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Kollagenbiosynthese und -reifung in Lungenfibrose

Collagen biosynthesis and maturation in lung fibrosis

Habilitationsschrift (kumulative Habilitation)

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INHALT

1 INTRODUCTION

1.1 Interstitial lung disease

Interstitial lung disease (ILD) is a collective term for various lung conditions which are characterized by thickening of the alveolar walls by inflammation or fibrosis. ILD can be caused by exposure to noxious substances as organic antigens, air pollutants, or drugs, but can also occur as result of systemic or autoimmune disease where multiple organs, not only the lung, are damaged. Prolonged ILD often leads to interstitial scarring, *i.e.* lung fibrosis.

Hypersensitivity pneumonitis (HP), for example, also referred to as extrinsic allergic alveolitis (EAA), is typically caused by repeated inhalation of agricultural dusts (Selman *et al.*, 2012). If HP is diagnosed at an early stage and the responsible harmful compound identified, then avoiding exposure usually results in attenuation or even reversion of disease (Fernandez Perez *et al.*, 2013). However, chronic exposure can lead to severe impairment of lung function and distortion of lung structure so that ultimately transplantation may be the only effective therapeutic option (Selman *et al.*, 2012, Grunes *et al.*, 2013). Another prominent cause of ILD is medication. Numerous drugs have been associated with ILD as adverse side effect including anti-inflammatory, antimicrobial, cardiovascular, and cytotoxic drugs (Schwaiblmair *et al.*, 2012). Also in these cases, early diagnosis and discontinuation of the drug usually lead to improvement of lung fibrosis and even full recovery is possible. However, if the diagnosis is performed at a later stage with a significant degree of established lung fibrosis, mortality rates can be as high as 40% (Schwaiblmair *et al.*, 2012). Similarly, systemic disease can result in interstitial lung fibrosis: Sarcoidosis, for instance, is a systemic primarily inflammatory disease with unknown cause which can involve multiple organs, but mostly affects the lung. Here, nodules of inflamed tissue, so-called granulomas, form which may resolve without irreversible damage (Valeyre *et al.*, 2014), but which, in 20–25% of the patients, result in pulmonary fibrosis and permanent lung dysfunction (Patterson *et al.*, 2013). Classical autoimmune diseases, *e.g.* scleroderma, rheumatoid arthritis, and systemic lupus erythematosus, also often manifest in fibrosis of the lung and other organs and pulmonary involvement has a major impact on morbidity and mortality of patients (Kronbichler *et al.*, 2013, Vij *et al.*, 2013).

Idiopathic pulmonary fibrosis (IPF) is the most fatal ILD with a five-year survival rate of 30-50% and few treatment options (Kim *et al.*, 2006). Two recently FDA-approved drugs for IPF therapy, pirfenidone and nintedanib, decelerate but do not stop disease progression in IPF

patients by decreasing lung function decline. They also show considerable side effects and their mechanisms of action are incompletely understood (King *et al.*, 2014, Richeldi *et al.*, 2014). Hence, considering the prognosis of IPF and other forms of pulmonary fibrosis at advanced stage, there is a clear need to identify novel drug targets for late-stage ILD, in particular for IPF.

1.2 IPF pathogenesis

The etiology of IPF is poorly understood. The current belief is that an aberrant wound-healing response is initialized by repeated micro-injuries to the lung epithelium. This leads to secretion of fibrotic mediators including transforming growth factor β (TGF-β) which results in an accumulation of (myo-)fibroblasts in the alveolar region (Selman *et al.*, 2001, Blackwell *et al.*, 2014, Renzoni *et al.*, 2014, Selman *et al.*, 2014, Wolters *et al.*, 2014). The latter overgrow the delicate alveolar lung tissue and secrete increased amounts of extracellular matrix (ECM) proteins.

1.2.1 The airway epithelium

Traditionally, research on IPF pathogenesis has focused on alveolar epithelial-mesenchymal cross-talk as disease-initiating mechanism and fibroblast foci as final manifestation of disease (Selman *et al.*, 2001, Renzoni *et al.*, 2014, Wolters *et al.*, 2014). Alveolar epithelial cells have been shown to activate TGF-β from latent complexes in the ECM (Tatler *et al.*, 2012, Henderson *et al.*, 2013, Sheppard, 2015), release TGF-β-containing exosomes (Borges *et al.*, 2013), secrete connective tissue growth factor (CTGF) and sonic hedgehog (SHH), and release Wnt1-inducible signaling protein (WISP1) (Konigshoff *et al.*, 2009), all contributing to activation of (myo-)fibroblasts. Importantly, the discovery that a *MUC5B* promoter polymorphism was associated with increased risk of developing familial and sporadic IPF strongly suggested a causal involvement also of the bronchial or the distal bronchiolized epithelium (Seibold *et al.*, 2011, Hunninghake *et al.*, 2013, Seibold *et al.*, 2013). This prompted a number of studies aiming to describe the cellular composition of the bronchial epithelium and proliferative bronchiolar lesions which are characteristic for IPF and include hyperplasia and metaplasia of bronchiolar and bronchiolo-alveolar junctions, bronchiolization, and honeycombing (Plantier *et al.*, 2011, Seibold *et al.*, 2013, Smirnova *et al.*, 2016). Still, the role of these structures in IPF pathogenesis remains elusive.

1.2.2 Excessive production of extracellular matrix by the interstitial (myo)fibroblast

Multiple progenitor cells may contribute to the myofibroblast population, but the most well-established source is the interstitial fibroblast (Rock *et al.*, 2011). Myofibroblasts synthesize and deposit excessive amounts of extracellular matrix (ECM) proteins, like collagen type I, III, V, and fibronectin (Fernandez *et al.*, 2012). The resulting accumulation of ECM in the alveolar region is the ultimate pathological feature of lung fibrosis, leading to progressive lung function decline (Selman *et al.*, 2001, Wolters *et al.*, 2014). Notably, recent studies highlight that collagens are the main components of the ECM including newly synthesized ECM in a fibrotic response (Decaris *et al.*, 2014, Beachley *et al.*, 2015).

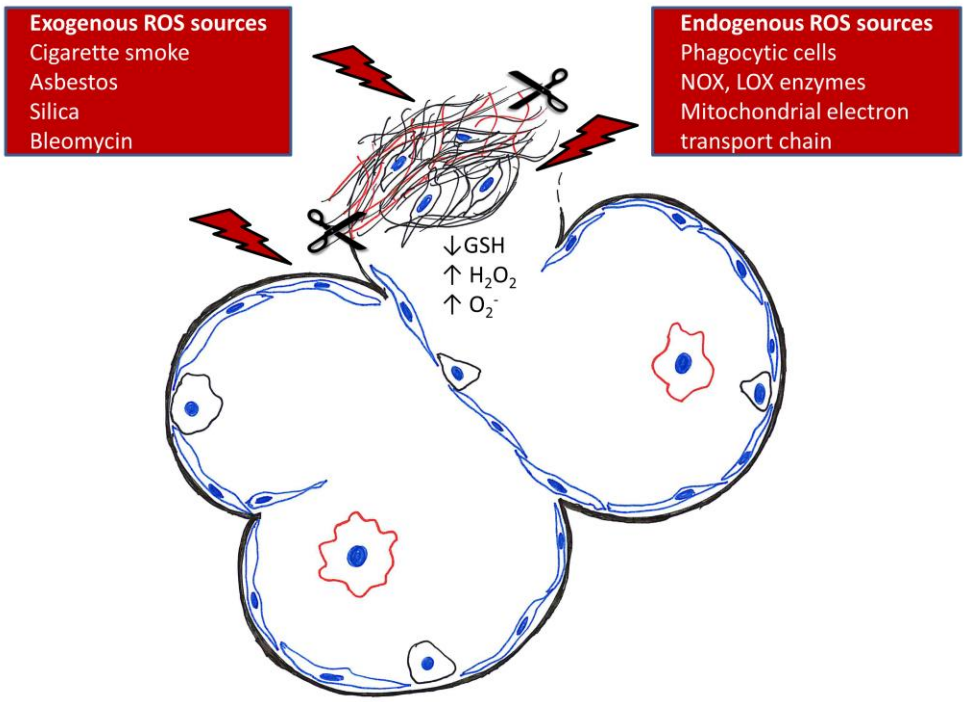

Figure 1: Excessive deposition of extracellular matrix (ECM) in the alveolar region and increased oxidative stress are two central pathological features in pulmonary fibrosis. Exogenous and endogenous sources cause increase of reactive oxygen species (*e.g.* hydrogen peroxide, H_2O_2, and superoxide anion, O_2^-) and lead to depletion of glutathione (GSH). In response, the alveolar basement membrane as well as the interstitial extracellular matrix (ECM) can be modified and damaged, *e.g.* resulting in increased shedding (✂) of ECM components. Little is known about the localization of potentially protective antioxidant proteins in the extracellular space.

1.2.3 Oxidative stress in interstitial lung disease and effects on the extracellular matrix

Several lines of evidence support the involvement of oxidative stress in fibrotic lung disease (Liu *et al.*, 2010, Cheresh *et al.*, 2013). For instance, markers of increased oxidative stress have been detected in exhaled air and bronchoalveolar lavage fluid (BALF) of patients suffering from IPF, sarcoidosis and HP (Maier *et al.*, 1991, Rahman *et al.*, 1999, Rottoli *et al.*, 2005, Psathakis *et al.*, 2006, Bargagli *et al.*, 2007). Furthermore, depletion of glutathione, the most abundant low-molecular-weight antioxidant, has been reported in the epithelial lining fluid (ELF) of IPF, sarcoidosis, and HP patients (Cantin *et al.*, 1989, Meyer *et al.*, 1994, Behr *et al.*, 2000, Boots *et al.*, 2009). Finally, numerous endogenous and exogenous agents implicated in the aetiology of pulmonary fibrosis cause levels of reactive oxygen species (ROS) to increase. Extrinsic sources include cigarette smoke, asbestos, silica, and bleomycin, all well-known risk factors for pulmonary fibrosis. Endogenous ROS sources include superoxide and hydrogen peroxide-producing phagocytic cells, but also intra- and extracellular enzymatic systems which produce ROS, as *e.g.* members of the NADPH oxidase (NOX) family, the mitochondrial electron transport chain, or extracellular lysyl oxidase activity in collagen crosslinking (Kagan *et al.*, 2003, Winterbourn, 2008, Hecker *et al.*, 2009, Carnesecchi *et al.*, 2011, *cf.* Figure 1).

Importantly, the composition of the ECM is affected by oxidative stress (Kliment *et al.*, 2010). Several *in vivo* studies in mouse models of lung fibrosis have shown that ECM components as *e.g.* collagen, heparan sulphate, syndecan, and hyaluronic acid are increasingly fragmented or shed from the cell surface in response to oxidative stress (Fattman *et al.*, 2003, Kliment *et al.*, 2008, Kliment *et al.*, 2009, Zelko *et al.*, 2010). In line with these observations, increased levels of collagen III, hyaluronic acid, and syndecan-1 have also been reported in BALF from IPF and HP patients (Bjermer *et al.*, 1989, Cormier *et al.*, 1993, Kliment *et al.*, 2009, Kliment *et al.*, 2010). A number of extracellular antioxidant proteins have been described to localize to the ECM of the lung, suggesting a potential protective role in presence of oxidative stress (Kinnula, 2005), in particular, glutathione peroxidase 3 (GPX3) and extracellular superoxide dismutase (EC-SOD or SOD3) (Kliment *et al.*, 2008, Kliment *et al.*, 2009, Zelko *et al.*, 2010, Burk *et al.*, 2011, Yamada *et al.*, 2012). While the role of EC-SOD in pulmonary fibrosis has received considerable attention (Gao *et al.*, 2008), the regulation and distribution of GPX3 in this context had not been studied in detail.

1.3 Collagen biosynthesis and maturation

Collagen biosynthesis is a highly complex process starting with transcription of collagen genes followed by translation and translocation of the nascent polypeptide chain to the rER, cotranslational modification and folding, trafficking across the Golgi network, secretion, and finally, extracellular processing and maturation (Ishikawa *et al.*, 2015, *cf.* Figure 2).

Folding in the rER requires several enzymes and molecular chaperones essential for post-translational modifications (PTMs) and the folding of procollagen chains into triple helices, where one of the rate-limiting steps is the *cis-trans* isomerization of proline residues catalyzed by rER resident peptidyl prolyl isomerases (PPIases, Ishikawa *et al.*, 2013). Two ER proteins participating in this multistep process are the collagen chaperones FK506-binding protein 10 (FKBP10, see 1.3.2) and heat-shock protein 47 (HSP47, also called SerpinH1, Ishikawa *et al.*, 2015). PTMs like hydroxylation of lysyl or prolyl residues (*cf.* Figure 3), or glycosylation of hydroxylysines are essential for proper stability, assembly and secretion of procollagen, as well as for the final supramolecular structure of these molecules (Hudson *et al.*, 2013). For instance, hydroxylation of proline residues in position 4 (4-hydroxyproline, *cf.* Figure 3) is known to increase thermodynamic stability of the triple helix (Ishikawa *et al.*, 2013). Also, the degree of lysine hydroxylation in the collagenous and telopeptide regions of fibrillar collagens defines the extent, type, and stability of extracellular intermolecular collagen crosslinks (Eyre *et al.*, 2008). At the same time, the functions of the comparatively rare prolyl-3-hydroxylation (3-hydroxyproline, *cf.* Figure 3) and O-glycosylations are far less understood.

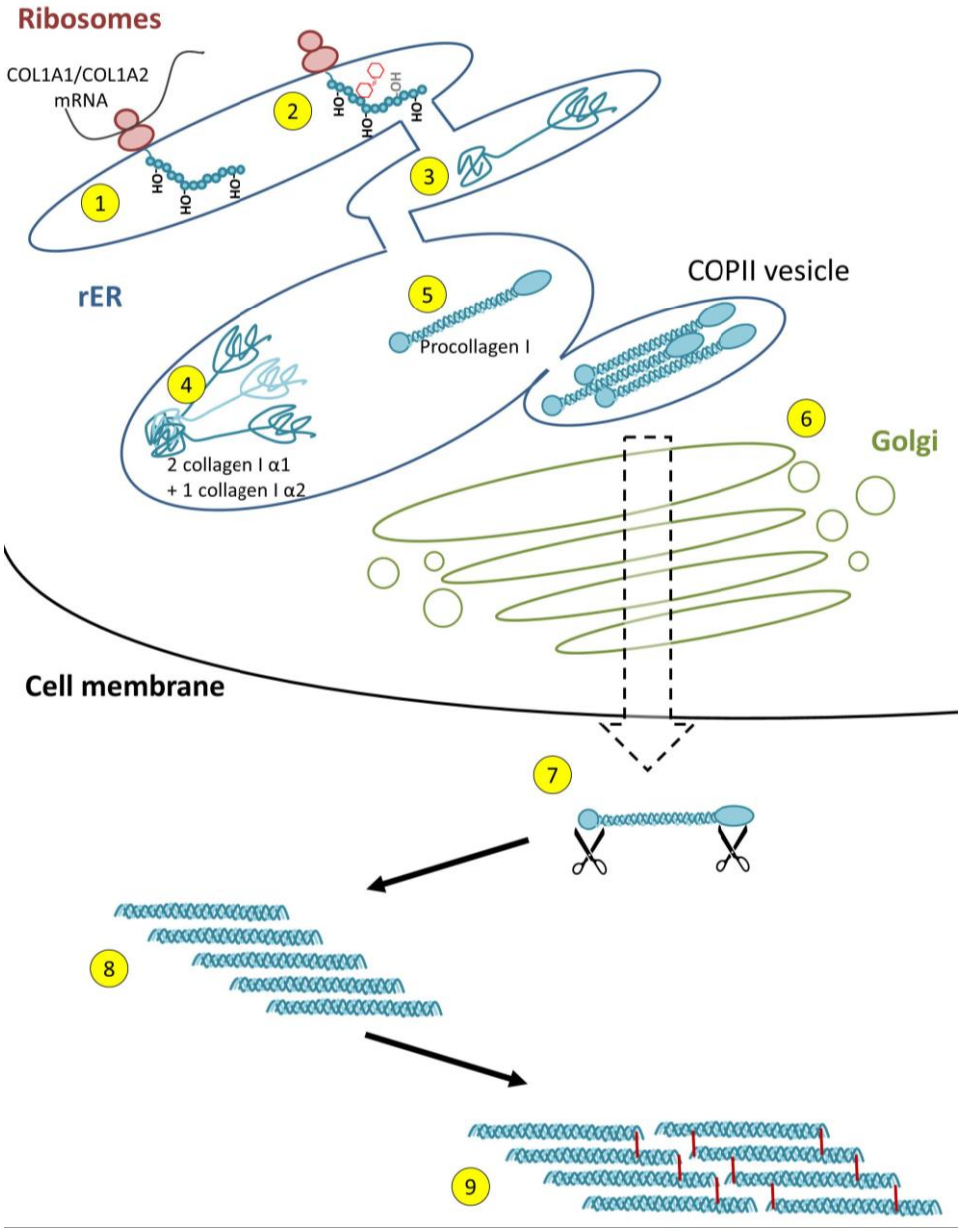

Figure 2: Intracellular collagen biosynthesis and extracellular maturation of collagen I. 1) Cotranslational prolyl-4- and lysyl-hydroxylation of the nascent collagen polypeptide chain in the rough endoplasmic reticulum (rER) is followed by 2) glycosylation and prolyl-3-hydroxylation and 3) folding of the C- and N-terminal propeptides. 4) For collagen I, two properly folded α1 chain C-propeptides assemble with one α2 chain C-propeptide, forming the triple helix nucleus. 5) Triple helix formation occurs in a zipper-like fashion and is dependent on peptidyl-prolyl isomerases and collagen chaperones, including FKBP10. 6) After packaging into COPII vesicles, collagen triple helices are transported via the trans-Golgi network and finally secreted into the extracellular space. 7) In the extracellular space, propeptide cleavage involving at least three proteases triggers 8) auto-assembly of collagen fibrils, an entropy-driven process. 9) Finally, fibrils are stabilized by crosslinking.

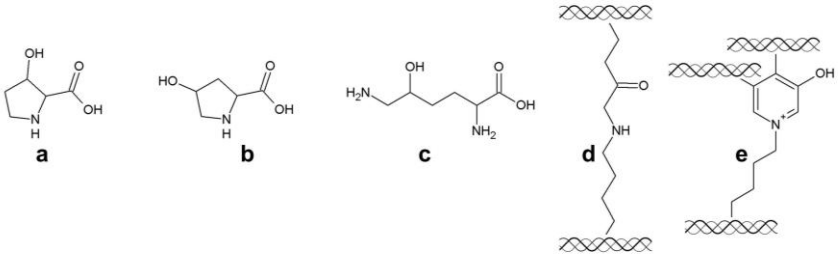

Figure 3: Examples for collagen post-translational modifications. Structures of (a) 3-hydroxyproline, (b) 4-hydroxyproline, (c) 5-hydroxylysine, (d) the divalent collagen crosslink hydroxyl-lysinoketonorleucine, and (e) the trivalent collagen crosslink lysyl pyridinoline.

Following trans-Golgi trafficking and secretion, collagen is secreted into the extracellular space where spontaneous fibril assembly and collagen crosslinking mediated by enzymes of the lysyl oxidase (LOX) family occurs.

1.3.1 Collagen biosynthesis and maturation in idiopathic pulmonary fibrosis

It is incompletely understood how collagen biosynthesis and modification are altered in fibrosis and how such changes might affect extracellular collagen PTMs, adherent cell function, and disease progression. The concept of targeting extracellular collagen crosslinking as an anti-fibrotic strategy has received considerable attention in the past years, fueled by the discovery of the collagen crosslinking enzyme lysyl oxidase-like 2 (LOXL2) as a potential drug target for pulmonary fibrosis (Barry-Hamilton *et al.*, 2010). However, a phase II study with a monoclonal anti-LOXL2 antibody has been terminated due to lack of efficacy (http://www.gilead.com/news/press-releases/2016/1/gilead-terminates-phase-2-study-of-simtuzumab-in-patients-with-idiopathic-pulmonary-fibrosis). One must bear in mind that lysyl oxidases perform the final step of collagen maturation, acting on extracellular fibrillar collagen (Robins, 2007). Fibrillar collagen is resistant to most extracellular proteases (Manka *et al.*, 2012, Panwar *et al.*, 2013), suggesting that inhibition of LOXL2, acting after spontaneous fibril formation (Chen *et al.*, 2009, Steplewski *et al.*, 2012), might have limited effects on the reduction and resolution of collagen-rich fibrotic areas. Therefore, it is conceivable that collagen synthesis and processing must be inhibited at an earlier step.

1.3.2 FK506-binding protein 10 (FKBP10)

The family of FK506-binding proteins (FKBPs) belongs to the class of immunophilins, peptidyl-prolyl cis/trans isomerases (PPIases) with the ability to bind immunosuppressive

drugs. FKBPs bind FK506 (tacrolimus) and rapamycin via their FK506-binding domains (FKBD), which also exhibit the PPIase activity of the protein. Binding of FK506 or rapamycin typically results in inhibition of the PPIase activity. The nomenclature for the FKBPs is ambiguous as their gene names do not match with their traditional protein names, which reflect the molecular weight. Here, the gene name FKBP10 will be used consistently also for the protein (as recommended by the protein database Uniprot, http://www.uniprot.org/), even if in the literature FKBP65 is used as protein name to a large extent.

FKBP10 contains four FKBDs, but only one FKBD can be inhibited by FK506 and surprisingly also by cyclosporine A, which is not known to inhibit PPIase activity in any other FKBP (Coss *et al.*, 1995, Zeng *et al.*, 1998). The current literature supports a major function of FKBP10 in folding and post-translational modification of collagen I. Mutations in the FKBP10 gene have been associated with recessive type XI of *osteogenesis imperfecta* and Bruck´s syndrome, collagen-related disorders characterized by growth deficiency, bone fragility and deformity (Alanay *et al.*, 2010, Barnes *et al.*, 2012). In OI patients with FKBP10 deficiency, extracellular collagen crosslinking is dramatically reduced, caused by absent hydroxylation of the collagen telopeptide lysine. FKBP10 has therefore been suggested to be essential for lysyl hydroxylase activity (Barnes *et al.*, 2012). Supporting this idea, two recent studies have independently provided evidence that FKBP10 modulates collagen lysyl hydroxylase 2 activity. Therefore, FKBP10 indirectly affects the generation of collagen hydroxylysines and thus the extent and stability of the extracellular collagen crosslinks introduced by enzymes of the lysyl oxidase (LOX) family (Gjaltema *et al.*, 2016, Duran *et al.*, 2017).

2 AIMS OF HABILITATION

Currently approved options for treatment of IPF, *i.e.* nintedanib and pirfenidone, merely decelerate lung function decline in patients and do not stop progression of disease, let alone reverse fibrosis. The overall aim of this thesis is therefore to identify novel therapeutic strategies for IPF, with a focus on modulating properties of the extracellular matrix, most importantly collagen.

More specifically, the aims of this habilitation project were the following:

- Investigate association of antioxidant proteins with the extracellular matrix in ILD
- Investigate expression and function of FKBP10 in pulmonary fibrosis
- Establish human-derived *in vitro* culture models for lung fibrosis for functional analysis
- Elucidate the effects of current IPF therapeutics on collagen biosynthesis and fibril formation

3 MATERIAL AND METHODS

3.1 Patient samples

Resected human lung tissue and lung explant material for analysis and isolation of primary human cells was obtained from the BioArchive CPC-M for lung diseases at the Comprehensive Pneumology Center (CPC), Helmholtz Zentrum München, Munich, Germany (Papers I-IV). All participants have given written informed consent and the study was approved by the local ethics committee of Ludwig-Maximilians University of Munich, Germany (333-10). For Paper I, additional patients were enrolled at the Thomayer Hospital, Prague, Czech republic. This study was approved by the Ethics Committee of Thomayer Hospital and the Institute of Clinical and Experimental Medicine, Prague, Czech Republic. All procedures were carried out in accordance with the approved guidelines.

3.2 The mouse model of bleomycin-induced lung fibrosis

The model of bleomycin-induced lung fibrosis is the best-established and most widely used mouse model for lung fibrosis (Moeller *et al.*, 2008, Peng *et al.*, 2013). Intratracheal instillation of bleomycin into mice leads to an initial acute lung injury, followed by an inflammatory response and lung fibrosis, which in our hands peaks at day 14 post-instillation and resolves over the next four weeks (Schiller *et al.*, 2015, Staab-Weijnitz *et al.*, 2015, Fernandez *et al.*, 2016). Typical read-outs include lung function (flexiVent, SCIREQ), fibrotic markers on transcript and protein level (*e.g.* hydroxyproline content, collagen 1A1 biosynthesis, collagen deposition, fibronectin, α-smooth muscle actin), histology (Masson Trichrome, immunofluorescence), and analysis of bronchoalveolar lavage (BAL). All animal experiments were conducted under strict governmental and international guidelines.

3.3 Primary human lung fibroblasts as an *in vitro* model for fibrosis

For expansion, primary human lung fibroblasts (phLF) from different donors are cultured in DMEM/F12 (Life Technologies) supplemented with 20% FBS (Pan Biotech) and penicillin/streptomycin. To mimic fibrotic conditions and allow for efficient biosynthesis of collagen, phLF are treated with transforming growth factor β1 (TGF-β1) and cultured in presence of 2-phospho-L-ascorbic acid. Typical readouts used in this work are collagen biosynthesis on transcript and protein level, collagen secretion, collagen post-translational modifications, and expression of proteins of the ER-resident collagen biosynthesis machinery.

3.4 Primary human bronchial epithelial cells

Isolation, culture and differentiation of primary human bronchial epithelial cells (phBECs) from explants in our lab is established according to a published protocol (Fulcher *et al.*, 2005, Schamberger *et al.*, 2014). While submerged serum-free culture of these cells at low passages preserves basal cell-like properties, lifting cells to an air-liquid interface allows for organotypic culture of a pseudostratified epithelium composed of all epithelial cell types of a typical bronchial epithelium including basal cells, ciliated cells, Goblet cells, and Clara cells.

3.5 Precision-cut lung slices

Human precision-cut lung slices (PCLS) from lung resections or explants are being increasingly used as *ex vivo* models for chronic lung disease (Lauenstein *et al.*, 2014, Uhl *et al.*, 2015, Mercer *et al.*, 2016). To prepare PCLS at our institute, a cannula is inserted into a visible airway and the lung segments are filled with warm agarose (3 %). Lung segments are cooled on ice to allow solidification of the agarose and then cut into 500 µm thick sections with a vibratome (Hyrax V55; Zeiss, Jena, Germany). The resulting PCLS are cultivated in culture medium, where they float on the surface and are viable for up to 5 days (Uhl *et al.*, 2015). Importantly, PCLS preserve much of the cellular composition of normal tissue, in contrast to de- and recellularized tissue scaffolds, which are also being explored as alternative model systems in the lung field (Parker *et al.*, 2014, Wagner *et al.*, 2014).

3.6 Quantitative label-free mass spectrometry and solubility profiling

The proteome analysis pipeline including quantitative detergent solubility profiling (QDSP) has been established by Schiller *et al.* (2015). For proteome analysis of murine and human tissue samples, typically ~100mg of fresh frozen lung tissue (wet weight) is homogenized in phosphate-buffered saline, followed by centrifugation and collection of the soluble proteins. For extraction of proteins from the insoluble pellet, the pellet is sequentially incubated with buffers of increasing sodium deoxycholate and sodium dodecylsulphate concentrations, *i.e.* increasing solubilization stringency, resulting in three protein fractions which are highly enriched for matrisome-associated and ECM proteins (Naba *et al.*, 2012, Schiller *et al.*, 2015). Protein fractions are digested using a combination of LysC protease and trypsin and the resulting peptides subjected to analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Quadrupole/Orbitrap type mass spectrometer (Q-Exactive, Thermo Scientific). MS raw files are analyzed with the MaxQuant software (Cox *et al.*, 2008) and the

Andromeda search engine is used to match results with the Uniprot FASTA database (Cox *et al.*, 2011).

3.7 Other methods

For detailed description of more conventional methods for quantification of gene expression, imaging, and functional analysis, I would like to refer to the attached original papers and manuscripts and references therein.

4 RESULTS AND DISCUSSION

4.1 Glutathione peroxidase 3 associates with collagen in the extracellular matrix in interstitial lung disease (Paper I) (Schamberger *et al.*, 2016)

Aberrant antioxidant activity and excessive deposition of extracellular matrix (ECM), in particular collagens, are hallmarks of interstitial lung diseases (ILD). It is known that oxidative stress alters the ECM, but extracellular antioxidant defence mechanisms in ILD are incompletely understood. We therefore hypothesized that in ILD, lung cells secrete extracellular antioxidant proteins which associate with the ECM, in particular with collagen, and potentially protect the ECM from oxidative damage.

Therefore, we initially extracted abundance and detergent solubility of known extracellular antioxidant enzymes from a proteomic dataset of bleomycin-induced lung fibrosis in mice (Schiller *et al.*, 2015). We found that superoxide dismutase 3 (Sod3), Gpx3, and selenium-dependent Gpx activity were increased in mouse BALF during bleomycin-induced lung fibrosis. In lung tissue homogenates, Gpx3, but not Sod3, was upregulated and detergent solubility profiling indicated that Gpx3 associated with ECM proteins. Immunofluorescence analysis showed that Gpx3 was expressed by bronchial epithelial cells and localized to the basement membrane in normal mouse lung. In response to bleomycin, Gpx3 was additionally found expressed in interstitial fibroblasts and in association with collagen I in the interstitial fibrotic ECM. In agreement, we found that phBECs und phLFs express GPX3 in culture. While previous studies have shown expression of GPX3 by bronchial epithelial cells and presence in the ELF (Avissar *et al.*, 1996, Comhair *et al.*, 2001) as well as localization to the basement membrane (Olson *et al.*, 2010, Burk *et al.*, 2011), expression by fibroblasts and association to interstitial collagen in the lung has not been reported before. In addition, presence of Gpx3 in the lung has been largely attributed to serum Gpx3 supplied by the kidneys (Olson *et al.*, 2010, Burk *et al.*, 2011, Yamada *et al.*, 2012). Our study highlights that GPX3 is expressed and secreted by mouse and human lung structural cells under physiological and pathological conditions and suggests that locally expressed and secreted GPX3 significantly contributes to extracellular lung GPX3.

As to human ILD samples, BALF of some patients contained high levels of GPX3, in particular patients with HP and sarcoidosis, *i.e.* ILDs with an inflammatory component. In addition, GPX3 protein was upregulated in lung homogenates from IPF patients. This lead us to investigate whether GPX3 expression in phBECs and phLFs was regulated by

proinflammatory or profibrotic stimuli, or by the oxidative stress inducer menadione. Unexpectedly, we found that GPX3 expression and secretion was consistently downregulated by TNF-α in both cell types. Regulation by TGF-β1 and menadione was cell-type-specific: In phBECs, TGF-β1 moderately upregulated GPX3 while menadione had no effect. In phLFs, also TGF-β1 downregulated GPX3, while menadione increased *GPX3* expression and secretion.

In conclusion, the antioxidant enzyme GPX3 is expressed by bronchial epithelial cells and secreted in active form into the ELF. GPX3 is upregulated in the mouse model of bleomycin-induced lung fibrosis and localizes to lung ELF and the ECM. While only some HP and sarcoidosis patients, but not IPF patients, showed high levels of GPX3 in BALF, we found significant upregulation of GPX3 in total tissue homogenates from IPF patients. Importantly, we could show colocalization of GPX3 with collagen I in the interstitial fibrotic ECM, suggesting that GPX3 may protect collagen I in this compartment from oxidative damage.

4.2 FK506-binding protein 10 is upregulated in ILD including IPF and localizes to lung fibroblasts (Paper II) (Staab-Weijnitz *et al.*, 2015)

Increased abundance and stiffness of the extracellular matrix (ECM), in particular collagens, is a general characteristic of organ fibrosis, including fibrosis of the lung. FKBP10 is a collagen chaperone, mutations of which are described to lead to reduced collagen crosslinking and ECM stiffness, *e.g.* in *osteogenesis imperfecta*. Here we hypothesized that FKBP10 is upregulated in lung fibrosis.

To this end, we initially analyzed *Fkbp10* expression in the mouse model of bleomycin-induced lung fibrosis using qRT-PCR, Western Blot, and immunofluorescence. We found that *Fkbp10* expression was increased up to five-fold 14 days after bleomycin instillation, the time point where fibrosis peaks in our model (Schiller *et al.*, 2015, Staab-Weijnitz *et al.*, 2015, Fernandez *et al.*, 2016), both at transcript and protein level. Immunofluorescence analysis using the myofibroblast marker α-smooth muscle actin (α-SMA) and the alveolar epithelial cell markers podoplanin (T1α), or thyroid transcription factor 1 (TTF1) demonstrated expression in interstitial (myo-)fibroblasts, but not in alveolar type I and type II epithelial cells, respectively (Staab-Weijnitz *et al.*, 2015).

Then, we analysed microarray data from 99 IPF patients and 43 control subjects derived from samples of the Lung Tissue Research Consortium (LTRC), a resource program of the U.S. National Heart Lung and Blood Institute. FKBP10 was strongly and highly significantly

upregulated in IPF lung samples. Western Blot Analysis of lung homogenates derived from 6 IPF patients and 5 control subjects from our in-house CPC BioArchive confirmed upregulation of FKBP10 in an independent cohort. Similar to fibrotic mouse lung, FKBP10 was found expressed in interstitial (myo-)fibroblasts and not in alveolar epithelial cells in IPF lung tissue. In addition, we found FKBP10 localized in $CD68^+$ macrophages (Staab-Weijnitz *et al.*, 2015). We also compared baseline expression in phLFs with baseline expression levels and, for some, also the TGF-β-induced fold change of FKBP10 induction in the following cell types and lines: PhBECs, differentiated at the air-liquid interface (Schamberger *et al.*, 2015), the human bronchial epithelial cell line 16-HBE, the human alveolar epithelial cell line A549, primary human alveolar type II (phAT2) cells (courtesy of Dr. Kathrin Mutze and Prof. Dr. Dr. Melanie Königshoff, CPC Munich), and the murine alveolar macrophage line MH-S. In agreement with our results from our immunoflourescence-based colocalization studies, phLFs had by far the highest baseline expression levels and also showed the greatest ability to upregulate FKBP10 expression (about 3-fold) in response to TGF-β1 (see Figure 4, unpublished data).

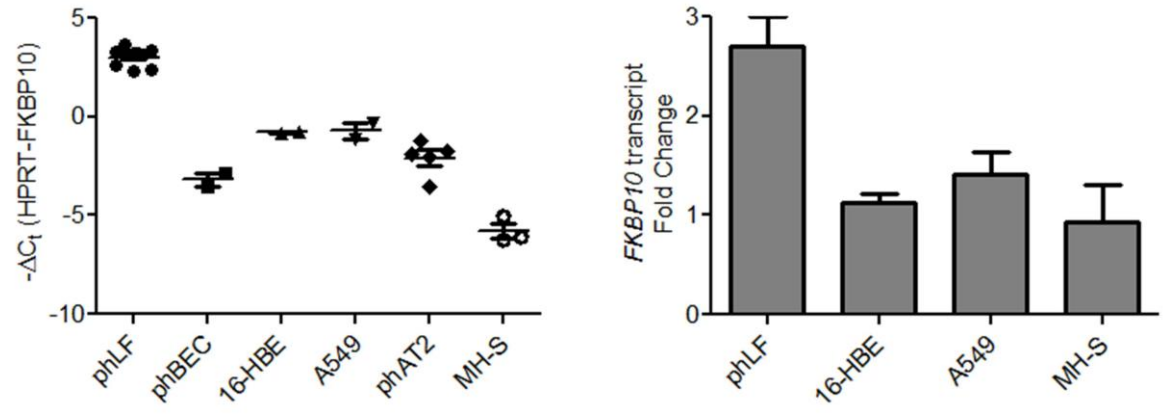

Figure 4: Baseline expression of *FKBP10* and induction by TGF-β1 in selected cell types. *Left-hand panel*: Baseline expression of *FKBP10* normalized to expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) demonstrates highest expression levels in primary human lung fibroblasts (phLFs). *Right-hand panel*: In response to 2 ng/ml TGF-β1, phLFs show the greatest ability to further induce *FKBP10* expression.

Finally, a recent proteomic analysis confirmed upregulation of FKBP10 in end-stage ILD, including IPF, but also non-IPF ILD such as HP, suggesting that FKBP10 overexpression is a general characteristic of lung fibrosis, irrespective of cause. In contrast, FKBP10 was not increased in fibrotic skin samples from scleroderma patients (Schiller *et al.*, in revision). In collaboration with Dr. Lange-Sperandio (Hospital of the Ludwig-Maximilian University of

Munich, Germany), we have observed that Fkbp10 is induced in the unilateral ureteral obstruction (UUO) mouse model for kidney fibrosis (unpublished data, not shown). Taken together, these findings suggest that FKBP10 overexpression may be specific for fibrosis of internal organs.

4.3 Primary human lung fibroblasts and human precision-cut lung slices are suitable *in vitro* models for lung fibrosis (Papers II, III, and IV) (Staab-Weijnitz *et al.*, 2015, Alsafadi *et al.*, 2017, Knüppel *et al.*, 2017)

Lung fibrosis is a complex process involving infiltration and differentiation of several cell types including cells of the immune compartment and, clearly, animal models for lung fibrosis are indispensable to study disease pathogenesis in the whole organism. Nevertheless, there is no animal model that recapitulates all characteristics of human pulmonary fibrosis (Moore *et al.*, 2008, Antoniu *et al.*, 2009). Moreover, it is very well-acknowledged that considerable species-species differences exist between human and the most frequently used experimental animal, the mouse. Ultimately, this is reflected by the fact, that unfortunately many therapeutic approaches which work in mouse models are not effective in patients (Mestas *et al.*, 2004, Martignoni *et al.*, 2006). Therefore, human *in vitro* approaches must be developed and applied in the quest for novel drug targets.

Frequently, lung cell lines are used for *in vitro* studies of collagen biosynthesis in the context of lung fibrosis research. These include fetal fibroblast lines like HLF, MRC5 or the mouse lung fibroblast line CCL206 as well as epithelial lung cell lines, as *e.g.* the adenocarcinoma cell line A549 (Hisatomi *et al.*, 2012, Liu *et al.*, 2013, De Langhe *et al.*, 2014, Yang *et al.*, 2014). These cell lines are easy-to-handle, provide an unlimited supply of comparatively homogeneous cell material, and circumvent ethical issues associated with material derived from patients. However, it has been shown that fetal fibroblasts differ dramatically in gene expression and response to TGF-β1 (Rolfe *et al.*, 2007, Navarro *et al.*, 2009). A549 cells produce at best a tenth of the collagen produced by phLFs in our hands, and CCL206 cells, even if they have comparable COL1A1 transcript levels, show substantially less collagen secretion and do not upregulate collagen secretion in response to TGF-β1 (unpublished observations).

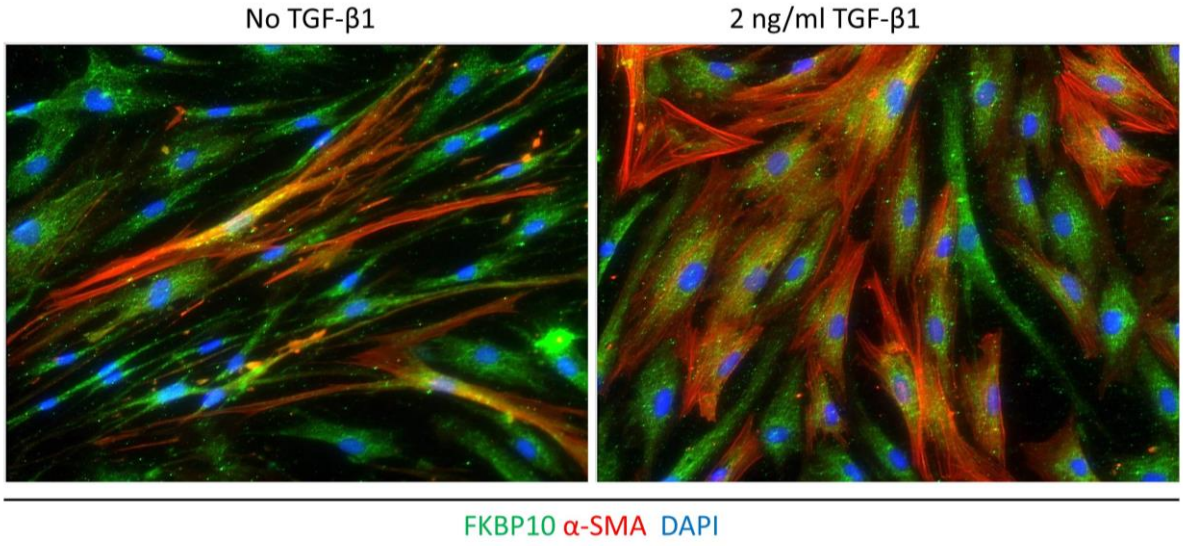

Figure 5: Primary human lung fibroblasts differentiate to myofibroblasts in response to TGF-β1. Treatment of phLFs with 2 ng/ml TGF-β1 results in cell hypertrophy, increased expression of α-smooth muscle actin (α-SMA), and generation of stress fibers, all characteristics of the myofibroblast. FKBP10, FK506-binding protein, see text.

Therefore, we established an *in vitro* model of lung fibrosis using phLFs, derived from IPF patient explants, healthy donor lungs, or normal histology regions from lung tumor resections. We included physiological concentrations (2 ng/ml) of TGF-β1 as central profibrotic cytokine which is well-known to induce differentiation to myofibroblasts and collagen gene transcription (*cf.* Figure 5). Another crucial component of the cell culture medium was 2-phosphoascorbate, a stable form of ascorbate, which is an essential cofactor of all collagen lysyl and prolyl hydroxylases, but often omitted in *in vitro* studies of collagen biosynthesis (Hata *et al.*, 1989, Chen *et al.*, 2009). Under these conditions, independent of cell origin, phLF consistently and reproducibly secreted high and easily detectable levels of secreted collagen, including total collagen, collagen I, and collagen III (Staab-Weijnitz *et al.*, 2015, Knüppel *et al.*, 2017). Notably, in prolonged fibroblast culture, we could collect enough collagen from cell culture supernatant to allow for fractionation, purification and analysis of the different collagen types via polyacrylamide gel electrophoresis, in gel digestion, and MS/MS analysis. Furthermore, by electron microscopy, we were able to detect deposited collagen fibrils under these conditions (Knüppel *et al.*, 2017), which frequently is limited in *in vitro* systems to study fibrogenesis (Chen *et al.*, 2009). Collectively, this demonstrates that

culture of phLFs in presence of 2-phosphoascorbate and TGF-β1 represents a suitable model to study collagen biosynthesis, secretion, and deposition in the context of lung fibrogenesis.

A drawback of human cell culture is certainly the fact that normal tissue architecture and cell composition is not maintained. This can be bypassed by tissue culture approaches. Therefore, we took advantage of a technology established at the institute which allows for the generation of precision-cut lung slices (PCLS) from human lung (Uhl *et al.*, 2015). IPF explant-derived PCLS have been used previously to evaluate a potential novel drug for IPF (Mercer *et al.*, 2016). Here, we reasoned that incubation of PCLS derived from normal histology human lung tissue with key profibrotic mediators would result in increased ECM deposition and aberrant tissue repair (Alsafadi *et al.*, 2017).

We chose to use a cocktail of the profibrotic mediators TGF-β1, platelet-derived growth factor AB (PDGF-AB), tumor necrosis factor-α (TNF-α), and lysophosphatidic acid (LPA) for the following reasons: TGF-β1 is a profibrotic cytokine secreted by macrophages, epithelial cells, endothelial cells, and mesenchymal cells, which is well-known to play a central role in fibrogenesis of all organs, by recruiting and activating fibroblasts, inducing differentiation to myofibroblasts, and increasing ECM production (Agostini *et al.*, 2006, Fernandez *et al.*, 2012, Rockey *et al.*, 2015). PDGF is secreted by macrophages, fibroblasts, epithelial cells, and endothelial cells upon injury and induces fibroblast proliferation in the lung (Antoniades *et al.*, 1990, Bonner, 2004, Agostini *et al.*, 2006, Noskovicova *et al.*, 2015). The proinflammatory cytokine TNF-α, secreted by macrophages, lymphocytes, epithelial cells, and endothelial cells, is present in fibrotic areas of the lung and has been shown to induce further profibrotic cytokine secretion and also fibroblast proliferation (Zhang *et al.*, 1993, Agostini *et al.*, 2006). Finally, LPA was added in order to take into account the more recently established role of LPA signaling for the aberrant wound healing response in lung fibrosis, including induction of epithelial cell apoptosis, recruitment of fibroblasts, and activation of latent TGF-β (Tager *et al.*, 2008, Shea *et al.*, 2012).

PCLS from each patient were treated with this fibrosis cocktail (FC) and vehicle control in parallel. Incubation of PCLS with FC did not affect viability of PCLS in the analyzed time frame (5 days) relative to vehicle control. We analyzed gene expression of various markers of lung remodeling as well as secretion and deposition of collagen I, fibronectin, and tenascin-C as representative ECM proteins. In agreement with our hypothesis, gene expression of *CTGF*, *SERPINE1*, and *MMP7*, encoding the tissue remodelling factors connective tissue growth

factor (CTGF), plasminogen activator inhibitor-1 (PAI1), and matrix metallopeptidase 7 (MMP7) was upregulated in response to 48h and 120h incubation with FC. The same was true for *ACTA2* encoding α-smooth muscle actin (α-SMA), a known marker of myofibroblast differentiation, and *COL1A1*, *FN1*, and *TNC*, coding for the ECM proteins collagen I (α1 chain), fibronectin, and tenascin-C, respectively. On the protein level, we observed increases of the mesenchymal marker vimentin, of fibronectin, tenascin-C, and collagen I in the majority of PCLS, but mostly unchanged levels of α-SMA. These changes were accompanied by significant decreases of alveolar epithelial cell markers including surfactant protein C and E-cadherin as well as by downregulation of *HOPX* expression (Alsafadi *et al.*, 2017). As both the SPC-positive AT2 cells and the HOPX-positive cell population represent alveolar progenitor cells (Jain *et al.*, 2015), these results suggest depletion of the alveolar progenitor cell pool resulting in deficient epithelial regeneration.

Collectively, these findings demonstrate that incubation of PCLS with FC induces central events in lung fibrogenesis, most importantly increased synthesis and deposition of ECM proteins and inhibition of alveolar repair. With most changes occurring already after 48 h of treatment, this model clearly has potential to allow for evaluation of putative therapeutics in the remaining viability window of 3 days.

4.4 Downregulation of FKBP10 by RNA interference decreases collagen biosynthesis and secretion in primary human lung fibroblasts (Paper II) (Staab-Weijnitz *et al.*, 2015)

Following up on our observation that, in lung fibrosis, FKBP10 was mostly expressed by interstitial fibroblasts (see 4.2), we used our phLF-based *in vitro* model of lung fibrosis (*cf.* 3.3 and 4.3) to assess subcellular localization, regulation, and function of FKBP10. Subcellular localization of FKBP10 was assessed by immunofluorescent stainings and subcellular fractionation, collectively demonstrating association with the endoplasmic reticulum, in agreement with previous reports (Patterson *et al.*, 2000, Lietman *et al.*, 2014). *FKBP10* expression in phLF was upregulated by TGF-β1, but downregulated by two mechanistically independent ER stress inducers, tunicamycin and thapsigargin. Finally, the function of FKBP10 was analyzed in phLF by siRNA-mediated loss-of-function-experiments followed by assessment of collagen biosynthesis and secretion as well as other markers of lung fibrosis. Knockdown of FKBP10 attenuated expression of pro-fibrotic mediators and effectors, including collagens I and V and α-smooth muscle actin, on the transcript and

protein level. Importantly, loss of FKBP10 expression significantly suppressed collagen secretion by phLF. The effect on collagen secretion was compared with those of the currently approved IPF therapeutics and found to be equally strong as submicromolar concentrations of nintedanib and even superior to millimolar concentrations of pirfenidone. We therefore concluded that FKBP10 may be a novel drug target for IPF (Staab-Weijnitz *et al.*, 2015).

4.5 Nintedanib is more effective in downregulating collagen biosynthesis and secretion than pirfenidone (Paper III) (Knüppel *et al.*, 2017)

Nintedanib and pirfenidone decelerate lung function decline in IPF, but their underlying mechanisms of action are poorly understood. The aim of this study was to elucidate the effects of these two IPF drugs on several regulatory levels of collagen biosynthesis and maturation in our *in vitro* model system for lung fibrosis (*cf.* 3.3 and 4.3), using phLFs derived from IPF patients as well as from healthy donor controls. By and large, effects of both drugs were similar on IPF and control phLFs, thus independent of cell origin. We found that both drugs acted on important regulatory levels in collagen synthesis and processing. Overall, nintedanib was more effective in downregulating profibrotic gene expression and collagen secretion: For instance, nintedanib consistently downregulated transcript and protein levels of basal and TGF-β1-induced collagen I and III in IPF phLF, while pirfenidone only marginally reduced COL1A1 and COL3A1 transcripts, effects which did not translate to the protein level. In addition, only nintedanib decreased expression of FKBP10 and fibronectin, another ECM protein. Further highlighting the superior efficacy of nintedanib in this system, a concentration of 0.5 to 1.0 mM pirfenidone, *i.e.* exceeding the effective concentration of nintedanib at least 500 times, was necessary to achieve the described minor effects on collagen transcript levels. Notably, serum levels of pirfenidone during standard therapy of IPF patients (three daily doses of 801 mg pirfenidone) do not exceed 100 μM (Wollin *et al.*, 2015), a concentration at which we did not observe any effect on phLF gene expression.

4.6 Both nintedanib and pirfenidone inhibit collagen fibril assembly (Paper III) (Knüppel *et al.*, 2017)

Electron microscopy of fibroblast cultures treated with nintedanib or pirfenidone revealed fewer, shorter and thinner collagen fibril bundles compared with untreated controls. Thickness of collagen fibrils was quantified and found to be significantly smaller for fibrils formed in presence of nintedanib or pirfenidone. Expression of collagen V, a minor collagen important for initiation of extracellular fibrillogenesis, was downregulated by both drugs. Finally, both

drugs considerably delayed fibril formation of purified collagen I in a dose-dependent manner.

These results put forward the inhibition of extracellular fibril formation as a completely new mechanism of action for both drugs and collectively suggest two independent underlying mechanisms: First, as type V collagen is a component of collagen type I fibrils, with an important role in fibrogenesis and regulation of fiber size regulation (Wenstrup *et al.*, 2004, Parra *et al.*, 2006, Vittal *et al.*, 2013), its downregulation by both drugs very likely contributes to the phenomenon of fewer and thinner fibrils in the extracellular space of phLFs described here. Importantly, collagen V is heavily overexpressed compared in IPF lungs to normal lungs (Vittal *et al.*, 2013).

Second, we could show that both drugs have a direct effect on the entropy-driven auto-assembly process underlying collagen I fibril formation (Kadler *et al.*, 1996). We studied this process in a direct manner using purified pepsin-digested collagen I (Ishikawa *et al.*, 2014) and found that low micromolar concentrations of both drugs inhibited collagen I fibril formation with comparable efficiencies in a dose-dependent manner. The exact molecular mechanisms remain obscure, but it can be speculated that pirfenidone and nintedanib directly bind to collagen triple helices and mask or alter interaction sites due to changes in hydrophobicity or charges on the surface of the triple helix.

Inhibition of fibril assembly has been proposed by others as anti-fibrotic therapeutic strategy (Chung *et al.*, 2008), however, without receiving much attention thus far. The discovery that both effective IPF therapeutics act on this level provides new leverage to this concept and calls for the development of therapeutics that specifically target collagen fibril formation.

5 CONCLUSIONS AND OUTLOOK

In conclusion, the presented data within this habilitation thesis propose several novel concepts for pathogenesis and treatment strategies of IPF. First, GPX3 is secreted by lung structural cells and associates with the ECM, including the basal membrane and collagen I in the interstitial fibrotic matrix. At this point, the physiological relevance of this observation is unclear. Oxidative damage of extracellular collagen may affect collagen turnover in the lung, as oxidative post-translational modifications could alter or mask binding sites of proteases. Also, ECM signaling pathways may be disrupted by changing binding affinity of collagen adhesion receptors as *e.g.* integrins or discoidin domain receptors. Finally, such oxidative post-translational modifications may represent neo-epitopes that trigger autoimmune processes, which in turn can aggravate disease progression (Shum *et al.*, 2013). Therefore, it is plausible that GPX3 is protective in lung fibrosis, but more research is required to clarify this.

Second, inhibition of the collagen chaperone FKBP10 and/or extracellular collagen fibril formation may qualify as novel therapeutic strategies for the treatment of IPF. Considering that the ECM appears to play a key role in IPF disease progression (Parker *et al.*, 2014) and that collagen constitutes the prevalent ECM component in normal and fibrotic ECM (Decaris *et al.*, 2014, Beachley *et al.*, 2015), the primary aim in IPF therapy must be to stop ECM deposition. Inhibition of extracellular collagen crosslinking as therapeutic strategy for IPF treatment as proposed by Barry-Hamilton *et al.* (2010) has not shown efficacy in phase II studies (http://www.gilead.com/news/press-releases/2016/1/gilead-terminates-phase-2-study-of-simtuzumab-in-patients-with-idiopathic-pulmonary-fibrosis). Therefore, it can be argued that inhibition of collagen deposition must happen at an earlier stage of collagen maturation, as *e.g.* intracellular biosynthesis or fibril formation. Nevertheless, this is an understudied field. Luo *et al.* (2015) have reported collagen prolyl-4-hydroxylase 3, an ER-resident enzyme that hydroxylates collagen prolines in the 4-position, as potential target for lung fibrosis. This habilitation work highlights two more points of possible intervention, namely inhibition at the level of triple helix folding in the ER and inhibition at the level of extracellular fibril assembly, which precedes collagen crosslinking. The observation that both IPF therapeutics nintedanib and pirfenidone act on fibril formation as well as that nintedanib downregulates FKBP10, argues for the concept of directly targeting the collagen biosynthesis/deposition pathway. Future work will aim at establishing a screening system to discover new specific drugs for these novel targets.

Animal models are indispensable for the study of lung fibrogenesis in the whole organism, but nevertheless limited in how well they reflect human lung physiology and pathology. In addition, recent revisions of legislation and guidelines for animal experimentation compelled by the EU Directive 2010/63/EU reflect a growing public opposition to the use of animals in medical research, emphasizing the need of the continued development of *in vitro* methods to reduce and replace animal experiments. In this work, we applied *in vitro* model systems with patient-derived cells to study regulation and function of target genes found to be overexpressed in disease. Notably, optimization of our fibroblast culture conditions to include ascorbate and TGF-β1 allowed for efficient collagen biosynthesis and extracellular maturation. Finally, we have established an *ex vivo* model for IPF, where PCLS from surgical resections of normal regions of human lung tissue are incubated with a "fibrosis cocktail" including TGF-β1 and other cytokines which efficiently induce a fibrotic phenotype. It is anticipated that both models will be useful for evaluating therapeutic approaches for lung fibrosis in the future.

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8.1 Originalarbeiten als Erst- oder Letztautor

1. Knüppel L, Ishikawa Y, Aichler M, Heinzelmann K, Hatz R, Behr J, Walch A, Bächinger HP, Eickelberg O, **Staab-Weijnitz CA** (2017); A novel antifibrotic mechanism of nintedanib and pirfenidone: Inhibition of collagen fibril assembly. *Am J Resp Cell Mol Biol*, in press, doi: 10.1165/rcmb.2016-0217OC (**IF** 2015: **4.08,** 0 Zitierungen)

2. Schamberger AC, Schiller HB, Fernandez IE, Sterclova M, Heinzelmann K, Hennen E, Hatz R, Behr J, Vašáková M, Mann M, Eickelberg O, **Staab-Weijnitz CA** (2016); Glutathione peroxidase 3 localizes to the epithelial lining fluid and the extracellular matrix in interstitial lung disease. *Sci Rep,* **6**:29952 (**IF** 2015: **5.23**, 0 Zitierungen)

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8.3 Übersichtsartikel

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8.4 Sonstige Veröffentlichungen

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9 VERSICHERUNG AN EIDES STATT

Die vorliegende wissenschaftliche Arbeit wurde von mir selbstständig verfasst und die Herkunft des verwendeten und zitierten Materials wurde ordnungsgemäß kenntlich gemacht.

Es wurde an keiner anderen Hochschule ein Habilitationsgesuch eingereicht, es wurde kein akademischer Grad entzogen und es ist kein Verfahren anhängig, das die Entziehung eines akademischen Grades zur Folge haben könnte.

München, 22.03.2017

Claudia Staab-Weijnitz

PhD, Dipl.-Biochem. Univ.

10 ORIGINALARBEITEN DER KUMULATIVEN HABILITATIONSSCHRIFT

Diese Habilitationsschrift basiert auf den folgenden publizierten Artikeln, auf die im Text durch die entsprechenden römischen Ziffern verwiesen wird.

Paper I Schamberger AC, Schiller HB, Fernandez IE, Sterclova M, Heinzelmann K, Hennen E, Hatz R, Behr J, Vašáková M, Mann M, Eickelberg O, **Staab-Weijnitz CA** (2016). Glutathione peroxidase 3 localizes to the epithelial lining fluid and the extracellular matrix in interstitial lung disease. *Sci Rep*, 6:29952 doi:10.1038/srep29952

Paper II **Staab-Weijnitz CA**, Fernandez IE, Knüppel L, Maul J, Heinzelmann K, Juan-Guardela BM, Hennen E, Preissler G, Winter H, Neurohr C, Hatz R, Lindner M, Behr J, Kaminski N, Eickelberg O (2015). FK506-Binding Protein 10, a Potential Novel Drug Target for Idiopathic Pulmonary Fibrosis. *Am J Respir Crit Care Med*, 192(4):455-67 doi:10.1164/rccm.201412-2233OC

Paper III Knüppel L, Ishikawa Y, Aichler M, Heinzelmann K, Hatz R, Behr J, Walch A, Bächinger HP, Eickelberg O, **Staab-Weijnitz CA** (2017). A novel antifibrotic mechanism of nintedanib and pirfenidone: Inhibition of collagen fibril assembly. *Am J Resp Cell Mol Biol*, 57(1):77-90 doi:10.1165/rcmb.2016-0217OC

Paper IV Alsafadi HN, **Staab-Weijnitz CA**, Lehmann M, Lindner M, Peschel B, Königshoff M, Wagner DE (2017). An *ex vivo* model to induce early fibrosis-like changes in human precision-cut lung slices. *Am J Physiol Lung Cell Mol Physiol*, 312(6):L896-L902 doi:10.1152/ajplung.00084.2017