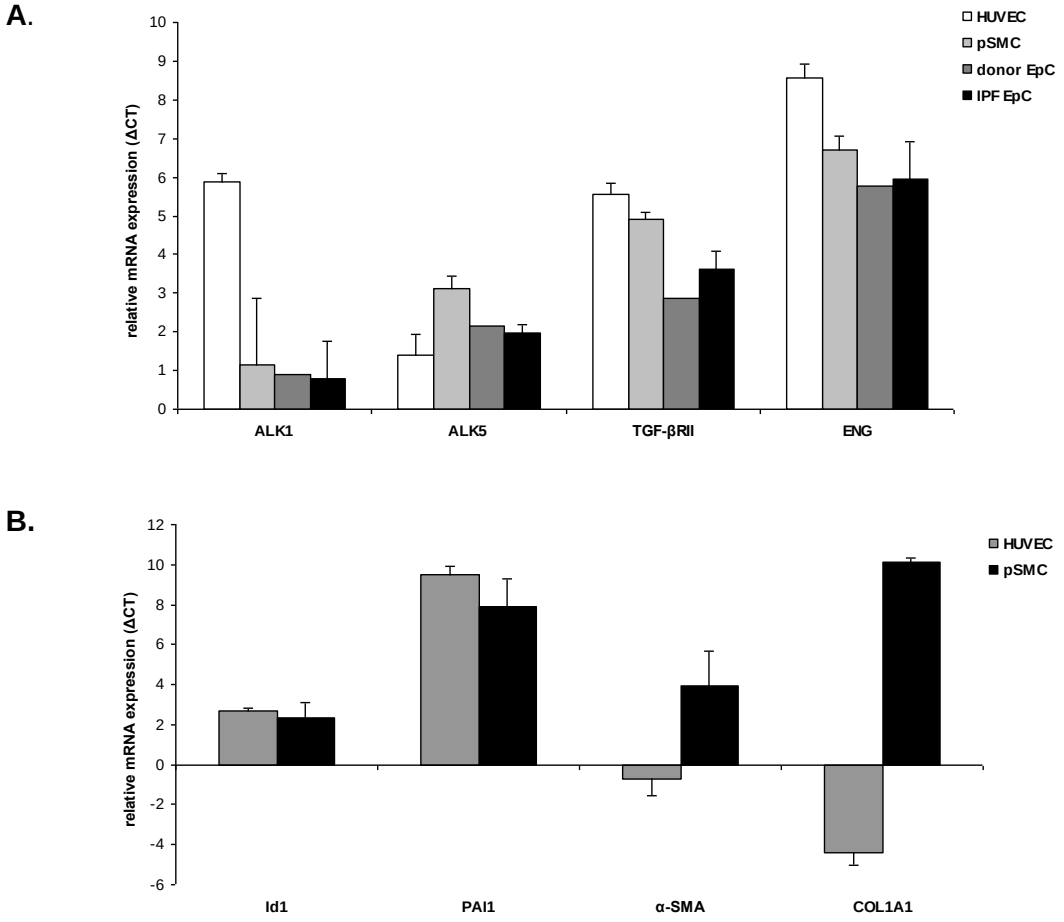


Figure 4.7. Endogenous mRNA expression analysis of TGF-β receptors and target genes in pulmonary smooth muscle cells, human umbilical vein endothelial cells and epithelial cells from transplant donors and IPF patients.

The mRNA expression levels of the TGF-β receptors (**A**) and the TGF-β target genes (**B**) were analysed by quantitative real-time PCR in the pSMC, EpC. Human umbilical vein endothelial cells serve as the positive control for ALK1 and endoglin mRNA expression. Values represent mean ± S.E.M. (n=4), except for donor EpC (n=1).

The expression of ALK1 in the lung pSMC and EpC is generally low (ΔCt of 1.1, 0.9 and 0.8 in pSMC, healthy EpC and fibrotic EpC, respectively) compared to HUVEC (ΔCt of 5.9) which are known to have a very high level of endogenous ALK1 and endoglin. The other receptors are expressed at the high levels implying that the TGF-β signalling receptors are present in all these cells. The level of Id1 (ΔCt of 2.7 and 2.3 in HUVEC and pSMC, respectively) is not as high as expected when compared to the level of ALK1 in this cell type. The low levels of α-SMA (ΔCt of -0.9) and COL1A1 (ΔCt of -4.5) mRNA expression in HUVECs in comparison to pSMC (ΔCt of 3.9 for α-SMA and 10.1 for COL1A1) confirms that HUVECs does not play such a big role in ECM production as pSMC do.

4.3.2. Expression analysis of TGF-β system components in human cell lines

4.3.2.1. Expression analysis of TGF-β system components in IMR-90 cells

Due to the limited amount of the freshly isolated primary fibroblasts, the mRNA expression of TGF-β system components was analysed in the human lung fibroblast cell lines, IMR-90 and HFL1. The IMR-90 cell line is a human lung fibroblast cell line derived from a caucasian female. The mRNA expression level of TGF-β type I receptors, TGF-β type II receptor, endoglin and TGF-β target genes in IMR-90 cells was analysed by quantitative real-time PCR (Figure 4.8.).

As expected, the mRNA expression levels of most of these genes were similar to those seen in primary fibroblasts, except for endoglin and Id1. Endoglin expression (ΔCt of 2.4 and -3.4 in IMR-90 and donor primary fibroblasts, respectively) was higher in IMR-90 than in primary fibroblasts, and Id1 expression (ΔCt of -4.0 and 1.8 in IMR-90 and donor primary fibroblasts, respectively) was very low in IMR-90 in comparison to the expression level seen in primary fibroblasts.

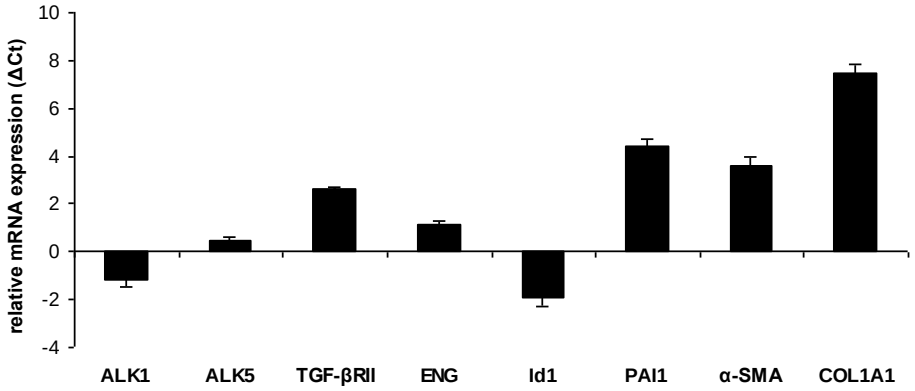


Figure 4.8. Endogenous mRNA expression analysis of TGF-β receptors and target genes in the human lung fibroblast cell line, IMR-90.
The mRNA expression levels of the TGF-β receptors and the TGF-β target genes were analysed by quantitative real-time PCR in IMR-90 cell. Values are represented as mean ± S.E.M. (n=3).

4.3.2.2. Expression analysis of TGF-β system components in HFL1 cells

The mRNA expression levels of TGF-β system components were analysed in another human lung fibroblast cell line, HFL1, which are foetal lung fibroblasts. The mRNA expression levels of ALK1, ALK5, TGF-β type II receptor, endoglin and TGF-β target genes in HFL1 cells were analysed by quantitative real-time PCR (Figure 4.9.). The expression levels of investigated genes displayed the same pattern in HFL1 as the mRNA expression levels in IMR-90 cells.

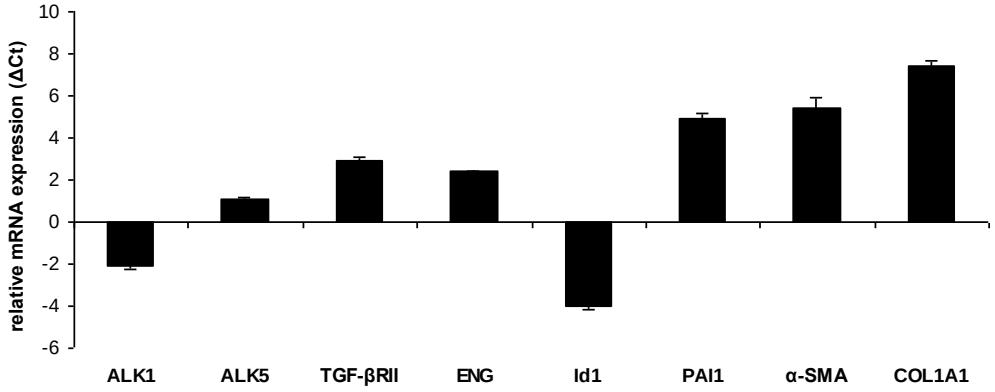


Figure 4.9. Endogenous mRNA expression analysis of TGF-β receptors and target genes in the human lung fibroblast cell line, HFL1.
The mRNA expression levels of the TGF-β receptors and the TGF-β target genes were analysed by quantitative real-time PCR in the HFL1 cells. Values are represented as mean ± S.E.M. (n=3).

4.4. TGF-β signalling activity in HFL1 and IMR-90 fibroblast cell lines

4.4.1. Analysis of the phosphorylation of Smads in HFL1 and IMR-90 fibroblast cell lines

Previous results displayed the presence of TGF-β signalling components in IMR-90 and HFL1 cell lines at the mRNA level. The activation of TGF-β signalling leads to the phosphorylation of the downstream mediators: the Smads. To investigate a signalling in IMR-90 and HFL1 cells, cells were stimulated with TGF-β1 or BMP2 ligands for 30 s or 2 h, and phosphorylated Smad proteins were analysed by immunoblotting (Figure 4.10.).

Both TGF-β and BMP signalling pathways are activated in IMR-90 and HFL1. The phosphorylation of Smad2 and Smad1/5/8 occurs at the earliest time point, after 30 s of TGF-β1 and BMP2 stimulation, respectively. Surprisingly, after TGF-β1 stimulation the phosphorylation of BMP-specific Smads was visible, indicating the presence of an additional pathway, ALK1/Smad1/5/8.

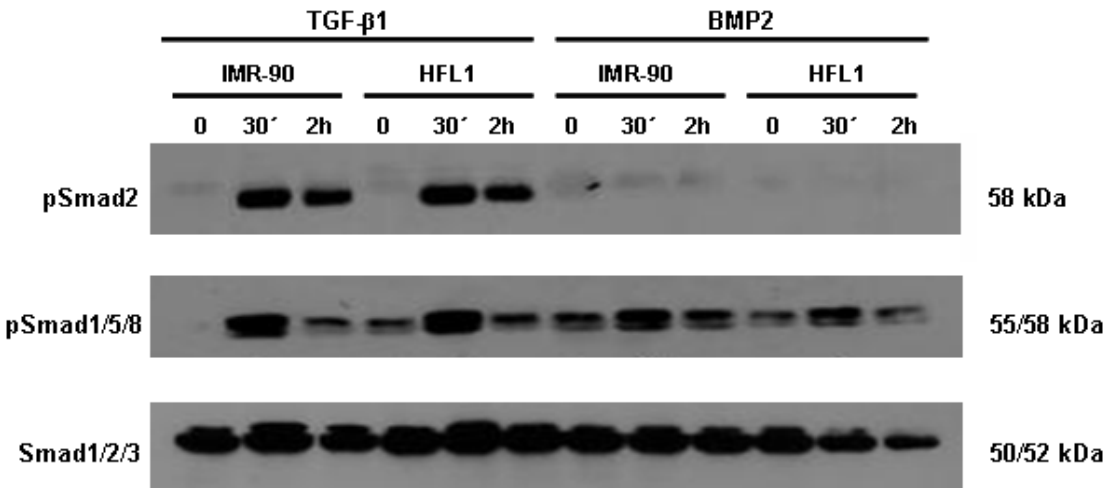


Figure 4.10. TGF-β signalling activity in human fibroblast cell lines.
Phosphorylation of Smad2 and Smad1/5/8 in the human fibroblast cell lines, IMR-90 and HFL1, after TGF-β1 and BMP2 stimulation for 30 s and 2h. Smad1/2/3, total amount of Smad1/2/3 proteins level, serves as a loading control. The images are representative of three independent experiments.

4.5. siRNA knockdown of ALK1 and ALK5 in HFL1 and IMR-90 fibroblast cell lines

4.5.1. Knockdown of ALK1 in HFL1 and IMR-90 fibroblast cell lines

Attenuated levels of ALK1 were observed in the fibrotic samples of the total lung homogenates and the primary fibroblasts, as well in the lung fibroblast cell lines. To investigate, whether the downregulation of ALK1 might cause the excessive ECM production leading to the IPF, siRNA-mediated knockdown of the TGF-β type I receptor was performed. The fibroblast cell lines were transfected with increasing amounts of siRNA directed against human ALK1. The knockdown efficiency was analysed by quantitative real-time PCR (Figure 4.11.) and the percent gene knockdown was calculated as described in the chapter 3.2.11.4.

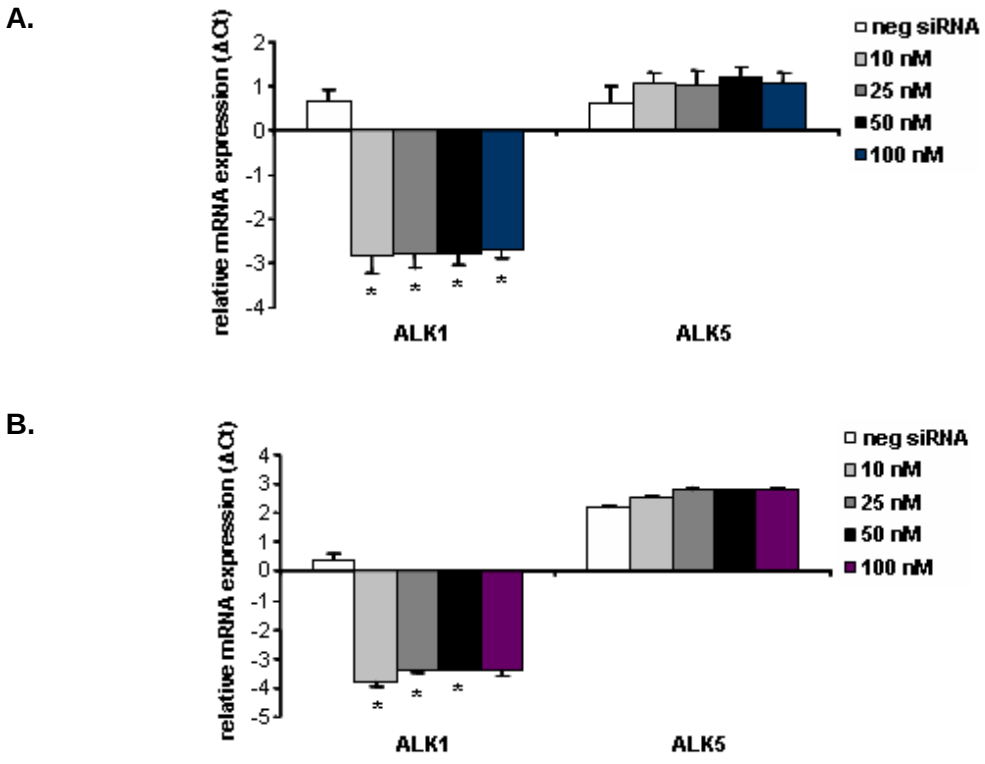


Figure 4.11. Analysis of siRNA-mediated knockdown of ALK1.
The cells were transfected with the increasing concentrations of siRNA specific for the ALK1 gene (10, 25, 50 and 100 nM) for 36 h and the ALK1 and ALK5 mRNA expression levels were analysed by quantitative real-time PCR in the human fibroblasts cell lines, HFL1 (**A**) and IMR-90 (**B**). Values represent mean ± S.E.M. (n=3). Statistically significant differences of $p \leq 0.0005$ in A and $p \leq 0.005$ in B (by Student's t-test) are marked by *.

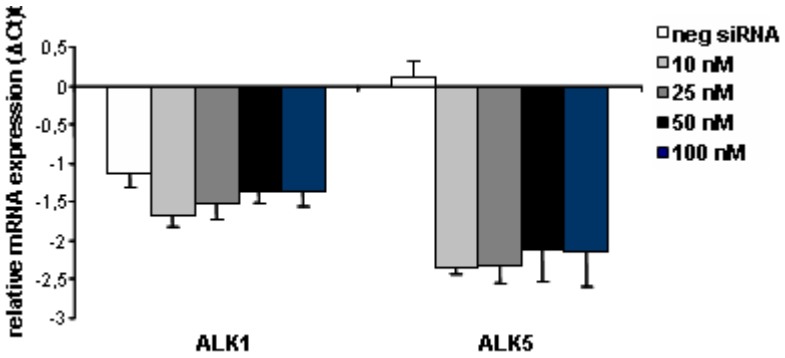
Activin receptor-like kinase 1 mRNA expression was significantly downregulated by siRNA knockdown technology, in both IMR-90 and HFL1 cells. The best knockdown (91.2%) in HFL1 cells was seen at a concentration of 10 nM of siRNA, but the other concentrations were also effective (25 nM – 90.7%, 50 nM – 90.7% and 100 nM – 90.1%). Similarly, in IMR-90 cells the best knockdown was observed at a siRNA concentration of 10 nM (94.4%). The percent knockdown with other siRNA concentrations was as effective as in HFL1 cells, at a concentration of 25 nM – 92.9%, 50 nM – 92.6% and at 100 nM – 92.7%. Activin receptor-like kinase 5 mRNA expression was not disturbed by any of siRNA targeting ALK1.

4.5.2. Knockdown of ALK5 in HFL1 and IMR-90 fibroblast cell lines

Activin receptor-like kinase 5 is a receptor co-existing with ALK1 and as reported [79]; both play well-balanced roles in endothelial cells. The siRNA-mediated gene knockdown of ALK5 was performed to investigate the role of the second TGF-β type I receptor in contrast to ALK1 in the process of ECM production. The IMR-90 and HFL1 were transfected with increasing amounts of siRNA directed against human ALK5. The knockdown efficiency was analysed by quantitative real-time PCR (Figure 4.12.) and the percent gene knockdown was calculated as mentioned in section 3.2.11.4.

Activin receptor-like kinase 5 mRNA was significantly downregulated by siRNA oligonucleotides, in both IMR-90 and HFL1 cells. In HFL1, the best concentration was 10 nM (81.9% of gene knockdown), but other concentrations were equally good at knocking down the expression of ALK5, for example 10 nM. At 25 nM of siRNA the percent knockdown was 81.6%, at 50 nM – 79.0% and at 100 nM – 79.2%. In IMR-90 cells, the siRNA effectiveness was calculated as follows: 10 nM – 85.9%, 25 nM – 78.2%, 50 nM – 84.7% and 100 nM – 84.7%. In both cases, the minimal concentration of 10 nM was significant and sufficient to knockdown more than 80% of the ALK5 mRNA expression, similar to the effect of ALK1 siRNA knockdown. Activin receptor-like kinase 1 mRNA expression was not changed during the siRNA treatment of ALK5 in both fibroblast cell lines.

A.



B.

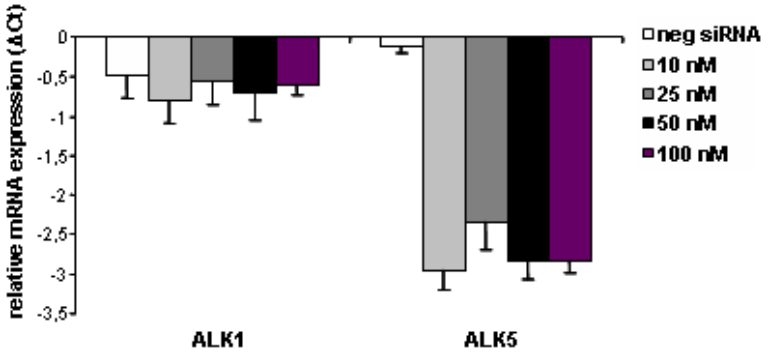


Figure 4.12. Analysis of siRNA-mediated knockdown of ALK5.
The cells were transfected with the increasing concentrations of siRNA specific for the ALK5 gene (10, 25, 50 and 100 nM) for 36 h and the ALK1 and ALK5 mRNA expression levels were analysed by quantitative real-time PCR in the human fibroblasts cell lines, HFL1 (**A**) and IMR-90 (**B**). Values represent mean ± S.E.M. (n=3). Statistically significant differences of $p \leq 0.005$ (by Student's t-test) are marked by *.

4.6. Effect of siRNA-mediated downregulation of ALK1 on ECM deposition in HFL1 and IMR-90 fibroblasts

4.6.1. Expression analysis of TGF-β receptors and target genes after knockdown of ALK1 in fibroblast cell lines

Whether a direct causal relationship existed between downregulation of ALK1 and the process of ECM deposition in human fibroblasts was next examined. From the previous results, there is a very effective siRNA-mediated knockdown of both, ALK1 and ALK5, in HFL1 and IMR-90 fibroblasts. The HFL1 and IMR-90 cells were transfected with

10 nM of siRNA specific for human ALK1 for 48 h and stimulated with TGF-β1 for an additional 24 h. The mRNA expression levels of ALK1, ALK5, TGF-β type II receptor, endoglin and TGF-β target genes in HFL1 cells were analysed by quantitative real-time PCR (Figure 4.13.).

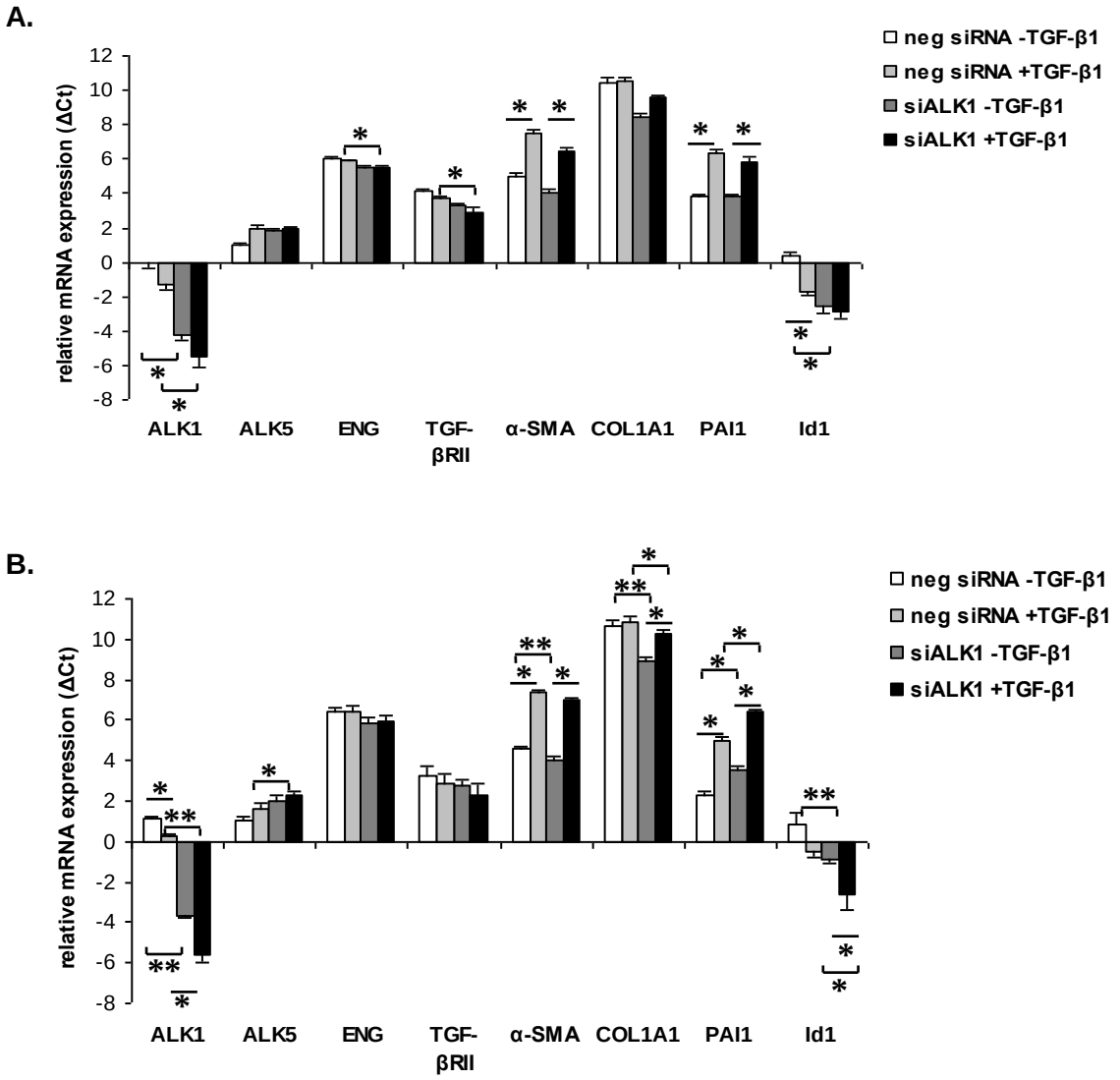


Figure 4.13. Expression analysis of TGF-β receptors and target genes after knockdown of ALK1 in fibroblast cell lines.

Cells were transfected with 10 nM of siRNA specific for the ALK1 gene for 48 h and stimulated with TGF-β1 for an additional 24 h. The mRNA expression levels of the TGF-β receptors and the TGF-β target genes were analysed by quantitative reverse transcriptase PCR in the human fibroblasts cell lines, HFL1 (**A**) and IMR-90 (**B**). Values represent mean ± S.E.M. (n=4). Statistically significant differences of $p \leq 0.05$ and $p \leq 0.005$ (by Student's t-test) are marked by * and **, respectively. The knockdown level was more than 80% in both cell lines.

As shown previously, there was a significant knockdown of ALK1 without affecting the ALK5, TGF-βRII and endoglin mRNA levels. COL1A1 mRNA expression in IMR-90 cells was significantly downregulated after siRNA treatment (ΔCt of 10.6 and 8.9 in negative siRNA and siALK1 without TGF-β1 stimulation, respectively), and was partially rescued by TGF-β1 stimulation, but was still significantly lower in comparison to the negative siRNA-treated cells (ΔCt of 10.9 and 10.2 in negative siRNA and siALK1 with TGF-β1 stimulation, respectively). The results obtained with HFL1 cells were not significant, but reveal the same pattern of COL1A1 gene expression without TGF-β1 stimulation (ΔCt of 10.4 and 8.4 in negative siRNA and siALK1, respectively), as well as after TGF-β1 treatment (ΔCt of 10.6 and 9.6 in negative siRNA and siALK1, respectively). The mRNA expression levels of PAI1 and α-SMA were not altered by siRNA-mediated knockdown of ALK1, although TGF-β1 was a very potent stimulator of expression of these genes independently of siALK1. The Id1 mRNA level was significantly changed during the siRNA transfection and TGF-β1 stimulation, reflecting exactly the level of ALK1 mRNA during the same treatments.

4.6.2. Effect of ALK1 knockdown on TGF-β1-induced collagen deposition in fibroblast cell lines

Increased collagen deposition, largely mediated by enhanced TGF-β1 signalling, represents the pathological hallmark of fibrosis [19]. Additionally, TGF-β1 induces the differentiation of myofibroblasts and the collagen production by these cells [31]. Previous results indicated the significantly increased level of COL1A1 expression upon TGF-β1 stimulation and ALK1 knockdown. Therefore, an investigation was performed to determine collagen deposition in the presence of the ALK1 siRNA in human fibroblast cell lines. Cells were transfected with siRNA against ALK1 or ALK5 for 48 h and subsequently stimulated with TGF-β1 (2 ng/ml) for up to 24 h, and cellular collagen content was analysed by Sircol assay (Figure 4.14.).

The TGF-β1-induced increase in collagen production (35.12 ng/ml and 42.85 ng/ml of collagen content in neg siRNA treated cells without or with TGF-β1 stimulation, respectively) was significantly attenuated by siRNA-mediated knockdown of ALK1 in HFL1 cells (42.85 ng/ml and 32.81 ng/ml of collagen content in neg siRNA or siALK1 treated cells after TGF-β1 stimulation, respectively). No changes in COL1A1 levels upon

siRNA treatment were detected in IMR-90 cells. The collagen production was not altered by ALK5 knockdown in both cell types.

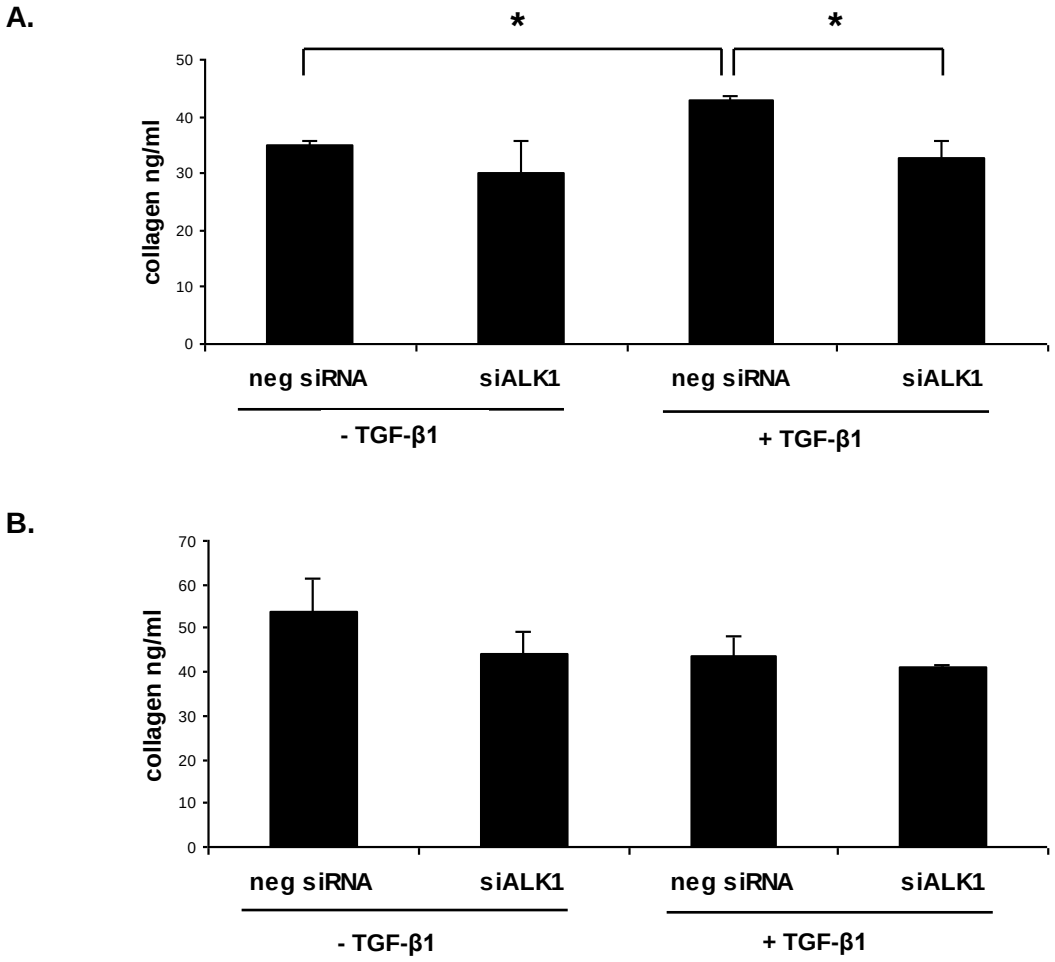


Figure 4.14. Effect of ALK1 siRNA knockdown on TGF-β1-induced cellular collagen production in fibroblast cell lines.

The cells were transfected with 10 nM of siRNA specific for the ALK1 gene for 48 h and stimulated with TGF-β1 for an additional 24 h. Cellular collagen content was analysed by Sircol assay in the human fibroblast cell lines, HFL1 (**A**) and IMR-90 (**B**). Values represent mean ± S.E.M. (n=3). Statistically significant differences ($p \leq 0.05$, student's t-test) are marked by *.

The collagen deposition is characterised not only by cellular collagen production, but also by collagen secretion into the extracellular space leading to the excessive ECM protein accumulation in the fibrotic lung tissue [40]. Therefore, the determination of secreted collagen level in the presence of the ALK1 siRNA in human fibroblast cell lines was performed. Cells were transfected with ALK1 siRNA for 48 h and stimulated with TGF-β1 (2 ng/ml) for an additional 24 h, and cell culture media were collected. Collagen content in the cell culture supernatant was analysed by Sircol assay (Figure 4.15.).

The collagen content in the cell culture media of HFL1 cells did not reveal any changes after ALK1 ablation, and treatment with TGF-β1. However, the collagen levels in the cell culture supernatants of IMR-90 cells was decreased by ALK1 knockdown, and was significantly reversed after TGF-β1 stimulation (32.88 ng/ml and 65.31 ng/ml collagen in the cell culture supernatants of the cells transfected with siALK1 without or with TGF-β1 stimulation, respectively).

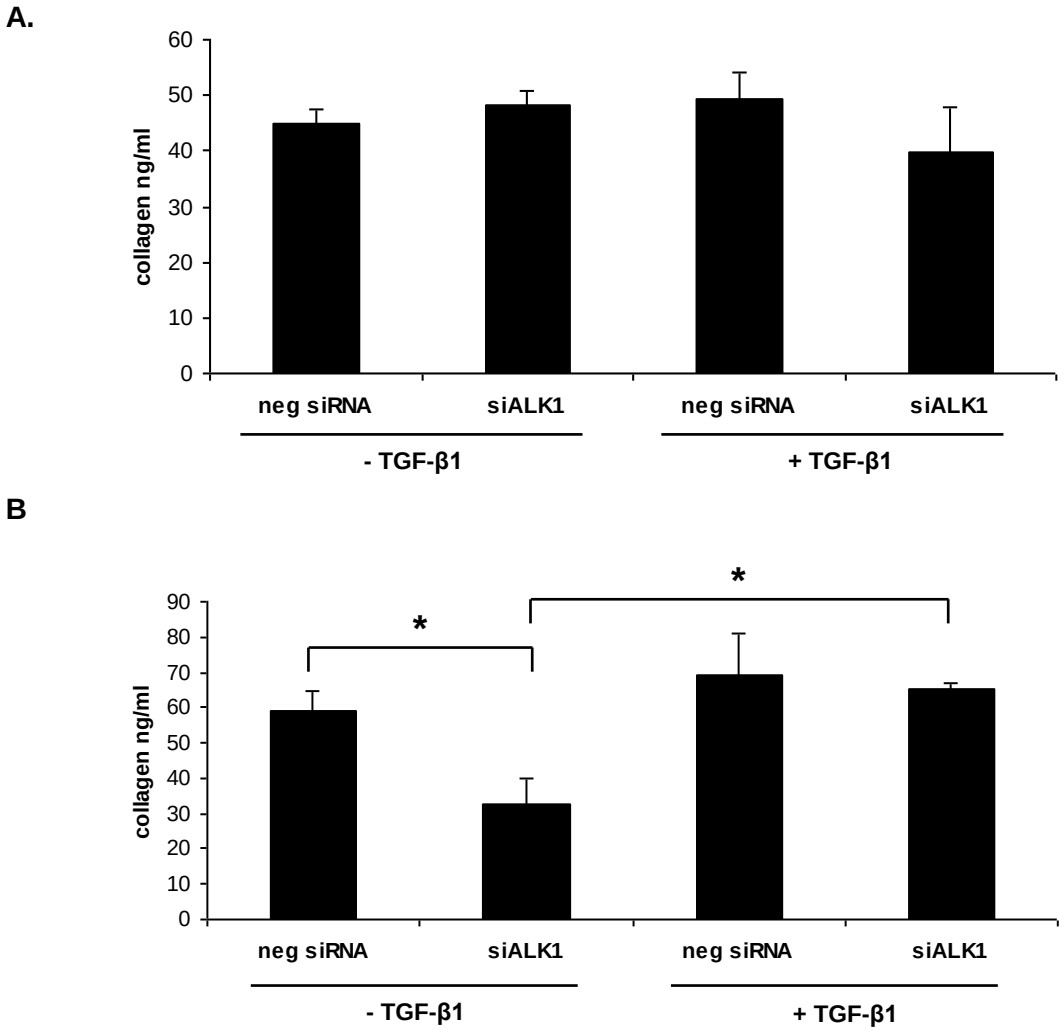


Figure 4.15. Effect of ALK1 siRNA knockdown on TGF-β1-induced collagen secretion by fibroblast cell lines.

The cells were transfected with 10 nM of siRNA specific for the ALK1 gene for 48 h and stimulated with TGF-β1 for an additional 24 h. Cell culture media from the HFL1 (**A**) and IMR-90 (**B**) cultures were collected and secreted collagen content was analysed by Sircol assay. Values represent mean ± S.E.M. (n=3). Statistically significant differences of $p \leq 0.05$ (by Student's t-test) are marked by *.

5. Discussion

5.1. Involvement of ALK1 in lung diseases

Recently, many reports have shown an interest in ALK1 expression and activity, in the cooperation of ALK1 and ALK5 receptors, but few publications have addressed the impact of ALK1 on lung diseases. Activin receptor-like kinase 1 is linked to the autosomal dominant vascular disorder, haereditary hemorrhagic telangiectasia (HHT) type 2 [103, 104]. Haereditary hemorrhagic telangiectasia is a multisystemic vascular dysplasia characterised by dilated vessels or telangiectases on mucocutaneous surfaces and arteriovenous malformations (AVMs) in the lung, liver and brain [105]. The *ACVRL1* (*ALK1*) gene was found to be mutated in HHT2. Haereditary hemorrhagic telangiectasia results in secondary pulmonary artery hypertension (PAH) [106]. The *ALK1* mutations associated with primary pulmonary hypertension (PPH) are also of varying types and found throughout the gene [107]. Hypertension causes structural changes in the arteries, including hypertrophy of vascular smooth muscle cells, collagen and fibronectin accumulation, and destruction of elastic fibers. In the lung, in vascular smooth muscle cells, endothelial cells, and fibroblasts, TGF-β1 increases the synthesis of ECM proteins, such as fibronectin, α-SMA, collagens, and PAI1. All this information points to a specific role for TGF-β signalling in the ECM protein production, specifically for the type I receptors, ALK1 and ALK5, which are found to be the main players in the homeostasis of endothelial cells and vascular fibrosis [67], but nothing is known about the role of ALK1 in the pulmonary fibrosis.

5.2. TGF-β signalling components in fibrotic human lungs

5.2.1. TGF-β receptors, Smads and target gene expression patterns

In the present study, the TGF-β signalling components, such receptors, intracellular mediators and target genes, were analysed in the context of IPF. All TGF-β members investigated were present in the lungs of healthy subjects and patients with

IPF, and no changes between them were found (Figure 4.1., 4.2., 4.3.). All aspects of the fibrotic processes have been shown to be regulated by TGF-β [108]. No other growth factor has as many different activities contributing to matrix accumulation, as does TGF-β. The TGF-β is present in the normal lungs, suggesting a role in the regulation of normal physiological processes to maintain lung homeostasis [109]. To a large extent, TGF-β mediates tissue remodelling and repair [110-112], and is present in lung diseases and systemic diseases, for example giant-cell interstitial pneumonia, histiocytosis, scleroderma, systemic lupus erythematosus, cystic fibrosis [113], chronic obstructive lung disease [114], and idiopathic pulmonary fibrosis [115-118]. Transforming growth factor β signalling is a very prominent and one of the most studied pathway in the context of lung fibrosis.

The Smad3 RNA expression was not changed when assessed by quantitative PCR, despite the fact that Smad3 was downregulated when assessed by semiquantitative PCR (Figure 4.2. and 4.3.). These discrepancies might be explained by the technical difficulties performing the semiquantitative PCR. Generally, it is known that Smads are involved in the regulation of COL1, COL3 and COL4 gene expression, and coordination of increased levels of these collagens in the media. Overexpression of Smad2, Smad3 and Smad4 increases the production of all collagen types [119] Although it is reported that in skin fibrosis, treatment of myofibroblasts with TGF-β1 inhibits expression of Smad3, but not Smad2 [120]. Conversely, the TGF-β/Smad3 pathway is the key regulator of the progression of IPF, because the progression of IL-1β-induced inflammation toward fibrosis is Smad3 dependent, and it develops only in wild type control mice and not in Smad3 null mice [121]. In another study, mice deficient in Smad3 demonstrated suppressed type I procollagen mRNA expression and reduced hydroxyproline content in the lungs, compared with wild-type mice treated with bleomycin. Furthermore, loss of Smad3 greatly attenuated the morphological fibrotic responses to bleomycin in the mouse lungs [122]. Surprisingly, however, production of ECM was unaffected during cutaneous wound healing in Smad3 null-mice, suggesting the existence of a Smad3-independent mechanism involved in the tissue repair by dermal fibroblasts [123].

No evidence has been provided concerning the implication of ALK3 and ALK6 in the pathogenesis of lung fibrosis. It is known that the BMP7 ligand has an anti-fibrotic effect in renal fibrosis, which is mediated by p38/Smad1 pathways in a dose-dependent manner [124, 125]. BMP7 drives its signal via ALK6, but the exact molecular mechanism of this action in the lung has not yet been elucidated.

However, significant changes were observed at the ALK1 mRNA expression level. There are no publications reporting the role of this specific type I TGF-β receptor in IPF. One of the characteristics of fibrotic lesions is the aberrant expression of TGF-β receptors. For instance, an increased ratio of TGF-βRI to TGF-βRII expression levels has been observed in liver fibrosis [126] and scleroderma [127], while increases in the expression of TGF-βRI has been observed in kidney fibrosis [128]. In addition, a previous study has demonstrated that constitutively elevated expression levels of CTGF in scleroderma fibroblasts are Smad2/3-independent [129]. These findings suggest that overproduction of matrix in fibrosis might be, at least in part, due to the alternative activation of TGF-β signalling.

Recently, a study in scleroderma fibroblasts was performed, and the authors found that collagen protein production in these cells was resistant to inhibition by ALK5 kinase inhibitors. Additionally, it was concluded that TGF-βRI-dependent induction of fibrotic processes does not involve Smad2/3, and is activated and mediated by ALK1/Smad1 and ERK1/2 pathways [130]. Similarly, TGF-β signalling via activation of ALK1/Smad1 pathway and subsequent upregulation of the Id1 gene has been shown to contribute to transdifferentiation of hepatic stellate cells into myofibroblast during fibrosis processes in the liver [131]. These recent studies strongly suggest that activation of ALK1/Smad1 signalling may play an important role in the development of organ fibrosis, and needs further investigation.

According to many reports, the expression of COL1 and α-SMA is highly upregulated during IPF pathogenesis [30, 66, 72, 132], and it has been shown that mRNA levels of COL1A1 and α-SMA has been significantly increased (Figure 4.4.). Similar results were obtained with studies on the mRNA expression of plasminogen activator inhibitor 1 (PAI1). The PAI1 is known to be the major physiological inhibitor of tissue-type and urokinase-type plasminogen activator. These two molecules convert inactive plasminogen into its fibrin-degrading form, plasmin. Plasma and tissue concentrations of PAI1 are extremely low under normal circumstances, but increase under pathological conditions, for example organ fibrosis. This increase is mediated by many factors, including TGF-β [133-136]. Moreover, Id1 mRNA levels were significantly lower in fibrotic samples compared to healthy subjects. The Id1 protein is a helix-loop-helix protein inhibitor of differentiation 1, which lacks the basic DNA binding domain and functions as naturally-occurring dominant-negative inhibitor of gene transcription, by forming nonfunctional heterodimers with basic helix-loop-helix proteins [137]. It has been found that mice lacking the gene for Id1 have increased susceptibility to bleomycin-induced lung

fibrosis, demonstrated by elevated levels of collagen deposition and fibrogenesis [138]. However, Id1 was found to be highly expressed by myofibroblasts within fibroblastic foci in response to TGF-β1, and further behaved like a typical immediate-early gene [139].

5.3. ALK1 expression in fibroblasts

5.3.1. Cell type localisation of ALK1

Activin receptor-like kinase 1 was reported to be specifically expressed in endothelial cells, at the active sites of angiogenesis and the specific sites of epithelial-mesenchymal cells [80, 91]. Recently, the ALK1 expression was shown to be present in the human chondrocytes [140].

The immunohistochemical analysis of the ALK1 localisation in paraffin-embedded human tissue sections (Figure 4.5.) demonstrated the presence of ALK1 in bronchial and alveolar epithelial cells, smooth muscle cells, endothelial cells, and the interstitial areas, which were α-SMA positive, suggesting activated interstitial fibroblasts. Activated fibroblasts, called myofibroblasts, are α-SMA positive and posses a contractile phenotype [141]. These results are of importance since the α-SMA-positive interstitial regions are the places of the activated (myo)fibroblasts and the localisation of ALK1 to that cell type is novel, and has not been reported previously. Activin receptor-like kinase 5 was stained in all lung cell types, as it has been indicated that ALK5 receptor is expressed predominantly in different tissues and cell types, including lung. The immunohistochemical staining is a non-quantitative method; therefore, it has been difficult to quantify the expression level of ALK1 and ALK5 in healthy and fibrotic tissue sections

5.3.2. Lung cell type specific expression pattern of ALK1

5.3.2.1. ALK1 mRNA expression in primary human fibroblasts

The mRNA expression of ALK1 was significantly lower in primary human fibroblasts isolated from the lungs of fibrotic patients, compared with donor fibroblasts (Figure 4.6.). Moreover, the mRNA levels of other receptors and Smads were not altered, except for Smad1, which exhibited decreased mRNA levels in fibrotic fibroblasts. The Smad1, together with Smad5 and Smad8, is a specific mediator of ALK1/TGF-β signalling [142]. Nevertheless, the mRNA expression analysis does not reflect the activity of

the ALK1 pathway, which in this case might suggest the attenuation of ALK1/Smad1 signalling.

Activated fibroblasts during fibrogenesis lead to increased production of ECM proteins, as is shown by elevated levels of COL1A1 and α-SMA mRNA in fibrosis-derived fibroblasts (Figure 4.6.). The lack of statistical significance and large deviations in the case of donor fibroblasts might be explained as: a) there was no information about whether healthy patients were treated with some medication, b) it is known only that the patients were not smokers, c) there are no reports that these patients have other underlying lung diseases. Culture conditions and methods might effect some characteristic of the cells [143], for instance the absence of significant changes in α-SMA and COL1A1 between primary fibroblasts from healthy subjects and patients with IPF. Characteristic of fibroblasts *in vivo* are different from those of *in vitro* cultured cells. The number of passages is another factor that influences cells [144, 145]. Additionally, different results may be due to different characteristic of patients from whom the lung specimens were obtained, including the diagnosis of disease, age, and clinical status of the subjects. The pathological patterns of the cases used in the present study exhibited a UIP-type histopathology with the clinical manifestation of IPF.

It is known that under the stimulation of growth factors and of newly synthesised ECM components, the fibroblasts evolve toward the intermediate stage of proto-myofibroblasts, and further toward the differentiated myofibroblasts, which are characterised by the expression of α-SMA [146 147]. The importance of α-SMA expression as a marker of myofibroblastic modulation is currently well accepted. These α-SMA positive fibroblasts synthesise elevated levels of ECM components and matrix degrading proteases, which leads to excessive scarring in the diseased organ. The immunohistochemical study with antibodies specific for the propeptides of type I collagen have localised the cells extensively producing this collagen. In the normal lungs, the synthesis of collagen is sufficiently low that the fibroblasts remains unstained, whereas positive staining has identified fibroblasts activated to synthesise collagen type I as a consequence of disease [148].

The mRNA levels of Id1 and PAI1 in primary fibroblasts was similar to the mRNA levels of these genes in the human lung homogenates of healthy and fibrotic patients (Figure 4.4. and 4.6.). The Id1 was reported to be robustly, although transiently, induced in fibroblasts by TGF-β1, which might initially delay myofibroblast differentiation, and raises the possibility that Id1 plays a part in determining fibroblast behaviour in the setting of fibrosis [139]. The elevated PAI1 levels promote collagen deposition not by

inhibiting plasmin, but by stimulating migration of collagen-producing cells into the damaged tissue [149], and since the myofibroblasts are collagen-producing cells, the increased level of PAI1 mRNA appears in line with this idea.

5.3.2.2. ALK1 mRNA expression in the other lung cell types

The endogenous mRNA levels of TGF-β receptors and target genes in pulmonary smooth muscle cells (pSMC), and healthy and fibrotic epithelial cells (EpC) (Figure 4.7.) matched the immunohistochemical localisation of ALK1 and ALK5 (Figure 4.5.). The endogenous level of TGF-β target genes was verified in pSMC and HUVEC, but not in EpC due to the limitation of the freshly isolated primary cells.

The observed pattern of ALK1 expression was novel, since most data have indicated the presence of ALK1 exclusively in endothelial cells, therefore, HUVECs served as a positive control for ALK1 and endoglin expression. The HUVEC expressed COL1A1 and α-SMA at low levels, although these cells were reported to facilitate the collagen deposition by human periodontal ligament fibroblasts (HPLFs) in the model of co-culture of HPLFs with HUVECs [150]. Conversely, pSMC, which are involved in the remodelling processes in asthma, are α-SMA positive [151]. Smooth muscle cells involved in atherosclerosis change phenotype from a contractile to synthetic, and deposit type I and III collagens, elastin and fibronectin [152].

The mRNA level of ALK1 is very high in the HUVEC, whereas the level of Id1 is much more lower. It could be explained that Id1 is a specific, but not only one, target gene for ALK1/TGF-β signalling [153] and most importantly, we can not correlate directly the level of Id1 mRNA expression with mRNA level of ALK1 without knowing the activity level of that receptor.

5.3.2.3. Active ALK1/TGF-β signalling in human lung fibroblast cell lines

The ALK1 mRNA levels, as well as the other receptors and TGF-β pathway target genes, displayed a similar pattern of expression in both fibroblast cell lines (Figure 4.8. and 4.9.), as well as in the primary human fibroblasts (Figure 4.6.). Similarly, ALK1 was present at low level. However, both cell lines exhibited high levels of expression of the ECM genes COL1A1 and α-SMA, and the profibrotic PAI1.

Transforming growth factor β1 has been demonstrated to active TGF-β signalling pathways via the phosphorylation of Smad2 at 30 min after stimulation, and lasting for up

to 2 h in both cell lines, HFL1 and IMR-90 (Figure 4.10.). Conversely, BMP2 activated the BMP signalling by phosphorylation of Smad1/5/8 in both fibroblasts. Suprisingly, TGF-β1 was able to phosphorylate Smad1/5/8 as well, suggesting the presence of an active ALK1/TGF-β pathway in both cell lines in a similar fashion.

The canonical TGF-β pathway functions via ALK5, and leads to the phosphorylation of intracellular Smad2/3 [49]. There is also increasing evidence for the activation of other signalling pathway downstream of TGF-β, the ALK1/Smad1/5 pathway, shown mostly in the endothelial cells [79, 80]. Moreover, Smad-independent pathways involved in the TGF-β are active in many cell types and tissues [58, 154-156]. The canonical BMP pathway acts via the phosphorylation of Smad1/5/8 [157]. However, recent studies have established that activation of the Smad1 pathway may represent a novel aspect of profibrotic TGF-β signalling that functions independently of the activation of the canonical Smad2/3 pathway, at least in the *in vitro* model of systemic sclerosis [130]. Additionally, imatinib mesylate, an inhibitor of profibrotic effects of TGF-β in cultured cells and experimental models of fibrosis, was able to block TGF-β-induced activation of Smad1 and ERK1/2 in control fibroblasts and induce phosphorylation of Smad1 and ERK1/2 in systemic sclerosis fibroblasts [158].

Interestingly, there is a report demonstrating that in some cell types, TGF-β may induce Smad1 phosphorylation independently of the BMP type I receptors [159]. The authors have suggested that TGF-β can significantly induce Smad1 phosphorylation in several non-endothelial cell lineages using chemical inhibitors specific for TGF-β type I receptors (ALK4/5/7) and BMP type I receptors (ALK1/2/3/6).

A pathway for the canonical TGF-β signalling from the cell surface to the nucleus in response to the various ligands has been established. However, unexpected signalling outcomes, or even opposing biological outcomes of TGF-β signalling pathway, in the same cells, suggest that the signalling model is not quite as simple as first thought. The ALK1 pathway is one such examples, but signalling via the TGF-βRII/ALK1 complex is dependent on ALK5 kinase activity and endoglin, to result in Smad1/5 phosphorylation in response to TGF-β, and it has been proposed that ALK1 activation may inhibit ALK5/Smad2/3 signalling [80, 94]. The TGF-β can induce Smad1 phosphorylation in a number of epithelial cells and fibroblasts via ALK5 and ALK2 and/or ALK3, which suggests a dependence on a BMP type I receptors [160]. Another publication has presented similar results, where Smad1 phosphorylation in the mouse mammary epithelial cell line 4T1 was strictly dependent on ALK5 kinase acivity, and the authors ruled out the involvement of BMP receptors using shRNA [161]. Moreover, the specificity of type I

receptors for the canonical Smads is usually based on the L45 loop and phosphorylated GS motif, and on the L3 loop in the MH2 domain of the partner Smad [162-164]. The authors suggested that ALK5 can directly contact Smad1 despite the assumed incompatible L45-L3 pairing [159]. To conclude, the key pathway leading to the phosphorylation of Smad1 by TGF-β involves ALK1, but additionally, options to activate Smad1 signalling, which could be completely independent on ALK1, exist.

5.4. The effect of ALK1 on collagen deposition

5.4.1. Effective ALK1 knockdown in fibroblasts

To be able to mimic the situation in human lung homogenates and human primary fibroblasts, ALK1 and ALK5 mRNA expression was significantly and sufficiently silenced by siRNA technology (Figure 4.11. and 4.12.) in the HFL1 and IMR-90 fibroblast cell lines. Most importantly, the knockdown of ALK1 had no effect on the mRNA expression of ALK5, and ALK5 had no effect on ALK1 mRNA levels, suggesting that siRNA oligonucleotides used in the assay specifically recognise each type I receptor.

5.4.2. The effect of ALK1 on COL1A1 mRNA expession

From the previous results, the suggestion might be that the diminished levels of ALK1 could lead to the activation of fibroblasts, and subsequently to the elevated levels of collagen production and deposition. The ALK1 silencing lasted for 72 h and was more pronounced after TGF-β1 stimulation in both cell lines (Figure 4.13.) without affecting the mRNA level of ALK5, endoglin and TGF-βRII. The Id1, the specific target gene of ALK1, was downregulated in parallel with the knockdown of ALK1, and was more affected after TGF-β stimulation. The expression of PAI1 and α-SMA was significantly changed upon TGF-β1 stimulation, but independently of ALK1 siRNA-mediated knockdown. These alterations are also evident in the case of COL1A1 mRNA expression. The TGF-β1 had no effect on endogenous COL1A1 levels, but the siRNA specific for ALK1 attenuated the COL1A1 expression, which was restored almost to endogenous levels upon TGF-β1 treatment. This might imply that in the absence of ALK1, ALK5 signalling might by preferred to induce collagen gene transcription, although the lack of ALK1 abolished the amount of COL1A1 mRNA. To be able to distinguish precisely between the ALK1 and

ALK5 signalling, the same experiment with the knockdown of ALK5 might be of importance.

Collagen types I and II are the most abundant proteins found in the airways, blood vessels and alveolar septa. These collagens are synthesised by many cell types, but predominantly by mesenchymal cells: fibroblasts, myofibroblasts and smooth muscle cells [165]. There is considerable evidence implicating TGF-β1 in the pathogenesis of IPF [166]. Moreover, TGF-β is one of the most potent profibrotic mediators, produced by many cell types, which are known to promote fibroblast proliferation, migration, collagen synthesis and differentiation [165, 167, 168]. Surprisingly, COL1A1 mRNA was generally very highly expressed in the fibroblasts, but was not changed after TGF-β1 stimulation (Figure 4.13.). Conversely, PAI1 and α-SMA were very responsive to TGF-β1. The explanation to that confused results would be either the low concentration of ligand efficient to increase the already very high level of COL1A1, or the short stimulation time for the same action, although these concentration and stimulation time were always efficient in many other experiments. Longer stimulation with TGF-β1 caused cell death.

Recently it has been reported that ALK1 inhibits, while ALK5 potentiates, TGF-β1-induced Smad3-dependent transcriptional activity, and the expression of the ECM components PAI1, fibronectin and collagen type II in human chondrocytes [140]. Activin receptor-like kinase 1 may exert its inhibitory effect downstream of Smad3 phosphorylation. These ideas demonstrate that ALK1 opposes ALK5/TGF-β signalling in human chondrocytes similar to endothelial cells [79].

5.4.3. The effect of ALK1 on cellular and extracellular collagen deposition

Changes in mRNA expression do not always reflect the level of proteins production, or in the case of collagens and ECM, its deposition. In order to assess whether ALK1 can affect the process of lung fibrosis and collagen synthesis, further experiments were performed. From the presented results, ALK1 seems to be a player in fibroblast activation, although the mechanism of ALK1/ALK5 pathways was not specified yet. There were no obvious alterations in the cellular collagen production in HFL1 and IMR-90 cells, which could be ALK1-dependent (Figure 4.14.). Transforming growth factor β1-induced collagen production without any action of ALK1, and the attenuation of that production after siRNA knockdown of ALK1, were observed in HFL1 cells but not in

IMR-90. However, the changes occurred in the secretion of collagen by the IMR-90 cells into the culture media (Figure 4.15.). The ALK1 silencing displayed similar effects on collagen secretion, as was shown at the mRNA expression level of COL1A1 (Figure 4.13.) in the same cell line. The significant decrease in collagen secretion after ablation of ALK1 was rescued by TGF-β1 stimulation. The HFL1 and IMR-90 cells differ from each other in that they have different origins, and they were isolated from different aged foetuses, which could explain the divergent results obtained with collagen deposition.

The lack of ALK1 was associated with the fibrosis (Figure 4.3.) and seems to be involved in the activation of fibroblast (Figure 4.6.), but the details of such an action are not clear yet. The collagen deposition assay demonstrated that the lack of ALK1 leads to the decreased collagen production (Figure 4.15. and 4.16.), and more specifically to the decreased amount of COL1A1 mRNA (Figure 4.13). The opposite results could be explained by technical problems of the projects, as it has been mentioned that there is no information about the subjects from whom the lungs and primary fibroblasts were taken. Additionally, results were obtained with fibroblast cell lines, not primary cells, which could be the weak point of this study. The important point is that fibroblasts in the fibrotic lung tissue are constantly exposed to the action of TGF-β, which is a highly regulated profibrotic cytokine in IPF; therefore, cell culture of fibroblast cell lines without TGF-β does not reflect the fibrotic environment. Moreover, the data presented here were obtained *in vitro*, which perhaps can not be extrapolated to IPF in humans *in vivo.*

To date, no report have demonstrated ALK1 expression and action in lung fibroblasts. Although, as mentioned previously [130], the TGF-βRI-dependent upregulation of collagen and connective tissue growth factor (CTGF), a profibrotic cytokine upregulated in various fibrotic diseases, does not involve Smad2/3 activation, but is mediated by ALK1/Smad1 and ERK1/2 pathways. The inhibition of ALK5 either by a chemical inhibitor or using the ALK5 mutant cells have not associated with Smad3 phosphorylation, and potentially stimulated collagen protein levels. Furthermore, Smad1-specific siRNA abolished elevated level of collagen type I and CTGF expression in this model, pointing to the importance of ALK1/Smad1 signalling in the regulation of the profibrotic gene program in this model of fibrosis. The authors [130] used the scleroderma as the fibrosis model and dermal fibroblast isolated from diseases areas of skin, which might be the major feature different from the lung fibrosis.

Interestingly, recent studies have also demonstrated involvement of ALK1/Smad1 pathways in kidney and liver fibrosis. Elevated expression of Smad1 protein was observed in the human diabetic kidney, and in animal model of diabetic nephropathy [169-

[171]. In addition, Smad1 was shown to directly upregulate expression of the collagen type IV gene in mesangial cells [169]. Similarly, TGF-β signaling via activation of the ALK1/Smad1 pathway and subsequent upregulation of Id1 gene has been shown to contribute to transdifferentiation of hepatic stellate cells into myofibroblasts. The Id1 expression and Smad1 phosphorylation correlate with severity of bile duct ligation-induced liver fibrosis [131]. These recent studies strongly suggest that activation of ALK1/Smad1 may play an important role in development of organ fibrosis.

Moreover, the authors [130] demonstrated that activation of ERK1/2 pathway, which plays a relatively small role in TGF-β-dependent CTGF stimulation in dermal fibroblasts, was required for adenoviral transfected TGF-βRI-dependent persistent phosphorylation of Smad1 and subsequent elevated level of CTGF and collagen mRNA [130]. Similarly, activation of ERK1/2 was required for the phosphorylation of Smad1 in cardiomyocytes [172]. Moreover, concomitant activation of Smad1 and ERK1/2 pathways was observed in hepatic stellate cells in a bile duct ligation-induced liver fibrosis, suggesting that interaction between Smad1 and ERK pathways may be a general phenomenon [173].

In conclusion, these reports provides evidence for the existence of an alternative TGF-β-dependent, Smad3-independent signalling pathway that may operate during chronic stages of organ fibrosis. Fibroblasts from Smad3 null mice also exhibit a compensatory increase in activated MAPK levels in response to TGF-β [174], thus raising the possibility of a similar Smad3-independent pathway functioning *in vivo* during wound healing in this mouse model.

An altered ratio of TGF-β receptor subunits has also been observed in fibrotic diseases. Modulation of TGF-βRI/RII ratio take place in fibroblasts during wound healing processes [175]. The changes in the TGF-β receptor levels, together with the information about the pathways involved in fibrotic processes, suggest that the modulation of TGF-βRI and RII ratio might represent a novel model of diversity of TGF-β signalling and its biological effects in the normal and pathological conditions. The ALK1/Smad1 pathway might be of interest for the future potential target for the antifibrotic therapy in the organ fibrosis.

5.5. Conclusions and future perspectives

Activin receptor-like kinase 1, predominantly expressed in the endothelial cells, is a TGF-β type I receptor involved in angiogenesis and new tube formation, together with the widely-expressed second TGF-β type I receptor, ALK5. In endothelial cells, these

receptors function in a well-organised and controlled pattern to keep the balance during angiogenesis. The ALK1 has been recently demonstrated to play a role during organ fibrosis. In this study, for the first time, has been shown that ALK1 is present in human fibroblasts, and ALK1/Smad1/5/8 signalling is active. As fibroblasts are the major cells involved in the development of IPF, the potential role of ALK1 could be to influence collagen synthesis by these cells. Today, the pathway reported to lead to the excessive ECM production is the ALK5/Smad2/3 pathway. However, a recent publication has specified the involvement of ALK1/Smad1 signalling in fibroblast activation and collagen synthesis.

In this study, we hypothesised that expression of TGF-β system components are changed in the IPF, and the only changes were visible in the case of ALK1. ALK1 was downregulated in the lungs of fibrotic patients, as well as in the primary human fibroblasts isolated from such lungs. Additionally, siRNA-mediated silencing of ALK1 changed collagen deposition, as well as COL1A1 mRNA expression. To be able to specify the involvement of either ALK1 or ALK5 in the collagen synthesis, further experiments have to be performed. The knockdown of ALK5, or the inhibition of ALK5 by the commercially available inhibitors SB431542 and IN-1130, would give at least a partial answer to the question, if the collagen deposition were driven by ALK1 or ALK5 signalling pathway.

The present study was performed generally in human lung fibroblast cell lines, HFL1 and IMR-90. To have better correlation with the pathogenesis of the IPF in humans, the ALK1/ALK5 balance and their role in fibroblast activation and collagen deposition in the primary human fibroblasts would be of importance. The ALK1 and ALK5 are expressed in these cells, although TGF-β/BMP signalling activity was not studied in primary cells. The phosphorylation of Smads performed by immunoblotting is the best and easiest way to get the information about the activity of TGF-β. The TGF-β is one of the many other profibrotic mediators well-studied and most abundant in the fibrosis processes, and it is expressed by all the cells involved in this disease, so it might be possible to detect the active TGF-β signalling in the primary fibroblasts.

Methods such as microdissection would be a useful tool to collect material from the fibroblast foci, the specific places, which are the hallmark of fibrosis, cumulating the activated myofibroblasts. Using this method would be very important to get fibroblasts exactly from the diseased areas of the lung, and be sure the phenotype of these cells would not change during cultivation and culturing. The obtained RNA and proteins would represent the most interesting panel of any changes between the healthy and fibrotic patients.

Activin receptor-like kinase 1 heterozygous mice are available. The homozygous mice die at embryonic day 9.5 due to the lack of formation of branching capillary network and the dilation of the blood vessels [86]. The heterozygous mice are normal, and serves as a model of HHT type 2 [176-179]. The influence of ALK1 knockout on the lung phenotype and eventual involvement in the fibrotic processes has not yet been studied. However, there are publications demonstrating the correlation of HHT, and ALK1 and endoglin mutations, with the pulmonary hypertension [180, 181]. To investigate the fibrotic processes, such activation of fibroblasts and their differentiation, and collagen deposition, in the ALK1 heterozygous mice would be of important value.

As mentioned [130, 131, 158], ALK1 is involved in the upregulation of collagen type I independently of Smad2/3 and ALK5, but surprisingly, together with the ERK pathway. To be able to specify not only between ALK1 and ALK5 and the Smads involved in their signal transduction, but also between ALK1/Smad1 and the other pathways, which might be involved in the molecular mechanism of collagen deposition, further experiments must be done. It was shown that activation of the ERK1/2 (p44/42) MAPK pathway contributes to collagen upregulation in skin and lung fibroblasts from scleroderma patients [182, 183]. Tools exist which could be helpful to study this part of the project, for example, the specific antibodies for the phosphorylated forms of the MAPK kinases, commercially available inhibitors of kinases, such as UO126, the specific inhibitor of MEK, which all together would be possible to evaluate the activation of MAPK kinase pathways.

Furthermore, this would clarify whether manipulation of ALK1 activity in pulmonary fibrosis offers the intriguing possibility of interfering with the development of the disease. Reliable methods should be developed to target ALK1/Smad1 signalling function specifically in the lung, as a potential therapy for pulmonary fibrosis.

6. Appendix

6.1. List of primers used for PCR amplification

6.1.1. Quantitative RT-PCR

Quantitative RT-PCR				
GeneBank™ Accession number	**Forward Primer (5′-3′)**	**Reverse Primer (5′-3′)**	**Annealing temperature (°C)**	**Amplicon size (bp)**
ACVRL1 human NM_000020.2	GTGGAGTGTGTGG GAAAAGG	TCATGTCTGAGGCC ATGAAG	60	181
BMPRIA (ALK3) human NM_004329.2	TTTATGGCACCCAA GGAAAG	TGGTATTCAAGGGC ACATCA	60	156
BMPRIB (ALK6) human NM_001203.1	GGTTGCCTGTGGT CACTTCT	CCTTTCTGTGCAGC ATTCAA	60	110
BMPRII human NM_001204.5	GAAGACTGTTGGG ACCAGGA	TTGCGTTCATTCTG CTAGC	60	149
COL1A1 human NM_000088.2	CCAGAAGAACTGGT ACATCAGC	CGCCATACTCGAAC TGGAAT	60	94
ENG human NM_000118.1	GTGACGGTGAAGG TGGAACT	GATCTGCATGTTGT GGTTGG	60	114
ID1 human NM_181353.1	GTGGTGCGCTGTC TGTCTGA	AGTAACAGCCGTTC ATGTCG	60	129
PAI-1 human NM_000602.1	GAGAAACCCAGCA GCAGATT	TGGTGCTGATCTCA TCCTTG	60	125
PBGD human NM_000190.3	CCCACGCGAATCA CTCTCAT	TGTCTGGTAACGGC AATGCG	60	69
α-SMA human NM_001613.1	GAGAAGAGTTACGA GTTGCCTG	TGTTAGCATAGAGG TCCTTCCTG	60	180
SMAD1 human NM_005900.2	CAGTCTGTGAACCA TGGATTTG	TAACATCCTGGCGG TGGTAT	60	109
SMAD2 human NM_005901.4	GGAATTTGCTGCTC TTCTGG	TCTGCCTTCGGTAT TCTGCT	60	128
SMAD3 human NM_005902.3	CTGTGTGAGTTCGC CTTCAA	CGGGGAAGTTAGT GTTTTCG	60	181
SMAD4 human NM_005359.3	TCACAATGAGCTTG CATTCC	GGGTCCACGTATC CATCAAC	60	156
SMAD5 human NM_001001419.1	AAATGTCCAGAAAT CTGCCTCT	TCCCAGTTATTCGG GAGACT	60	99
SMAD8 human NM_005905.4	ATTTGGCTCCAAGC AGAAAG	GCTGAGCTGGGGG TTATATTC	60	117
TGFβRI (ALK5) human NM_004612.2	CAGCTCTGGTTGGT GTCAGA	ATGTGAAGATGGG CAAGACC	60	131
TGFβRII human NM_003242.5	TTTTCCACCTGTGA CAACCA	GGAGGAGCAGCAT CTTCCAG	60	185

6.1.2. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR				
GeneBank™ Accession number	**Forward Primer (5′-3′)**	**Reverse Primer (5′-3′)**	**Annealing temperature (°C)**	**Amplicon size (bp)**
ACVRL1 human NM_000020.2	ATCGCCTCAGACATGACCTC	TGATCCACACACACCACCTT	56-60	560
BMPRIA (ALK3) human NM_004329.2	TACACAGGAAACATTACAA	CTTTTAGTGATTCTCCAAC	60	628
BMPRIB (ALK6) human NM_001203.1	CAAGAAAGAGGATGGTGAG	ATAATCATAAAGGGAACCA	60	832
BMPRII human NM_001204.5	CTCGAATCCCCAGCCCTGAA	GCCGGGGCTCTTTTGTTGAA	56-61	525
HSP70 human NM_006597.3	TGTGTCTGCTTGGTAGGAATGGTGGTA	TTACCCGTCCCCGATTTGAAGAAC	58	387
α-SMA human NM_001613.1	GAGAAGAGTTACGAGTTGCCTG	TGTTAGCATAGAGGTCCTTCCTG	60	180
SMAD1 human NM_005900.2	GGAGACAGCTTTATTTCACCATATC	CAATAGTTTTCCAGAGGCAGATG	58	1491
SMAD2 human NM_001003652.2	GGGAGGTTCGATACAAGAGGCT	GGACCACACACAATGCTATGACA	58-60	1530
SMAD3 human NM_005902.3	AGCCATGTCGTCCATCCTG	CTTCTTCCTTGACAACAATGGG	59	1383
SMAD4 human NM_005359.3	TTCACTGTTTCCAAAGGATCAAAA	GTAGCCACCATCCTGATAACGTTAAG	58-59	1780
SMAD5 human NM_001001419.1	CTGTTCTTTCGGTAGCCACTGAC	GCATTATGAAACAGAAGATATGGGG	58	1459
TGFβRII human NM_003242.5	GGAAGCTCATGGACTTCAGC	CCCACTGTTAGCCAGGTCAT	56-60	632

6.2. List of antibodies

6.2.1. Primary antibodies

Antibody				
Primary	**Host**	**Dilution**		**Company**
		Immunoblotting	**Immunohistochemistry**	
anti-β-actin	rabbit	1:1000		Cell Signaling
anti-ALK1	goat		1:100	Santa Cruz

anti-α-SMA	mouse		1:1500	Sigma-Aldrich
anti-Smad1/2/3	mouse	1:1000		Santa Cruz
anti-Smad2/3	mouse	1:1000		BD Biosciences
anti-phospho Smad1/5/8	rabbit	1:500		Cell Signaling
anti-phospho Smad2	rabbit	1:500		Cell Signaling
anti-TGFβRI (ALK5)	rabbit	1:500		Cell Signaling
anti-TGFβRI (ALK5)	rabbit		1:100	Santa Cruz

6.2.2. Secondary antibodies

Antibody				
Secondary	**Host**	**Dilution**		**Company**
		Immunoblotting	**Immunohistochemistry**	
Biotinylated anti-mouse IgG	goat		Ready to use	Invitrogen
Biotinylated anti-goat IgG	goat		Ready to use	Invitrogen
Biotinylated anti-rabbit IgG	goat		Ready to use	Invitrogen
HRP-conjugated anti-mouse IgG	goat	1:3000		Pierce
HRP-conjugated anti-rabbit IgG	goat	1:3000		Pierce

6.3. Human siRNA sequences

Gene name	**ON-TARGETplus SMARTpool siRNA (5´-3´)**
ACVRL1 (NM_000020)	si#1 AGCCUAAAGUGAUUCAAA si#2 GAGCAGGGCGACACGAUGU si#3 GUCAAGAUCUUCUCCUCGA si#4 CGGGAGUGCUGGUACCCAA

TGFβRI (ALK5) (NM_004612)	si#1 GAGAAGAACGUUCGUGGUU si#2 UGCGAGAACUAUUGUGUUA si#3 GACCACAGACAAAGUUAUA si#4 CGAGAUAGGCCGUUUGUAU

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8. 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.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

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

- Fotini M Kouri, **Izabella Chrobak**, Oana V Amarie, Roxana Sandu, Walter Klepetko, Werner Seeger, Ralf T. Schermuly and Oliver Eickelberg. “Expression and Functional Analysis of the Transforming Growth Factor (TGF)-β superfamily in Pulmonary Arterial Hypertension: Role of PAI-1 in Disease Progression”. American Thoracic Society (ATS) International Conference, San Diego (USA),19-24 Mai 2006

- **Izabella Chrobak**, Oana V Amarie, Matthias Hecker, Ulrike Seay, Joanna K Jagielska, Werner Seeger, and Oliver Eickelberg. “Dysregulation of the TGF-β type I receptor Alk1 in Idiopathic Pulmonary Fibrosis” .14th International Colloquium on Lung ibrosis (ICLF), Schloss Reinhartshausen Kempinski Mainz (Germany), 7-10 September 2006

- Nils Banthien**, Izabella Chrobak**, Kamila Kitowska, Melanie Königshoff, Werner Seeger, Oliver Eickelberg. “Four-and-a-half-LIM-domain proteins: Essential regulators in pulmonary fibrosis?” 14th International Colloquium on Lung Fibrosis (ICLF), Schloss Reinhartshausen Kempinski Mainz (Germany), 7-10 September 2006

- **Izabella Chrobak,** Nils Banthien, Melanie Königshoff, Kamila Kitowska, Werner Seeger, and Oliver Eickelberg. “Imbalanced expression of the type I TGF-β receptors ALK-1 and ALK-5 in Pulmonary Fibrosis”. American Thoracic Society (ATS) International Conference, San Francisco (USA), 18-23 Mai 2007. International Trainee Travel Award (ITTA) 2007

- Kamila Kitowska, Dariusz Zakrzewicz, Melanie Königshoff, Matthias Hecker, **Izabela Chrobak**, Andreas Guenther, Werner Seeger, Patrick Bulau, and Oliver Eickelberg. “Increased Arginase Expression is a Novel Pathogenic Mechanism in Pulmonary Fibrosis”. American Thoracic Society (ATS) International Conference, San Francisco (USA), 18-23 Mai 2007

- Nils Banthien**, Izabella Chrobak**, Kamila Kitowska, Melanie Königshoff, Werner Seeger, Oliver Eickelberg. “Four-and-a-half-LIM-domain proteins: Essential regulators in pulmonary fibrosis?” American Thoracic Society (ATS) International Conference, San Francisco (USA), 18-23 Mai 2007

- Nils Banthien**, Izabella Chrobak**, Kamila Kitowska, Melanie Königshoff, Werner Seeger, Oliver Eickelberg. “Four-and-a-half-LIM-domain proteins: Essential regulators in pulmonary fibrosis?” European Respiratory Society (ERS) Annual Congress, Stockholm (Sweden), 15-19 September 2007

- **Izabela Chrobak,** Oliver Eickelberg. “Negative regulation of secreted Frizzled-related Protein 2 (sFRP2) by TGF-β1: Implications for tissue fibrosis”. Wnt Signaling in Development and Disease, Max Delbrück Communications Center, Berlin-Buch (Germany), 12-15 September 2007

- **Izabela Chrobak**, Nils Banthien, Melanie Königshoff, Kamila Kitowska, Werner Seeger, and Oliver Eickelberg. “Imbalanced expression of the type I TGF-β receptors ALK-1 and ALK-5 in pulmonary fibrosis”. Sektion Zellbiologie der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V., Munich (Germany), 12-13 October 2007; Sektion Zellbiologie der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V., Freiburg (Germany), 7-8 November 2008

- **Izabela Chrobak**, Oana V Amarie, Melanie Königshoff, Kamila Kitowska, Simone Becker, Werner Seeger, Oliver Eickelberg. “Imbalanced expression of the type I TGF-β receptor ALK-1 in pulmonary fibrosis”. 15th International Colloquium on Lung and Airway Fibrosis (ICLF), Sea Trail Resort, Sunset Beach, NC (USA), September 28 - October 1, 2008

- **Izabela Chrobak**, Oana V Amarie, Melanie Königshoff, Kamila Kitowska, Simone Becker, Werner Seeger, Oliver Eickelberg. “Dysregulation of the type I TGF-β receptor ALK-1 in pulmonary fibrosis”. 7th ERS Lung Science Conference, Estoril, Portugal, March 27-29, 2009

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