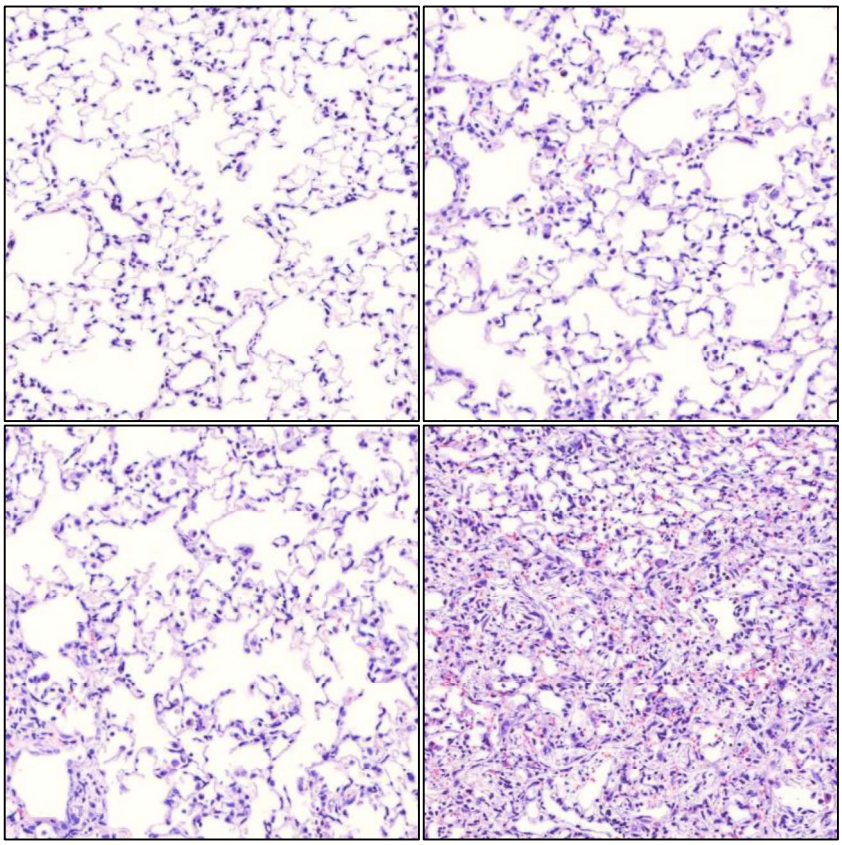
Matricellular Proteins in Pulmonary Fibrosis



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Matricellular Proteins in Pulmonary Fibrosis

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List of abbreviations

AE	Acute exacerbation
ALAT	Latin American Thoracic Association
ALI	Acute lung injury
ANGII	Angiotension II
APC	Adenomatous polyposis coli
ATI	Alveolar type I
ATII	Alveolar type II
ATS	American Thoracic Society
Ca^{2+}	Calcium $^{2+}$
CBP	CREB-binding protein
CCN	CyR61-CTGF-NOV
CK-1	Casein kinase-1
CTGF	Connective tissue growth factor
CT	C-terminal
CyR61	Cysteine-rich angiogenic inducer 61
DNA	Deoxyribonucleic acid
DQ	Desatinib and Quercetin
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ER	Endoplasmatic reticulum
ERS	European Respiratory Society
FGF	Fibroblast growth factor
FN1	Fibronectin 1
GSK3β	Glycogen synthase kinase 3 beta
HRCT	High resolution computer tomography

IGFBP	Insulin growth factor-binding protein
IL	Interleukin
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
JRS	Japanese Respiratory Society
kDa	Kilodalton
LEF	Lymphoid-enhancer factor
LTCs	Lung tissue cultures
miRNA	Micro RNA
MMP	Matrix metalloprotease
MUC5B	Mucin 5B
NF-κB	Nuklear factor kappa B
NOV	Nephroblastoma overexpressed
PAI1	Plasminogen activator inhibitor 1
PCLS	Precision cut lung slices
PDGF	Platelet-derived growth factor
phLFs	Primary human lung fibroblasts
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
SHH	Sonic hedgehog
SMAD	Sma (Caenorhabditis elegans)/ Mothers against decapentaplegic (Drosophila)
SPA	Surfactant protein A
SPC	Surfactant protein C
TCF	T-cell factor
TERC	Telomerase RNA component

TERT	Telomerase reverse transcriptase
TGFβ	Transforming growth factor beta
TGFβR	Transforming growth factor beta receptor
TIMP	Tissue inhibitor of metalloproteinases
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TOLLIP	Toll-interacting protein
TSP-1	Thrombospondin 1
UIP	Usual interstitial pneumonia
VILI	Ventilator-induced lung injury
VWC	Von-Willebrand factor, type C
WISP	WNT1-induced signaling protein
WNT	Wingless/Int1

Publications included in this thesis

Peer-reviewed publications

WISP1 mediates IL-6-dependent proliferation in primary human lung fibroblasts.
Klee S, Lehmann M, Wagner DE, Baarsma HA, Königshoff M. Sci Rep. 2016; 6: 20547, doi: 10.1038/srep20547

Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis *ex vivo*.
Lehmann M, Korfei M, Mutze K, **Klee S**, Skronska-Wasek W, Alsafadi HN, Ota C, Costa R, Schiller HB, Lindner M, Wagner DE, Günther A, Königshoff M. EurRespir J. 2017 Aug 3;50(2). doi: 10.1183/13993003.02367-2016

Introductory Summary

1. The lung

The lung is the primary organ for gas exchange. Every day, we in- and exhale roughly 10 000 liters of air, with a constant oxygen supply being the key to many physiological processes of the human body and its survival. The lung is separated in a left and a right lung, containing two and three lung lobes, respectively. It comprises a total area of about 130m^2 (Weibel *et al.* 1993), which is roughly comparable to half the size of a tennis court.

The lung has a very delicate architecture and is built by trachea, bronchi, bronchioles and most distally the alveoli, which represent the units responsible for the gas exchange in the lung. In total, the lung consists of roughly 400 million alveoli (Ochs *et al.* 2004). Overall, the lung contains at least 27 different cell types, including epithelial, endothelial, mesenchymal and immune cells (Figure 1) (Cardoso and Whitsett 2008, Franks *et al.* 2008).

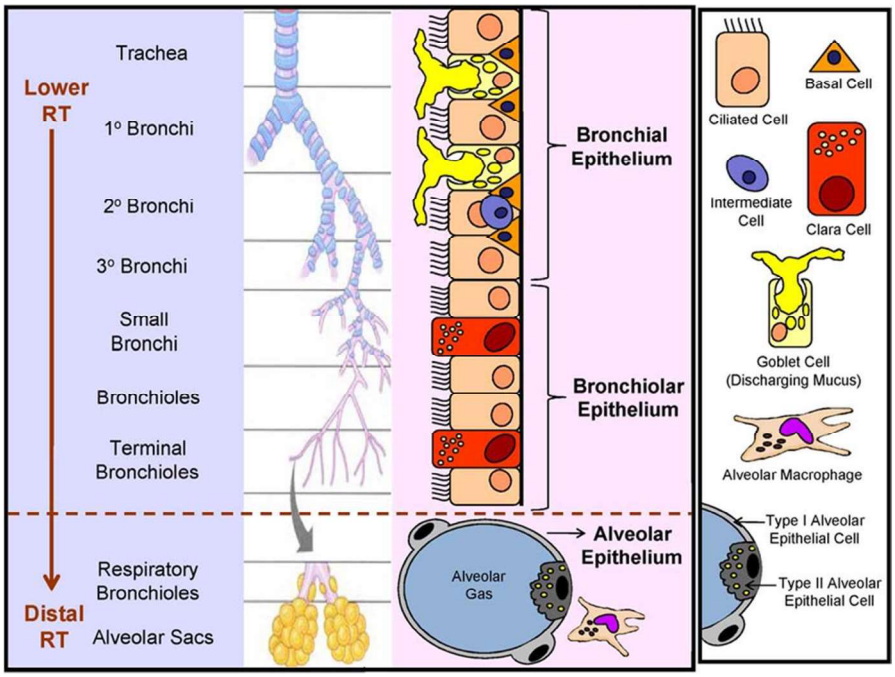

Fig. 1: Schematic representation of the lung tree and the corresponding (epithelial) cells in these areas (Berube *et al.* 2010)

2. Chronic lung diseases

Chronic lung diseases are one of the leading causes of death worldwide (World-Health-Organization 2007, Lozano *et al.* 2012). These diseases are usually highly complex and numerous factors can influence disease initiation and progression, including effects induced by genetic variants or environmental insults (Ramsey and Hobbs 2006, Dela Cruz *et al.* 2011, Ley and Collard 2013).

Currently, for most of the chronic lung diseases no treatment stopping or reversing the disease exists and the available therapies aim at symptomatic relief improving the patients` quality of life (Edmondson and Davies 2016, Barnes 2017, Liu *et al.* 2017). Therefore, there is a big unmet medical demand for therapeutic options, mainly because the underlying disease pathomechanisms or the interplay with other co-morbidities (e.g. cardio-vascular diseases) are only incompletely understood (Corlateanu *et al.* 2016, King and Nathan 2017, Margaritopoulos *et al.* 2017).

2.1. Idiopathic Pulmonary fibrosis (IPF)

IPF is a disease of the elderly, as patients are typically above 50 years of age at the time of diagnosis (Raghu *et al.* 2006, Garcia 2011). It is a progressive and devastating disease, which leads to irreversible changes of the lung architecture due to a dysfunctional wound repair (Strieter 2008, Betensley *et al.* 2016). This results in a continuous loss of breathing capacity (gas exchange), leading to reduced lung function and ultimately to the death of the patient. From the time of disease diagnosis, patients have a median survival of 3-5 years (Ley *et al.* 2011). Overall, the progression of the disease is highly variable. Although a number of molecular biomarkers have been found and linked to IPF, none of these is so far in clinical use to determine disease progression or therapy efficacy (Crestani 2013, Jenkins *et al.* 2015, Tzouvelekis *et al.* 2016, Guiot *et al.* 2017). IPF has a prevalence of 14 to 27.9 or 1.25 to 23 cases per 100 000 population and an incidence of 6.8 to 8.8 or 0.22 to 7.4 cases per 100 000 population in the USA and Europe, respectively (Nalysnyk *et al.* 2012). There are a number of potential risk factors contributing to disease development, including cigarette smoke, gastroesophageal reflux, microbiological agents like viruses of the human hepatitis virus family, and environmental exposures to metal or wood dust. Next to those, also a number of

potential genetic risk factors have been identified (Raghu *et al.* 2011). So far, therapeutic options are limited. Recently, two drugs have been approved in Japan, Europe and the USA, namely Pirfenidone (Esbrit®) and Nintedanib (Ofev®). Both drugs decelerate the loss of lung function compared to placebo-treated patients (King *et al.* 2014, Richeldi *et al.* 2014). Recently, it was shown that both Pirfenidone and Nintedanib have potential pro-survival effects (Fisher *et al.* 2017, Fleetwood *et al.* 2017, Nathan *et al.* 2017). Nevertheless, this requires further investigation in regard to follow-up time and a bigger cohort. Additionally, Pirfenidone and Nintedanib are only recommended for the treatment of mild-to-moderate IPF patients, leaving late-stage patients without approved pharmaceutical therapeutic options. Therefore, the only remaining treatment for end stage disease in IPF still is lung transplantation (Raghu *et al.* 2015). The amount of available donor lungs, however, is very restricted and only a certain percentage of IPF patients qualify for this option (Kreider and Kotloff 2009, Chaney *et al.* 2014, Kistler *et al.* 2014). It is thus of utter importance to develop new therapeutic options. Although still not fully elucidated, a number of pathophysiological mechanisms underlying disease development have been identified (King *et al.* 2011). They are summarized as hallmark features of the disease and include alveolar type (AT) II cell dysfunction, enhanced myofibroblast activation and proliferation, and increased parenchymal extracellular matrix (ECM) production (Todd *et al.* 2012, Moore and Herzog 2013, Camelo *et al.* 2014).

2.1.1. Pathophysiological features of IPF

IPF is a subgroup of interstitial lung diseases (ILD) with unknown disease cause. The diagnosis is based on the guidelines formulated by the American Thoracic Society (ATS), the European Respiratory Society (ERS), the Japanese Respiratory Society (JRS) and the Latin American Thoracic Association (ALAT) (Raghu, Rochwerg et al. 2015). Overall, diagnosis of IPF requires the exclusion of known causes of ILD and either a usual interstitial pneumonia (UIP) pattern on high resolution computer tomography (HRCT) (if the patient is not subjected to a surgical biopsy) or a specific combination of HRCT and surgical lung biopsy pattern (if the patient is subjected to the biopsy) (Raghu, Collard et al. 2011).

IPF is best described by its excessive scar tissue formation, which is initially found in the lower lung lobes (King *et al.* 2000). Normally, scar formation is required for the repair of injured tissue and is self-limiting (Beers and Morrisey 2011). However, in the case of IPF, scar

formation is a progressive feature of the disease, resulting in formation of disease-specific honeycomb structures, with so-called active fibroblast foci in close proximity (Selman and Pardo 2002, Kottmann *et al.* 2009, King, Pardo et al. 2011). Fibroblast foci are believed to be centers of high fibroblast activity, including fibroblast proliferation, activation towards myofibroblasts and subsequently enhanced and progressive ECM production, which contributes to the progressive formation of scar tissue and ultimately the progression of the disease (King, Pardo et al. 2011, Jones *et al.* 2016). Additionally, it was shown that an important cell type in the initiation and progression of lung fibrosis are the alveolar epithelial cells, namely the ATI and ATII cells (Sisson *et al.* 2010, Yang *et al.* 2013, Kulkarni *et al.* 2016). ATII cells have several functions, including the secretion of factors to maintain the alveolar structure (Castranova *et al.* 1988). Moreover, ATII cells also function as progenitor cells to ATI cells (Adamson and Bowden 1975), which are responsible for the gas exchange (Ward and Nicholas 1984). One of the recently highlighted risk factors for the development of IPF is the repetitive injury of the alveolar epithelium, leading to alterations in the phenotype and increased cell death of epithelial cells (King, Pardo et al. 2011, Camelo, Dunmore et al. 2014). In contrast to normal wound repair, a process that requires a tightly controlled interplay with the underlying mesenchymal cells (Crosby and Waters 2010, Akram *et al.* 2013), the damage is not appropriately repaired and thus can lead to the initiation of pro-fibrotic processes and subsequently fibrotic changes (Prasad *et al.* 2014, Chambers and Mercer 2015). Moreover, additional cell types of the lung have been reported to be involved in the initiation and progression of lung fibrosis, including endothelial cells and cells of the immune system. It was shown that also the endothelium is subjected to repetitive injury (Malli *et al.* 2013, Balestro *et al.* 2016, Hoyne *et al.* 2017). Moreover, the number of endothelial precursor cells is decreased in IPF patients (De Biasi *et al.* 2015), which in combination with the repetitive injuries results in an insufficient repair of the damaged sites and an endothelial leakage. This is accompanied by the secretion of fibrosis-associated factors like plasminogen activator inhibitor 1 (PAI1) or fibronectin 1 (FN1), but also soluble factors increasing the recruitment of immune cells (Leach *et al.* 2013). Usually, immune cells are recruited to sites of injury to remove excess of tissue debris and to kill potentially invading pathogens (Kulkarni *et al.* 2016, Wynn and Vannella 2016). However, they also secrete factors that stimulate further influx of inflammatory cells and toxic mediators that are harmful to the surrounding tissue. If this

process is not properly controlled, it exacerbates the injury, finally leading to progressive scar formation (Bringardner *et al.* 2008, Bagnato and Harari 2015, Byrne *et al.* 2016).

Besides the increased ECM formation and the continuous loss of respiratory capacity, about 5-10% of IPF patients suffer from so called acute exacerbations (AEs), which are defined as respiratory deteriorations leading to worsening of dyspnea in a time frame of less than 30 days for unidentifiable causes (Johannson and Collard 2013, Papiris *et al.* 2015, Collard *et al.* 2016). The AEs result in a drastic reduction in lung function and quality of life (Molyneaux *et al.* 2014). AE are often accompanied by prolonged hospitalization and an increased risk of death of up to 50% within the following 3 months after AE (Judge *et al.* 2012, Mura *et al.* 2012).

2.1.2. Pathomechanisms in IPF

Numerous factors are involved in the disease initiation and progression. Smoking, mechanic stress, environmental challenges to the lung like air pollution and the reactivation of various pathways involved in the lung development are shown to contribute to the disease (Baumgartner *et al.* 1997, Selman *et al.* 2008, Selman and Pardo 2014). Additionally, the repetitive injury to the endothelium and epithelium and its insufficient repair also play an important role in the disease onset (Magro *et al.* 2006, Strieter and Mehrad 2009, Elshazly *et al.* 2013). The repair processes are impaired in part due to an impaired crosstalk of different cell types in the lung that potentially lead to the induction and progression of the disease (Selman and Pardo 2002, Chapman 2011). Moreover, it has been shown recently, that senescence, a cellular mechanism that is associated with aging, can also contribute to the pathogenesis of IPF (Chilosi *et al.* 2013, Schafer *et al.* 2017).

2.1.2.1. Pathways deregulated in IPF

A number of signaling pathways, including transforming growth factor β (TGFβ) 1, tumor necrosis factor α (TNFα) and wingless/int1 (WNT) signaling, are deregulated in IPF (Konigshoff *et al.* 2008, Selman, Pardo et al. 2008, Fernandez and Eickelberg 2012). TGFβ1-mediated signaling is the best-studied pathway in respect to lung fibrosis. TGFβ1 has pleiotropic functions, including its involvement in embryogenesis, anti-inflammatory modulations and wound repair, but is also involved in numerous fibrogenic diseases and in cancer development (Kitisin *et al.* 2007, Wu and Hill 2009, Finnson *et al.* 2013, Meng *et al.* 2016). It was shown that

TGFβ1-induced signaling contributes to the development and progression of lung fibrosis and is potentially a main driver of the disease. The overexpression of TGFβ1 results in the development of lung fibrosis, whereas treatment antagonizing TGFβ1 signaling reduces the fibrotic burden in animal models (Giri *et al.* 1993, Sime *et al.* 1997, Varga and Pasche 2008). TGFβ1 is a member of the TGF superfamily, which in total comprises 23 family members. As for TGFβ1, it can induce intracellular signaling through at least three different TGFβ receptors (TGFβR), namely TGFβR1-3, which are ubiquitously expressed. TGFβ1 is expressed in a non-active form and requires activation prior to inducing signaling (Weiss and Attisano 2013). This can be achieved through different mechanisms, like physical processes (e.g. stretch or acidification), proteolytic cleavage by different enzymes (e.g. matrix-metalloproteases (MMPs) or plasmin) or the interaction with cell surface molecules (e.g. integrins or thrombospondin) (Shi *et al.* 2011, Horiguchi *et al.* 2012). Upon binding of TGFβ1 to its receptor, it mainly induces the phosphorylation and subsequent activation of Sma/Mothers against decapentaplegic (SMAD) 2 and 3, which in turn bind to the co-regulator SMAD4. This complex next translocates to the nucleus and induces target gene expression (Weiss and Attisano 2013). Specifically, it was shown that TGFβ1 can inhibit proliferation and induce apoptosis or epithelial-to-mesenchymal transition (EMT) in lung epithelial cells (Khalil *et al.* 1994, Kasai *et al.* 2005). On the other hand, TGFβ1 also influences lung mesenchymal cells, upregulating production of ECM proteins as well as inducing cell proliferation, in part via upregulating fibroblast growth factor 2 (FGF2) and platelet-derived growth factor (PDGF) (Fine and Goldstein 1987, Allen and Spiteri 2002, Xiao *et al.* 2012).

Another feature of IPF is a persistent inflammation. TNFα, a cytokine highly involved in inflammatory processes, is upregulated in IPF (Piguet *et al.* 1993, Kapanci *et al.* 1995). TNFα signals through the TNF receptor 1 and 2, inducing a range of different downstream cascades. The canonical pathway includes the activation of nuclear factor kappa B (NF-κB), which in turn translocates to the nucleus and, by interaction with cofactors such as p300 or cAMP response element-binding protein (CREB)-binding protein (CBP), induces the expression of mainly pro-inflammatory cytokines (Leong and Karsan 2000, Wajant *et al.* 2003, Bradley 2008). In the context of lung fibrosis, it was shown that blocking TNFα-induced signaling resulted in reduced fibrosis burden in an animal model. In this regard, overexpression of TNFα specifically in the lung resulted in the opposite effect, worsening the progression of lung fibrosis, which was

accompanied by increased ECM deposition (Piguet and Vesin 1994, Miyazaki *et al.* 1995, Sime *et al.* 1998). This underlines the involvement of TNFα in disease progression. A clinical trial was performed in human IPF patients using an inhibitor of TNFα, Etanercept (Raghu *et al.* 2008). However, despite positive results in animal models, the study was terminated as Etanercept did not show efficacy in IPF patients. Nevertheless, secondary analysis of the study data revealed a trend of improvement for IPF patients, though this effect was non-significant (Raghu, Brown et al. 2008).

It was also shown that developmental pathways crucial for lung morphogenesis are reactivated in IPF patients (Selman *et al.* 2006, Konigshoff, Balsara et al. 2008, Selman, Pardo et al. 2008, Bolanos *et al.* 2012). These pathways include Notch, Sonic Hedgehog (SHH) and WNT signaling. In particular, the WNT signaling pathway is important in a number of developmental processes during embryogenesis, including lung branching (Mucenski *et al.* 2003, De Langhe and Reynolds 2008). This is underlined by different studies using knockout mouse models of WNT pathway components showing that mice displayed an impaired lung organogenesis, which in some cases was embryonically lethal (Shu *et al.* 2002, Mucenski, Wert et al. 2003, Okubo and Hogan 2004). WNT signaling is subdivided into the canonical pathway, which signals through the activation of the cytosolic effector β-catenin, and the non-canonical pathways WNT/Planar cell polarity and WNT/Ca^{2+} signaling, which do not require the activation of β-catenin. As for IPF, it was shown that canonical WNT signaling is increased and contributes to a number of cell functions that are involved in the development and progression of lung fibrosis (Chilosi *et al.* 2003, Konigshoff, Balsara et al. 2008).

2.1.2.2. Senescence in IPF

IPF is a disease of the elderly. Aging is characterized by a number of hallmark features, including cellular senescence (Lopez-Otin *et al.* 2013). One of the features of senescence is the stable cell cycle arrest, which can be of advantage to protect against cancer development, but also detrimental when it comes to tissue repair (Campisi and d'Adda di Fagagna 2007). In contrast to quiescent cells that can resume proliferation upon reactivation of appropriate signaling cascades, senescent cells do not undergo mitotic processes or apoptosis.

It was recently shown that cellular senescence contributes to the pathogenesis of IPF (Minagawa *et al.* 2011, Yanai *et al.* 2015). Along with this, it was reported that lung epithelial

cells as well as lung fibroblasts undergo senescent changes in animal models of lung fibrosis and in patients suffering from IPF. Moreover, when comparing the amount of senescent cells in young and aged mice, 7% and 19% of senescent cells were found, respectively, showing an increased level of senescence due to aging (Wang *et al.* 2009). Along with this, aged mice are more susceptible to bleomycin-induced lung fibrosis (Stout-Delgado *et al.* 2016). With increasing age, the alveolar epithelium is challenged constantly by external insults like pathogens, which lead to microdamages and subsequently requires continuous repair of the affected sites. However, as more progenitor cells become senescent and are restricted in their proliferation and differentiation capacity, the wound repair is compromised, leading to incorrect repair mechanisms that can ultimately lead to the development of lung fibrosis (Plataki *et al.* 2005, Chilosi, Carloni et al. 2013). Moreover, senescence is also accompanied by the secretion of a variety of soluble mediators, which are called the "Senescence-Associated Secretory Phenotype" (SASP) (Campisi and d'Adda di Fagagna 2007). The SASP comprises, among other factors, pro-inflammatory cytokines like interleukin (IL) 6 as well as metalloproteinases like MMP7, which are shown to be upregulated in the disease of IPF and contribute to its progression (Zuo *et al.* 2002, Rosas *et al.* 2008, Zhou *et al.* 2010). Senescence can be induced e.g. by DNA damage or the insufficient repair of DNA telomeres or by overexpression of oncogenes (Campisi and d'Adda di Fagagna 2007, Campisi 2013). These features contribute to IPF, as e.g. fibroblasts secrete reactive oxygen species (ROS) (Waghray *et al.* 2005), which in turn damage the DNA of epithelial cells, driving these cells into senescence. Additionally, shortened telomeres, which contribute to cellular senescence, have been found in IPF patients compared to healthy, age-matched controls (Armanios 2009).

Recently, it has been shown that blocking senescence reduces fibrotic burden upon bleomycin challenge, underlining the importance of this cellular mechanism to disease pathogenesis (Schafer, White et al. 2017).

2.1.2.3. Genetic risk factors

A number of mutations in different genes have been attributed to an increased risk to develop IPF. These genes include telomerase RNA component (TERC) and telomerase reverse transcriptase (TERT), which are part of the telomerase machinery (Coghlan *et al.* 2014). Mutations of these genes were first observed in a subset of patients with familial pulmonary fibrosis (FPF) (Armanios *et al.* 2007), which were later also found in rare cases of spontaneous

IPF (Alder *et al.* 2008). Due to the mutations in TERT or TERC, telomere shortening can occur as found in several cell types in IPF patients (Alder, Chen et al. 2008, Naikawadi *et al.* 2016). It was reported that e.g. ATII cells undergo early apoptosis due to shortened telomeres and misfunction of enzymes like TERT leads to a further decrease in lung regeneration capacity upon injury, worsening the progression of IPF (Alder *et al.* 2015). Moreover, TGFβ1 was shown to contribute to telomere shortening, further contributing to ATII cell apoptosis via the mechanism of DNA damage (Chen *et al.* 2015). In addition to apoptosis, it has been shown in IPF that shortened telomeres can induce cellular senescence in affected cells (Schafer, White et al. 2017).

Other genes, in which mutations or polymorphisms were found to increase the risk of IPF development, include surfactant protein C (SPC), surfactant protein A (SPA) and Toll interacting protein (TOLLIP) or Mucin 5B (MUC5B), respectively (Wang *et al.* 2009, Crossno *et al.* 2010, Noth *et al.* 2013, Peljto *et al.* 2015). Mutations in SPC and or SPA can result in an aberrant accumulation of both proteins in the alveolar space. As these proteins are involved in alveolar stability and host defense, respectively, misexpression comprises their functions, leading to an accumulation of these proteins in the endoplasmatic reticulum (ER) and subsequently ER stress and unfolded protein response (UPR) in ATII cells (Bridges *et al.* 2006, Mulugeta *et al.* 2007, Wang, Kuan et al. 2009). This in turn contributes to increased apoptosis of ATII cells. Additionally, it was shown that a single nucleotide polymorphism (SNP) in the promoter of the MUC5B gene, associated with higher gene expression, predisposes to IPF development (38% of IPF patients, 34% of familial pulmonary fibrosis, 9% in healthy controls) (Seibold *et al.* 2011). MUC5B is a component of the mucus, which is required for pathogen clearance. Due to the increased expression of MUC5B, it is possible that the mucus transport is impaired leading to its accumulation in the bronchoalveolar regions and a subsequent chronic inflammation and injury (Peljto, Selman et al. 2015). Another component of the immune system that is associated with IPF is TOLLIP. It is an integral part of the toll-like receptor (TLR) signaling cascade and thereby also contributes to the host defense. A number of polymorphisms were found in the TOLLIP region in IPF patients, which correlated with increased disease and mortality risk. However, one polymorphism within the TOLLIP gene was identified to be protective against IPF development (Noth, Zhang et al. 2013). Taken together, this shows that the expression or function of a number of components of the immune system

is deranged and thereby contribute to the disease.

2.1.2.4. Impaired epithelial-mesenchymal crosstalk in IPF

In IPF, a number of different cell types are shown to contribute to the pathogenesis of the disease. Especially two distinct cell populations contribute to the pathogenesis of IPF, namely the epithelial and the mesenchymal cells. The interaction of both is required for a functional equilibrium within the lung and in case of an injury, for a sufficient but controlled repair of the damaged site (Hogan *et al.* 2014). Their interaction appears quite natural, because of their close proximity in the lung. As for the pathogenesis of IPF, the repetitive injury that the ATI and ATII cells are challenged with, results in an increased apoptosis of these cells which in turn results in an influx of underlying mesenchymal cells undergoing activation and increasing ECM production (Camelo, Dunmore et al. 2014). This results in further destruction of epithelial cells, which contributes to a vicious cycle and the progression of IPF (Camelo, Dunmore et al. 2014).

In addition to the above mentioned repetitive injury, other causes exist for epithelial cell death. Polymorphisms in genes like MUC5B or mutations in SPC, both associated with increased IPF risk, have been shown to affect epithelial survival, leading to increased apoptosis and finally to a damaged epithelium (Wolters *et al.* 2014). Moreover, surviving ATI and ATII cells were shown to alter their phenotype and thereby contribute e.g. to the pool of SASP secretion (Yang, Wheeler et al. 2013, Schafer, White et al. 2017). It was shown in several mouse models that by either inducing or preventing alveolar epithelial cell apoptosis, development of lung fibrosis could be either augmented or reduced, respectively (Kuwano *et al.* 1999, Thannickal and Horowitz 2006). This means that restoring the epithelial cell function in turn prevents mesenchymal cell-mediated accumulation of ECM. These findings underline the importance of the epithelial cells and the integrity of the epithelial cell barrier in the development of lung fibrosis.

Areas of fibroblast foci, the active sites of lung fibrosis, are often lined by ATI and ATII cells. Importantly, fibroblasts can affect the epithelial cells in a paracrine fashion. As mentioned above, fibroblasts potentially further contribute to alveolar cell apoptosis and thereby initiate a vicious cycle. Lung fibroblasts in response to TGFβ1 stimulation secrete ROS as well as Angiotensin II (ANGII) (Wang *et al.* 1999, Waghray, Cui et al. 2005). It was shown that both,

ANGII and ROS, induce alveolar epithelial cell apoptosis. In an *in vivo* mouse model, antagonizing ANGII reduced levels of epithelial cell apoptosis in response to bleomycin challenge compared to control animals (Li *et al.* 2007). Similarly, administration of superoxide dismutase, an antioxidant, resulted in reduced fibrosis burden in the bleomycin model (Tanaka *et al.* 2010).

It was shown in various *in vitro* assays that epithelial cells can stimulate the differentiation and proliferation of fibroblasts in a paracrine fashion (Yang, Wheeler et al. 2013, Yang *et al.* 2014). This in turn leads to an increased fibroblast pool and ECM production, which further drives progression of lung fibrosis. Epithelial cells were shown to secrete soluble mediators like TGFβ1 (which requires additional activation also induced by epithelial cells), connective tissue growth factor (CTGF/CCN2), WNT1-inducible signaling protein 1 (WISP1/CCN4), or SHH (Pan *et al.* 2001, Bhaskaran *et al.* 2007, Konigshoff *et al.* 2009). TGFβ1, as described above, has numerous effects on various cell types, including lung fibroblasts. In summary, TGFβ1 upregulates the production of ECM components like collagens and fibronectin, as well as induces fibroblast proliferation and resistance against apoptosis (Raghu *et al.* 1989, Xiao, Du et al. 2012). Additionally, the matricellular proteins CTGF/CCN2 and WISP1/CCN4 were shown to influence cell behavior either directly by binding to integrins, or by facilitating the binding and action of other mediators. Similar to TGFβ1, both CTGF/CCN2 and WISP1/CCN4 were shown to upregulate the production of ECM components by lung fibroblasts (Konigshoff, Kramer et al. 2009, Lin *et al.* 2013). Moreover, it was demonstrated that the presence of CTGF/CCN2 is required for full activity induced by TGFβ1. In CTGF/CCN2-deficient fibroblasts, TGFβ1-induced αSMA and collagen (Col) 1a1 levels were reduced. Similar effects as for TGFβ1, CTGF/CCN2 and WISP1/CCN4 were also reported for SHH (Sakai and Tager 2013).

3. WNT1-Inducible Signaling Protein 1 (WISP1)/CCN4

WISP1/CCN4 is a member of the **C**yR61/CCN1-**C**TGF/CCN2-**N**OV1/CCN3 (**CCN**) family (Brigstock 2003). Additional members of this protein family are WISP2/CCN5 and WISP3/CCN6. These matricellular proteins are integrated into the ECM and exhibit signaling/signaling-modulating rather than structural functions (Chen and Lau 2009). Proteins of the CCN family have been described to influence physiological processes like cell growth, differentiation and survival. Due to potential misregulation of their expression, they are also implicated in a vast array of pathophysiological processes including fibrosis, cancer and

metabolic diseases (Shimo *et al.* 2006, Jun and Lau 2011, Murahovschi *et al.* 2015, Kurundkar *et al.* 2016).

3.1. WISP1/CCN4 structure

The human WISP1 is located on chromosome 8 (8q24.1 – q24.3), giving rise to mRNA that codes for a protein of 376 amino acids with a predicted molecular mass of 40 kDa. Human and mouse WISP1 protein share 84% identical sequence. Besides the full-length variant of WISP1/CCN4, there is a splice variant lacking the third of five exons (Tanaka *et al.* 2001). It was originally identified in scirrhous gastric carcinoma cells and subsequently validated in different other cell types, including mesenchymal cells and various cancer cells (Cervello *et al.* 2004, Yanagita *et al.* 2007).

As mentioned above, all members of the CCN family were grouped due to their similarities in the protein structure (Perbal 2004). As shown in figure 2, all members contain a N-terminal export signal peptide, followed by 4 conserved units (Brigstock 2003, Perbal 2004). The only exception is WISP2/CCN5, which lacks the C-terminal domain. Each of the different four conserved units has unique properties (Chen and Lau 2009). Following the N-terminal export signal peptide, there is the Insulin-like Growth Factor Binding (IGFBP) domain, which function, though not fully understood, is involved in IGF binding. The second domain, the Von-Willebrand Factor C repeat (VWC), is potentially required to facilitate binding to different growth factors like TGFβ1 and BMPs and thereby alters their respective signaling. The third domain is the Thrombospondin repeat-1 (TSP-1) domain, which is suggested to be required for binding of the CCNs to the ECM and, furthermore, this domain is potentially involved in cell adhesion. The C-Terminal (CT) domain contains a cysteine knot, which is probably required for dimerization of CCNs to form homo- or heterodimers, or even oligomers. Moreover, due to its structure, it might be involved in ECM binding as well as in cell adhesion in concert with the TSP-1 domain.

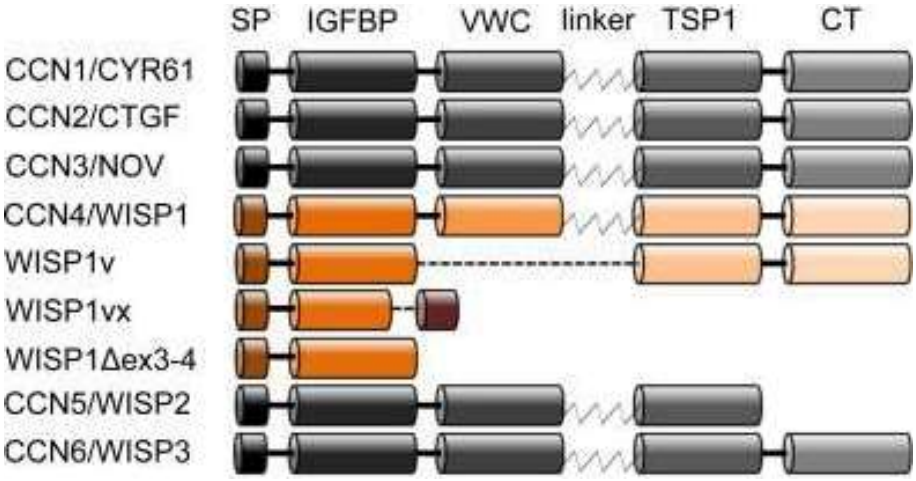

Fig. 2: Schematic overview of the structure of the CCN family proteins (Sakai and Tager 2013).

3.2. WISP1/CCN4 regulation and function

WISP1/CCN4 was originally discovered in a cancer cell line overexpressing WNT1, which gave the basis for its name (WNT1-inducible signaling protein 1) (Pennica *et al.* 1998). Moreover, it was shown that WNT3a, but not WNT4, is able to induce WISP1 expression, suggesting that WISP1/CCN4 is a downstream target gene of canonical WNT signaling (Pennica, Swanson et al. 1998, Berendsen *et al.* 2011). Additionally, the pro-fibrotic cytokines TGFβ1 and TNFα were shown to induce WISP1/CCN4 expression in various cell types (Venkatachalam *et al.* 2009, Jian *et al.* 2014). Interestingly, it was shown that TGFβ1 can induce WISP1/CCN4 in lung fibroblasts, a mechanism that is dependent on the miRNA-92a (Berschneider *et al.* 2014). This implies that WISP1 is a common downstream target of several pro-fibrotic pathways involved in lung fibrosis.

WISP1/CCN4 is involved in different physiological but also pathophysiological processes, ranging from angiogenesis, osteogenesis and wound repair to formation of cancer and fibrotic disorders (Berschneider and Konigshoff 2011, Maiese 2014).

Königshoff and colleagues showed that WISP1/CCN4 is upregulated in both experimental lung fibrosis as well as in IPF patients. Blocking WISP1/CCN4 in mice in the bleomycin-induced lung fibrosis model decreased fibrotic burden, accompanied by a decrease in a number of ECM marker genes, an improvement in lung function and most importantly, increased survival of these animals compared to the control group (Konigshoff, Kramer et al. 2009). Further *in vitro* studies showed that WISP1/CCN4 increases the proliferative capacity of ATII cells, but also

their capacity to undergo EMT. Moreover, WISP1/CCN4 treatment also affected mesenchymal cells, leading to an increased expression of ECM proteins Col1a1 and FN1 (Konigshoff, Kramer et al. 2009). Of note, it was also shown that WISP1 is upregulated in models of liver fibrosis, and neutralization of WISP1/CCN4 using a specific antibody against WISP1/CCN4 led to a reduction in liver fibrosis development, which was accompanied by reductions in TNFα, IL6 and p65 expression (Li *et al.* 2015).

Moreover, WISP1/CCN4 is also described to contribute to the development of other lung diseases, such as lung cancer, ventilator-induced lung injury (VILI), asthma and acute lung injury (ALI) (Li *et al.* 2012, Chen *et al.* 2016). WISP1/CCN4 is upregulated in lung cancer and polymorphisms in WISP1/CCN4 can predict cancer susceptibility and moreover, the effectiveness of chemotherapy (Soon *et al.* 2003, Chen *et al.* 2014, Chen *et al.* 2015). Mechanistically, WISP1/CCN4 contributes to the motility of cancer cells, explaining why especially patients with increased WISP1/CCN4 levels more frequently suffer from metastatic lesions compared to patients with primary tumors. In VILI, WISP1/CCN4 was shown to increase the alveolar capillary permeability and contribute to an increased TNFα release by macrophages (Li, Li et al. 2012). The latter effect is also observed in ALI, in which increased WISP1/CCN4 contributes to disease pathogenesis (Chen, Ding et al. 2016). However, it is also important to note that in a different model of ALI, WISP1/CCN4 had beneficial functions and was required for sufficient repair and restoration of the alveolar epithelium (Lawson and Blackwell 2013).

Therefore, although not entirely understood in a mechanistic way, it is becoming increasingly clear that WISP1/CCN4 has a function in inflammatory and remodeling processes of the lung. Moreover, the regulation of WISP1/CCN4 is of particular importance, as an imbalance appears to promote pathophysiological processes, whereas a controlled regulation seems to be required for appropriate repair mechanisms within the lung.

4. Hypotheses and Objectives

IPF is a progressive, fibrotic lung disease, characterized by the deregulation of a number of different hallmark features. One of those features is a deranged epithelial-mesenchymal cellular crosstalk mediated by a number of factors, including WISP1/CCN4, a secreted matricellular protein shown to influence cells like ATII cells and lung fibroblasts. Moreover, other secreted factors like interleukins and MMPs were found to be upregulated in the pathogenesis of IPF, with some being summarized in the so-called SASP, the secretome of senescent cells. In line with this, it was recently shown that cellular senescene is increased in IPF patients, however, its contribution to IPF is so far not clear and is being disscussed controversially. A third hallmark feature of IPF is the reactivation of a number of developmental pathways, including WNT/β-catenin, TGFβ1 and TNFα signaling. These pathways are implicated in different processes, which contribute to the progression of lung fibrosis. However, as these signaling pathways not only have pathological implications in disease progression, but also exhibit physiological properties required for normal cell and tissue functions, therapeutic targeting either did not show efficacy or resulted in offside effects negatively affecting the patients. Therefore, identifying shared downstream targets of these signaling pathways, which contribute to the development and progression of IPF, will provide potential valuable therapeutic targets. Moreover, understanding the role of cellular senescence and components of the SASP in IPF will potentially reveal additional targets in regard to single components of the SASP or the SASP as a whole in the search of a therapeutic strategy to cure IPF.

We hypothesized that WISP1/CCN4 is a common downstream target of several pro-fibrotic pathways and contributes to the development and progression of IPF. Moreover, we hypothesized that WISP1/CCN4 is a component of the SASP. As it was shown that the removal of senescent cells is live-prolonging in mice, we hypothesized that cellular senescence has detrimental functions in lung fibrosis, which at least in part is mediated through WISP1/CCN4, and removal of senescent cells will improve lung and pulmonary cell functions.

The aims of this study were to A) analyze the regulation of WISP1/CCN4 in the context of lung fibrosis *in vitro* and *in vivo*, B) investigate the functions induced by WISP1/CCN4 on lung fibroblasts *in vitro*, C) characterize which cells are affected by cellular senescence in lung

fibrosis, and D) study the effect of the depletion of senescent cells on epithelial cell function *in vitro* as well as in 3D lung tissue cultures (3D LTCs) *ex vivo* in the context of lung fibrosis.

5. Summary

IPF is a devastating fibrotic lung disease. The median survival of IPF patients is about 3-5 years, which is due to limited therapeutic options. Recently, two drugs (Nintedanib and Pirfenidone) have been approved for the treatment of mild-to-moderate IPF, however, these drugs only slow down the progression of the disease but neither halt nor reverse disease pathogenesis. A number of hallmark features have been described in the pathogensis of IPF including ATII cell dysfunction, (myo)fibroblast activation and proliferation as well as an increased ECM production. However, the processes involved in the initation and progression of IPF are still incompletely understood, which contributes to the limitation of available therapeutic options. In the first publication (Klee *et al.* 2016) we could show that WISP1/CCN4 is a common downstream target of TGFβ1- and TNFα-mediated signaling. Both pro-fibrotic cytokines induce WISP1/CCN4 mainly via NF-κB. Furthermore, we could show that WISP1/CCN4 is required for the expression of NF-κB-dependent downstream targets of TGFβ1 and TNFα. Here, especially the expression of IL6 was dependent on the presence of WISP1/CCN4. Additionally, we could show that the WISP1/CCN4 was required for fibroblast proliferation, a process that was in part mediated via the WISP1/CCN4-dependent IL6 expression.
In the second publication (Lehmann M *et al.* 2017) we analyzed the effect of cellular senescence in the context of IPF. We could show that senescence-associated markers are upregulated in epithelial cells of IPF patients as well as in experimental lung fibrosis. Furthermore, we could show that ATII cells derived from bleomycin-instilled mice exhibited an upregulation of SASP components and additionally, showed an increase in WISP1 secretion. Importantly, the clearance of senescent cells using the senolytic drugs dasatinib and quercetin resulted in a reduction of mesenchymal marker expression as well as a decrease in SASP component production in isolated murine ATII cells as well as in 3D LTCs. Two components found to be downregulated were WISP1/CCN4 and IL6. This shows that WISP1/CCN4 is a potential component of the SASP, and additionally, that the secretion of IL6 by ATII cells as well as in the 3D LTCs potentially depends on the presence of WISP1. Moreover, the treatment with the senolytic drugs dasatinib and quercetin increased epithelial marker expression in both systems.

Taken together, we could show that WISP1/CCN4 contributes to IPF via an upregulation of phLF proliferation. Moreover, clearance of senescent cells downregulated fibrosis-associated

markers, while it increases epithelial marker expression and potentially restores physiological epithelial cell functions.

6. Contribution

First publication (Klee *et al.* Sci Rep, 2016)

In vitro transfection of the WISP1 promoter construct and luciferase activity measurements (Fig 1B); *in vitro* treatment of phLFs with TGFβ1 (Fig 2 A-E, 4 A-D, Suppl Fig 2 A/B), TNFα (Fig 3 A-E, Fig 4 A/B/E/F, Suppl Fig 2 C/D, Suppl Fig 3 A/B) and IL6 (Fig 5 A, Fig 6 A/B, Suppl Fig 4 A); qPCR (Fig 2 A-D, Fig 3 A-D, Fig 4 A/C/E, Suppl Fig 1 A/B, Suppl Fig 2 A-D, Suppl Fig 3 A, Suppl Fig 4 A/B, Suppl Fig 5 A/B); WISP1 ELISA (Fig 2 E, Fig 3 E, Fig 4 B) and IL6 ELISA (Fig 4 D/F); Multiplex ELISA (Suppl Tab 1 and 2); siRNA transfection (Fig 4 A-F, Fig. 5 B-F; Fig 6 A, Suppl. Fig 2 A-D, Suppl Fig 4 B/C); Western Blot (Fig 5 C); immunoflourescence staining (Fig 5 D); *in vitro* experiments with neutralizing antibody (Fig 5 G, Fig 6 B); WST-1 assay (Fig. 5 A/E/F/G, Fig 6 A/B, Suppl Fig 4 C, Suppl Fig 5 C); *in vitro* treatment of phLF with pharmacological inhibitors (Suppl Fig 1 A/B, Suppl Fig 5 A-C); analysis of microarray (Suppl Fig 6 A/B); design of the experiments, preparation and editing of the figures and manuscript.

Second publication (Lehmann *et al.* Eur Respir J, 2017)

ELISA for murine IL6, WISP1 and SPC (Fig 5C/D/E) as well as human WISP1 (Fig 7C); animal model of bleomycin-induced lung fibrosis, orotracheal instillation of the mice with bleomycin (Fig 4, Fig 5, Fig 7).

7. Publication I

SCIENTIFIC REPORTS

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WISP1 mediates IL-6-dependent proliferation in primary human lung fibroblasts

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Idiopathic pulmonary fibrosis (IPF) is a progressive and fatal interstitial lung disease. IPF is characterized by epithelial cell injury and reprogramming, increases in (myo)fibroblasts, and altered deposition of extracellular matrix. The Wnt1-inducible signaling protein 1 (WISP1) is involved in impaired epithelial-mesenchymal crosstalk in pulmonary fibrosis. Here, we aimed to further investigate WISP1 regulation and function in primary human lung fibroblasts (phLFs). We demonstrate that WISP1 is directly upregulated by Transforming growth factor β1 (TGFβ1) and Tumor necrosis factor α (TNFα) in phLFs, using a luciferase-based reporter system. WISP1 mRNA and protein secretion increased in a time- and concentration-dependent manner by TGFβ1 and TNFα in phLFs, as analysed by qPCR and ELISA, respectively. Notably, WISP1 is required for TGFβ1- and TNFα-dependent induction of interleukin 6 (IL-6), a mechanism that is conserved in IPF phLFs. The siRNA-mediated WISP1 knockdown led to a significant IL-6 reduction after TGFβ1 or TNFα stimulation. Furthermore, siRNA-mediated downregulation or antibody-mediated neutralization of WISP1 reduced phLFs proliferation, a process that was in part rescued by IL-6. Taken together, these results strongly indicate that WISP1-induced IL-6 expression contributes to the pro-proliferative effect on fibroblasts, which is likely orchestrated by a variety of profibrotic mediators, including Wnts, TGFβ1 and TNFα.

Idiopathic pulmonary fibrosis (IPF) is a devastating and progressive interstitial lung disease with a median survival of 3 to 5 years and limited therapeutic options[1,2]. Recently, two drugs (Pirfenidone and Nintedanib) have been approved for the treatment of mild/moderate IPF, both of which significantly reduce lung function decline in IPF patients[3,4]. Therapies halting or reversing the disease progression are lacking and thus a more in-depth understanding of pathomechanisms driving IPF is needed. Histopathological features of IPF include alveolar epithelial cell injury and hyperplasia, (myo)fibroblast proliferation and differentiation, along with increased extracellular matrix (ECM) production and deposition[2,5,6]. Fibroblast foci are a key histologic characteristic of IPF and a major site of fibroblast proliferation[7]. As such, IPF is likely driven by impaired epithelial and mesenchymal cell communication. The Wnt1-inducible signaling protein 1 (WISP1) is a member of the CCN (**C**yR61, **C**TGF, **N**OV) family of matricellular proteins, which have been reported to be critically involved in epithelial-mesenchymal crosstalk[8,9]. WISP1 has been implicated in lung and airway remodeling[10–12]. Moreover, WISP1 is highly upregulated in patients with IPF as well as in experimental lung fibrosis[13–15]. Importantly, neutralizing antibodies against WISP1 attenuated the development of bleomycin-induced pulmonary fibrosis *in vivo*, thus demonstrating the potential of WISP1 as a therapeutic target for IPF[13].

Other cytokines involved in disturbed cellular crosstalk in IPF are Transforming growth factor β1 (TGFβ1) and Tumor necrosis factor α (TNFα). Both cytokines are highly upregulated in IPF and alter ECM production, survival and proliferation of distinct cell types in the lung, including alveolar epithelial cells and lung fibroblasts[2,16]. Consistently, knockout mice for TGFβ1, TNFα or components of their respective downstream signaling pathways attenuated development of experimental lung fibrosis[16,17].

We have recently reported that IPF fibroblasts display increased WISP1 levels and that miRNAs regulate WISP1 expression in TGFβ1-primed fibroblasts[15]. Here we aimed to further elucidate the upstream regulation of WISP1 in a profibrotic environment as well as its downstream functions in primary human lung fibroblasts. We demonstrate that WISP1 is directly upregulated by both TGFβ1 and TNFα in primary human lung fibroblasts

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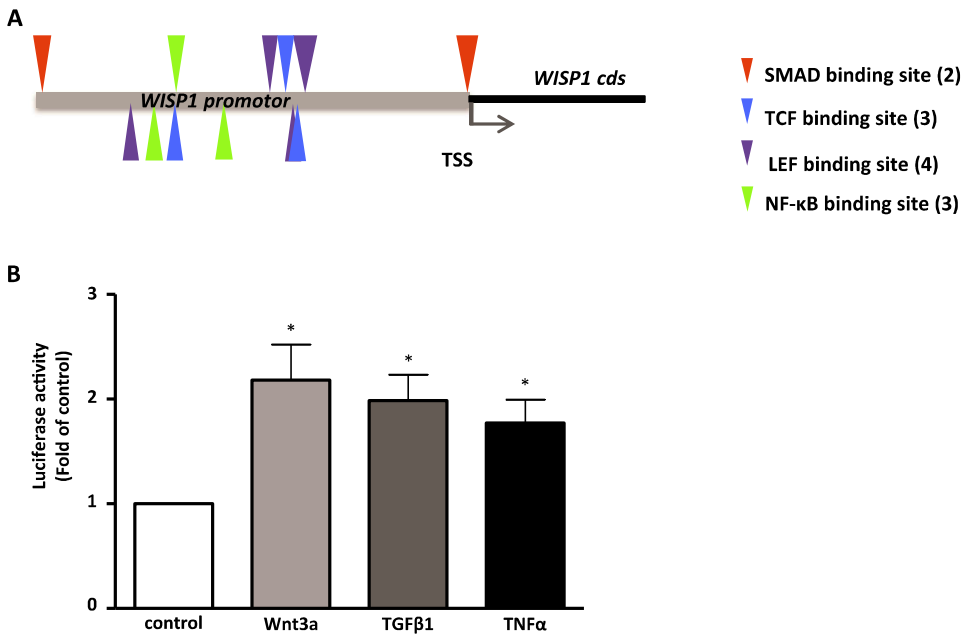

Figure 1. The WISP1 promoter region contains potential binding sites for transcription factors activated by profibrotic cytokines. (**A**) *In silico* analysis of the WISP1 promoter (2.5 kb upstream of the WISP1 transcription start site (TSS)) revealed potential binding sites for SMAD, TCF, LEF and NF-κB. (**B**) A reporter construct containing the WISP1 2.5 kb promoter region was transfected into primary human lung fibroblasts (phLFs). phLFs were treated with 100 ng/ml Wnt3a, 2 ng/ml TGFβ1 or 10 ng/ml TNFα for 24 hours followed by measurement of luciferase activity. Wnt3a, TGFβ1 and TNFα all significantly induced luciferase activity as compared to unstimulated conditions ($n = 4$, $^*p < 0.05$, 1-way ANOVA followed by Neuman-Keuls multiple comparison test).

and that the presence of WISP1 is required for TGFβ1- and TNFα-induced interleukin 6 (IL-6) production. Moreover, we show that WISP1-induced IL-6 contributes to increased fibroblast proliferation.

Results

WISP1 is a common downstream target of profibrotic signaling mediators. The matricellular protein WISP1 has recently been reported to be increased in IPF-derived lung fibroblasts[15]. Here, we addressed the question whether WISP1 expression is regulated by specific profibrotic mediators in primary human lung fibroblasts (phLFs). *In silico* analysis of a region of a total of 2.5 kb upstream of the WISP1 transcription starting site (here called WISP1 promoter region) revealed potential binding sites for transcription factors like T-cell factor (TCF) and lymphoid enhancer factor (LEF), SMADs, as well as nuclear factor kappa B (NF-κB), which are activated by canonical Wnt, TGFβ1 and TNFα signaling, respectively (Fig. 1A). In order to verify these potential mediators, we transfected phLFs with either a luciferase-based reporter plasmid containing the 2.5 kb WISP1 promoter element or a control plasmid and subsequently treated the phLFs with TGFβ1, TNFα, or Wnt3a. Wnt3a has been reported to exhibit profibrotic effects in the lung[18] and was further used as a positive control, since WISP1 has been described to be β-catenin dependent[13,19]. As shown in Fig. 1B, treatment with all three profibrotic cytokines induced a significant increase in luciferase activity, indicating that in addition to Wnt3a, TGFβ1 and TNFα directly induce WISP1 in phLFs.

TGFβ1 induces WISP1 expression and secretion in primary human lung fibroblasts. TGFβ1 is the main profibrotic cytokine active in IPF. It is involved in numerous processes including proliferation and ECM production by fibroblasts as well as epithelial cell reprogramming, which altogether ultimately drive lung fibrosis progression. We have recently shown that miRNAs regulate WISP1 expression in a TGFβ1-driven environment[15]. Here, we investigated the dynamics of WISP1 regulation by TGFβ1 in more detail. The induction of *WISP1* expression by TGFβ1 was time- and concentration-dependent (Fig. 2A,C; 24 hours: 1.92 ± 0.23 fold over control; 2 ng/ml TGFβ1: 3.14 ± 0.64 fold over control). Notably, the induction of *WISP1* was similar to the induction of *SERPINE1* (Fig. 2B,D), a direct target gene of TGFβ1. Next, we investigated the effect of TGFβ1 on WISP1 protein levels, and found significantly increased WISP1 secretion in phLFs as early as 24 hours upon TGFβ1 stimulation (Fig. 2E; 24 hours: control vs. 2 ng/ml TGFβ1: 24.02 ± 5.88 pg/ml vs. 75.52 ± 1.98 pg/ml; 48 hours: control vs. 2 ng/ml TGFβ1: 19.52 ± 1.38 pg/ml vs. 45.87 ± 7.63 pg/ml). Thus, TGFβ1 induces *WISP1* mRNA expression and secretion in a time- and concentration-dependent manner in phLFs.

WISP1 is regulated by TNFα in primary human lung fibroblasts. TNFα is a multi-faceted cytokine with numerous functions and is associated with lung fibrosis[20,21]. Our promoter studies suggested that WISP1 is a

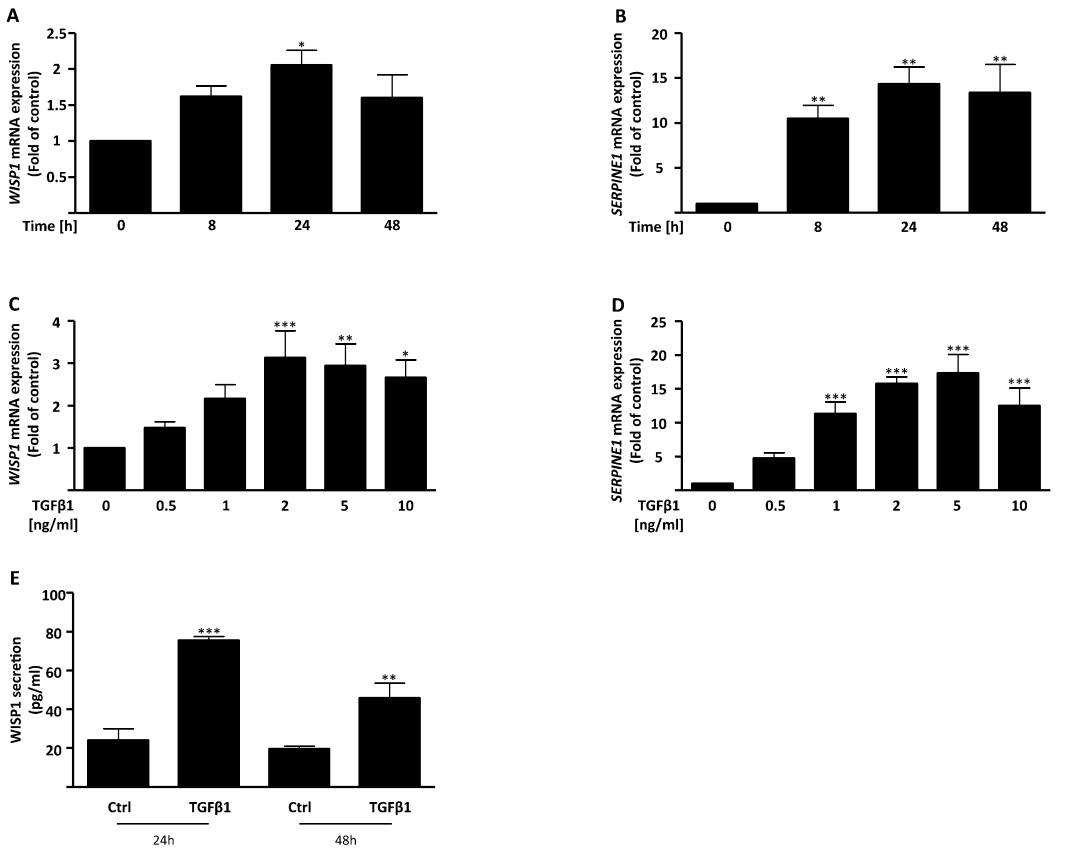

Figure 2. TGFβ1 induces WISP1 in primary human lung fibroblasts (phLFs). phLFs were treated with TGFβ1 (2 ng/ml) for 8, 24 and 48 hours and the expression of (**A**) *WISP1* and (**B**) *SERPINE1* were analysed using RT-qPCR. The phLFs were treated with TGFβ1 concentrations ranging from 0.5 to 10 ng/ml and the expression of (**C**) *WISP1* and (**D**) *SERPINE1* were analysed using RT-qPCR at 24 hours. *WISP1* was significantly upregulated using 2 ng/ml at 24 hours while *SERPINE1* was upregulated at 8 hours and at all further timepoints. (**E**) phLFs were treated with TGFβ1 (2 ng/ml) for 24 and 48 hours and WISP1 secretion was significantly upregulated as measured by ELISA ($n = 3$–6; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; 1-way ANOVA followed by Neuman-Keuls multiple comparison test; compared to respective control).

direct target of TNFα (Fig. 1). Next, we investigated the dynamics of WISP1 regulation by TNFα in more detail. We found that TNFα induced *WISP1* mRNA expression as early as 8 hours upon stimulation with a near-maximal induction of *WISP1* at 10 ng/ml (Fig. 3A,C; 8 hours: 4.61 ± 0.68 fold over control; 10 ng/ml: 3.41 ± 0.43 fold over control) accompanied by a similar trend of induction of the known TNFα target gene *IL-8* (Fig. 3B,D). Moreover, enhanced WISP1 protein secretion was observed at 24 and 48 hours upon TNFα stimulation (Fig. 3E; control vs. 10 ng/ml TNFα at 24 hours: 20.47 ± 3.66 pg/ml vs. 35.02 ± 3.63 pg/ml; control vs. 10 ng/ml TNFα at 48 hours: 20.99 ± 3.81 pg/ml vs. 44.7 ± 5.4 pg/ml).

We next sought to explore common downstream mechanisms by which TGFβ1 and TNFα might exert their effects to upregulate *WISP1* expression. We found that TGFβ1- and TNFα-mediated induction of *WISP1* was primarily NF-κB-dependent as shown by a significant reduction of *WISP1* in the presence of the IKKβ inhibitor (SC-514; Suppl. Fig. 1), but independent of extracellular signal-related kinase (MEK1/2; inhibitor: U0126) or c-Jun N-terminal kinase 1/2 (JNK1/2; inhibitor: SP600125), respectively.

WISP1 is required for IL-6 expression by the profibrotic cytokines TGFβ1 and TNFα. Since both TGFβ1 and TNFα induced WISP1 in phLFs, we next asked the question if WISP1 in turn is involved in TGFβ1- or TNFα-dependent cellular signaling and function. We analysed interleukin 6 (IL-6), which is induced by both TGFβ1 and TNFα in various cell types, e.g. via NF-κB[22–24]. IL-6 is a pro-inflammatory and pro-fibrotic cytokine reported to be involved in the pathogenesis of IPF[25,26]. To address the question if WISP1 is involved in *IL-6* induction in primary human lung fibroblasts, we used an siRNA-based approach to downregulate WISP1 prior to stimulation with TGFβ1 (2 ng/ml) or TNFα (10 ng/ml), respectively. *WISP1* was effectively downregulated upon specific siRNA knockdown by 86.2 ± 2.4% after 24 h in the unstimulated condition and importantly, also in the presence of TGFβ1- or TNFα-stimulation (Fig. 4A, baseline: −86.2 ± 2.4%, TGFβ1: −85.6 ± 3.7%, TNFα: −93.6 ± 0.5%; compared to siCtrl). We further validated the knockdown on protein level by analysing WISP1 secretion and found a strong reduction in WISP1 secretion upon siRNA-mediated knockdown in the presence of either TGFβ1 or TNFα (Fig. 4B; siCtrl + TGFβ1 vs. siWISP1 + TGFβ1: 37.02 ± 1.75 pg/ml vs. 7.62 ± 0.45 pg/ml; siCtrl + TNFα vs. siWISP1 + TNFα: 59.46 ± 10.82 pg/ml vs. 12.82 ± 2.45 pg/ml). We next examined the

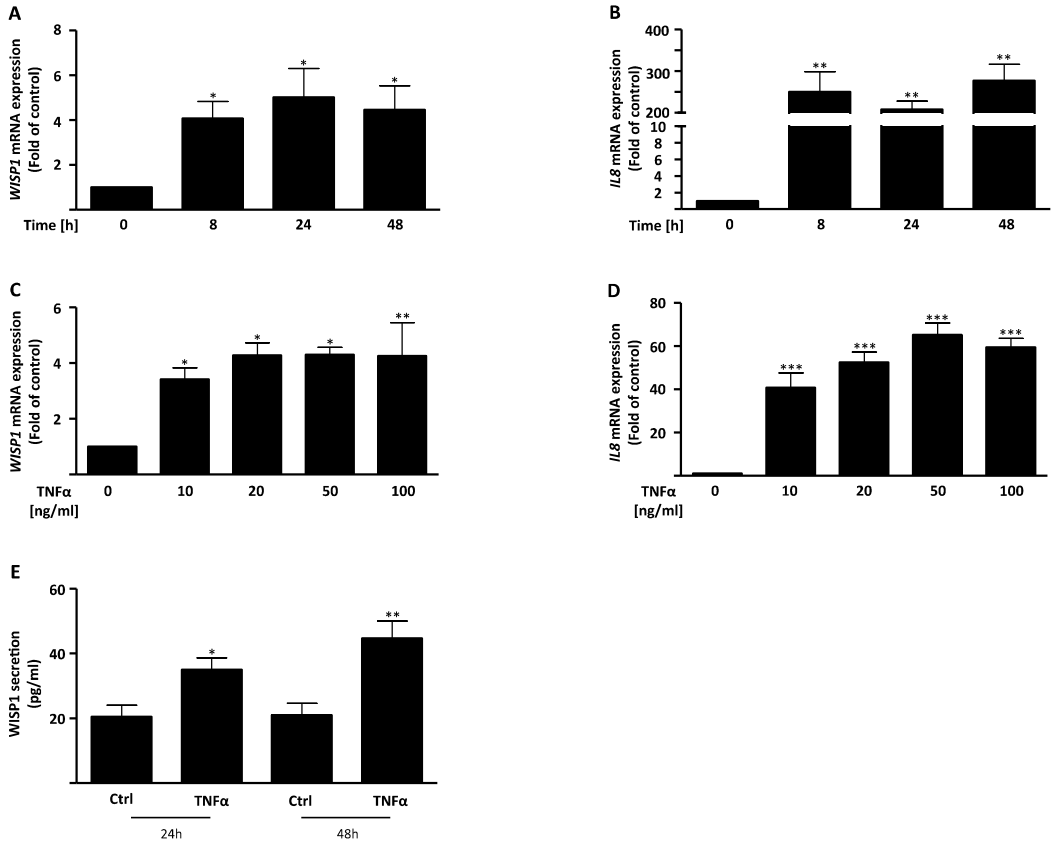

Figure 3. TNFα induces WISP1 in primary human lung fibroblasts (phLFs). The treatment of phLFs with TNFα (10 ng/ml) for 8, 24 and 48 hours was followed by the analysis of the expression of (**A**) *WISP1* and (**B**) *IL8* using RT-qPCR. (**C,D**) phLFs were treated with TNFα concentrations from 10 to 100 ng/ml and the expression of *WISP1* and *IL8* was analysed using RT-qPCR. *WISP1* and *IL8* were significantly increased after 8 hours of TNFα stimulation with 10 ng/ml (**E**) WISP1 secretion by phLFs after treatment with TNFα (10 ng/ml) for 24 and 48 hours was significantly increased as measured by ELISA ($n = 3$–4; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; 1-way ANOVA followed by Neuman-Keuls multiple comparison test; compared to respective control).

induction of IL-6 production by phLFs upon TGFβ1 or TNFα stimulation in the presence and absence of WISP1. Both TGFβ1 and TNFα treatments led to a significant upregulation of *IL6* expression in phLFs after 24 and 48 hours of stimulation (Fig. 4C,E; TGFβ1 at 48 hours: 1.81 ± 0.36 fold over control; TNFα at 48 hours: 5.04 ± 1.29 fold over control). In the absence of WISP1, however, IL-6 expression and secretion was significantly lower compared to the TGFβ1- or TNFα-treated cells transfected with control siRNA (Fig. 4C,E, respectively; siCtrl + TGFβ1 vs. siWISP1 + TGFβ1: *IL6* mRNA 1.81 ± 0.36 fold over control vs. 0.81 ± 0.1 fold over control; siCtrl + TNFα vs. siWISP1 + TNFα: *IL6* mRNA 5.04 ± 1.29 fold over control vs. 2.78 ± 0.81 fold over control). Importantly, these results were validated on the protein level by analysing IL-6 secretion (Fig. 4D,F, respectively; siCtrl + TGFβ1 vs. siWISP1 + TGFβ1 at 48 h: 2.09 ± 0.21 ng/ml vs. 0.46 ± 0.09 ng/ml; siCtrl + TNFα vs. siWISP1 + TNFα at 48 h: 5.13 ± 0.06 ng/ml vs. 3.69 ± 0.44 ng/ml). Importantly, we found similar results in phLFs derived from IPF patients, suggesting that the same mechanisms of WISP1 induction as well as WISP1-dependent *IL6* expression are present in IPF-derived phLFs (Suppl. Fig. 2). Notably, WISP1 specifically affected IL-6 production in phLFs, while the induction of IL8 by TNFα, as well as other cytokines, such as monocyte chemoattractant protein-1 (MCP-1) and interferon γ (IFNγ) were not affected by the loss of WISP1 as measured by a multiplex ELISA (Suppl. Fig. 3A,B and Suppl. Tables 1 and 2).

Loss of WISP1 reduces proliferation of primary human lung fibroblasts. IL-6 has been reported to exhibit pro-proliferative effects on lung fibroblasts[27,28]. In support of these findings, stimulating phLFs with IL-6 led to a concentration-dependent increase in cell proliferation (Fig. 5A), however, this effect was not mediated by an increase in WISP1 following IL-6 treatment since IL-6 did not induce *WISP1* expression in phLFs (Suppl. Fig. 4A). As WISP1-depleted cells produced less IL-6 and WISP1 has been shown to be pro-proliferative in non-lung cells[8] as well as in lung alveolar epithelial type II (ATII) cells *in vitro*[13], we investigated the effect of WISP1 on the proliferation of phLFs. Indeed, siRNA-mediated knockdown of WISP1 resulted in a significantly reduced number of phLFs and reduced expression of cyclin D1 as analysed by Western Blotting (Fig. 5B,C). Moreover, we found decreased immunofluorescent staining of Proliferating-Cell-Nuclear-Antigen (PCNA; Fig. 5D). In addition, we observed significantly reduced metabolic activity of phLFs in a WST-1 assay due to loss of WISP1 (Fig. 5E, reduction by 18.7 ± 3.5%, Suppl. Fig. 4B,C) and reduced cell numbers compared to control siRNA transfected cells

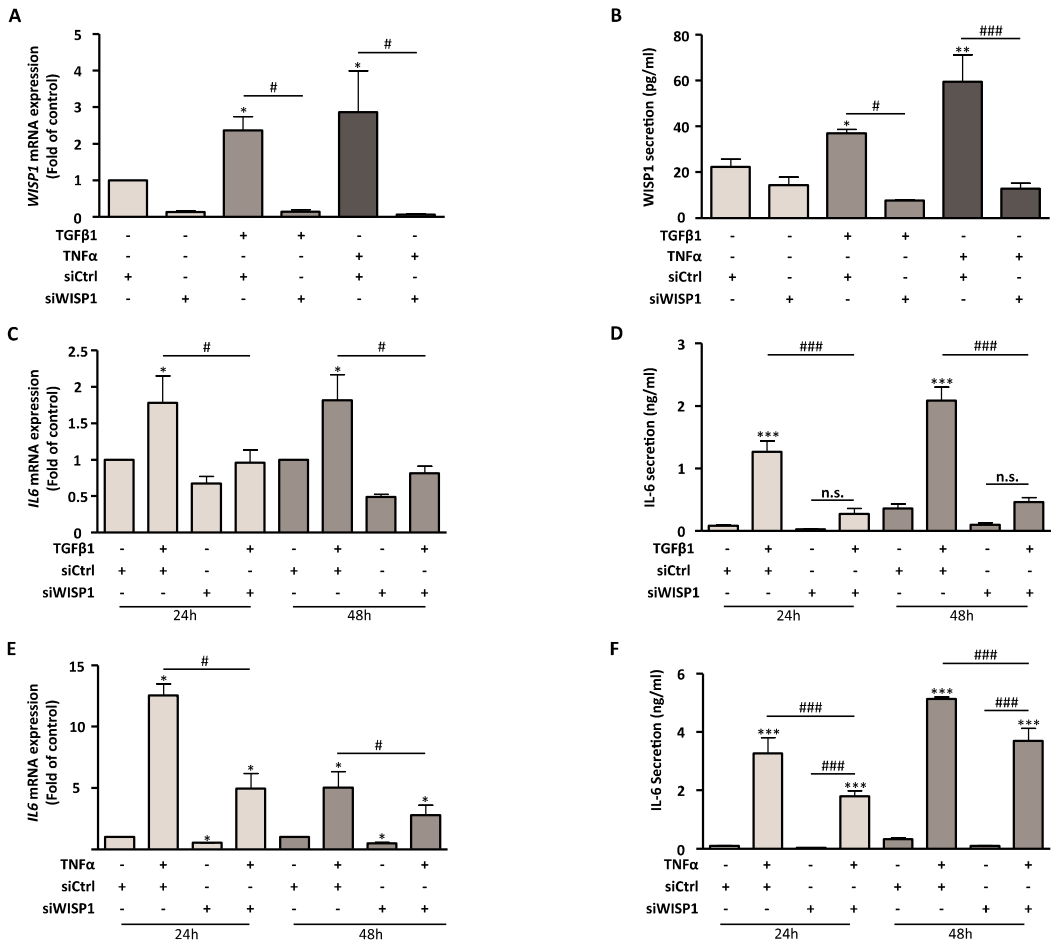

Figure 4. Loss of WISP1 results in reduced expression of TGFβ1- and TNFα-induced IL-6 in phLFs. phLFs were transfected with control (siCtrl) or WISP1-targeting (siWISP1) siRNAs and subsequently treated with TGFβ1 (2 ng/ml) or TNFα (10 ng/ml) for (**A,B**) 24 hours or (**C-F**) 24 and 48 hours. *WISP1* (**A**) mRNA was analysed by RT-qPCR and (**B**) secretion was measured by ELISA. siRNA treatment against *WISP1* results in significant loss of WISP1 at baseline and in the presence of TGFβ1 and TNFα treatment. IL-6 levels were analysed at 24 and 48 hours of 2 ng/ml TGFβ1 or 10 ng/ml TNFα treatment and (**C,E**) IL-6 mRNA was analysed by RT-qPCR and (**D,F**) IL-6 secretion was measured by ELISA. Loss of WISP1 results in loss of IL-6 induction even in the presence of TGFβ1 or TNFα treatment ($n = 4$; $^{*,\#}p < 0.05$; $^{**}p < 0.01$; $^{***,\#\#\#}p < 0.001$; 1-way ANOVA followed by Neuman-Keuls multiple comparison test).

(Fig. 5F; reduction by 22.9 ± 2.8%). Moreover, using either a neutralizing antibody targeting WISP1 (Fig. 5G) or an IKKβ inhibitor (SC-514) that downregulates *IL6* expression (Suppl. Fig. 5A,B), we further corroborated our results and observed a significantly decreased metabolic activity of phLFs upon αWISP1 treatment (Fig. 5G; reduction by 9.2 ± 2.4%) as well as upon NF-κB inhibition (Suppl. Fig. 5C; reduction by 23.5 ± 2.4%). Taken together, our data strongly indicate that WISP1 exhibits pro-proliferative effects on phLFs. To this end, we further found that *WISP1* expression levels in IPF tissue negatively correlated with lung function measurement parameters (%DL_{CO} and %FVC), which also have been shown to correlate to the number of fibroblast foci in IPF[29] (Suppl. Fig. 6A,B).

WISP1-induced IL-6 expression contributes to primary human lung fibroblast proliferation. Given our findings that the presence of WISP1 is required for the induction of IL-6 by TGFβ1 and TNFα and that IL-6 is able to induce proliferation in phLFs, we next hypothesized that the reduced proliferation of phLFs in the absence of WISP1 might be a result of reduced IL-6 levels. To address this question, we either transfected phLFs with siRNAs (siWISP1 and respective control) or treated cells with a neutralizing αWISP1 antibody and subsequently treated the cells with IL-6 (10 ng/ml; Fig. 6A,B). Notably, cells lacking WISP1 showed a significantly higher increase in the proliferative response to IL-6 compared to siCtrl transfected cells (Fig. 6A; 16.8% vs. 6.3%). Consistently, the decrease in WST1 by WISP1 siRNA was in part restored by IL-6 (Fig. 6A; untreated vs. IL-6 treated: −17.34 ± 3.5% vs. −12.32 ± 4.12%), indicating a partial rescue of the proliferation defect by IL-6 in cells lacking WISP1. Additionally, cells treated with the αWISP1 antibody and subsequently with IL-6 showed fully restored proliferation capacity compared to cells treated with an IgG control (Fig. 6B; untreated vs.

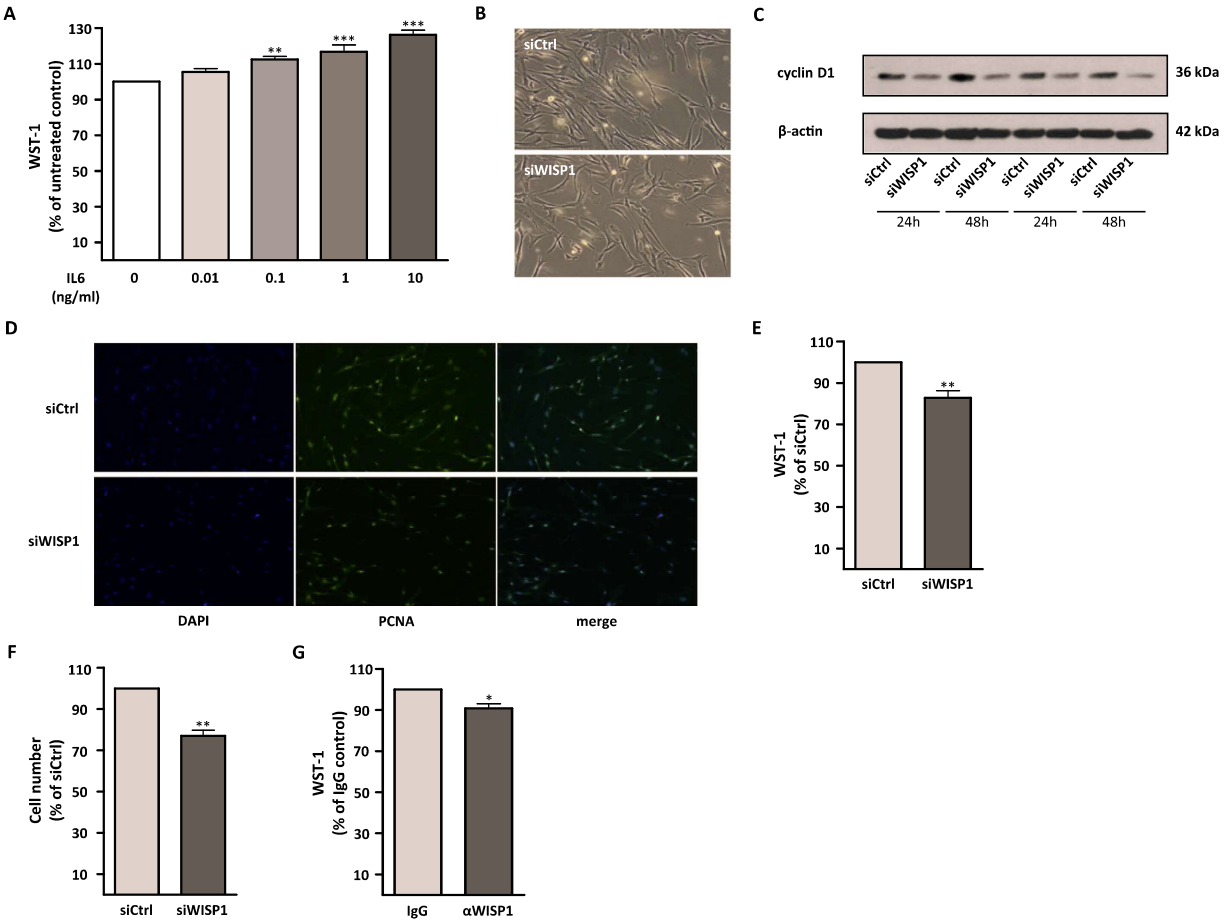

Figure 5. Loss of WISP1 decreases proliferation of phLFs. (**A**) The phLFs showed a dose-dependent increase in proliferation with different concentrations of IL-6 as measured by WST-1. (**B–F**) phLFs transfected with control (siCtrl) or WISP1-targeting (siWISP1) siRNA had decreased proliferation. (**B**) Representative bright field images of siCtrl and siWISP1 transfected cells (magnification: 100×). (**C**) Representative Western Blot of cyclin D1 levels in phLFs after 24 and 48 hours of treatment with siWISP1 and (**D**) immunofluorescence staining of PCNA (green) and DAPI (blue; magnification: 100×) shows qualitative decreases in cell number and PCNA staining. Decreased proliferation in siWISP1 conditions was measured by (**E**) WST-1 assay and (**F**) cell count. (**G**) Additionally, phLFs were treated with a neutralizing αWISP1 antibody and decreased proliferation was observed by WST-1 assay ($n = 3–7$; $^{*}p < 0.05$; $^{**}p < 0.01$; A: 1-way ANOVA followed by Neuman-Keuls multiple comparison test; **E–G**: Student's T-test).

IL-6 treated: $-9.2 \pm 2.4\%$ vs. $0 \pm 1.8\%$). Taken together, these results strongly indicate that WISP1-induced IL-6 expression significantly contributes to the pro-proliferative fibroblast function, which is likely orchestrated by a variety of profibrotic mediators, including Wnts, TGFβ1 and TNFα (Fig. 7).

Discussion

IPF is a chronic lung disease with poor outcome prediction[1,2]. Two recently approved drugs have been shown to reduce the progression of lung function decline in mild to moderate IPF, however, they have not been reported to halt or reverse pathological changes in lung architecture and lung function[3,4]. Thus, further understanding of the pathomechanisms involved in IPF development and progression is required to develop more effective therapeutic strategies. TNFα and TGFβ1 are highly upregulated in IPF and targeting profibrotic mediators induced downstream of TGFβ1 and TNFα represents a promising therapeutic approach for IPF[2,13]. Here, we show that both TGFβ1 and TNFα induce WISP1 expression and secretion in primary human lung fibroblasts. The WISP1 promoter contains transcription factor binding sites for TCF/LEF, SMADs, as well as NF-κB and our initial promoter studies indicate a direct control of *WISP1* expression by Wnt3a, TGFβ1, and TNFα. WISP1 is upregulated in IPF and has been shown to be an epithelial cell-derived mediator of impaired epithelial-to-mesenchymal crosstalk. Of note, neutralizing WISP1 led to a reduction of experimentally induced lung fibrosis[13]. WISP1 has further been described as a target gene of canonical Wnt signaling[30], a developmental pathway reactivated in IPF[31] and inhibition of which has been shown to prevent and reverse fibrotic changes in the murine lung[32,33]. We have recently reported that WISP1 is increased in IPF fibroblasts[15]. Here, we revealed a novel mechanism by which WISP1

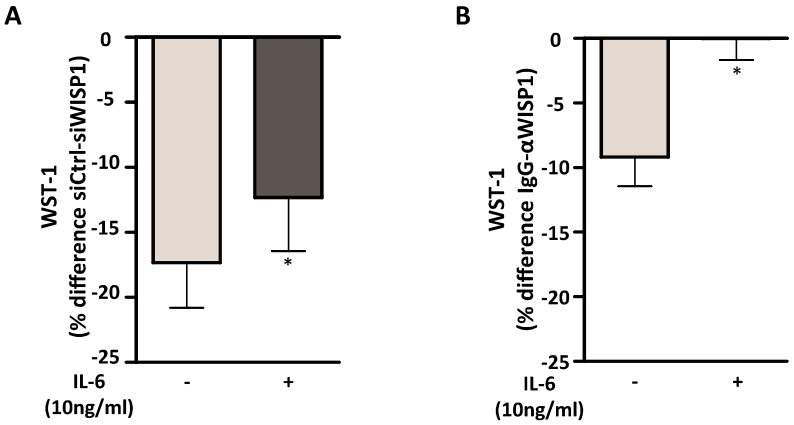

Figure 6. WISP1 increases proliferation of phLFs in part via IL-6. The phLFs were either (**A**) transfected with siCtrl or siWISP1 or (**B**) pre-incubated with a neutralizing αWISP1 antibody and treated with or without IL-6 (10 ng/ml). Metabolic activity of the phLFs was measured by WST-1 conversion and statistically significant increases were observed following IL-6 treatment demonstrating that IL-6 induction is, in part, responsible for mediating the pro-proliferative effects of WISP1 ($n = 7$; $^*p < 0.05$; Student's T-test).

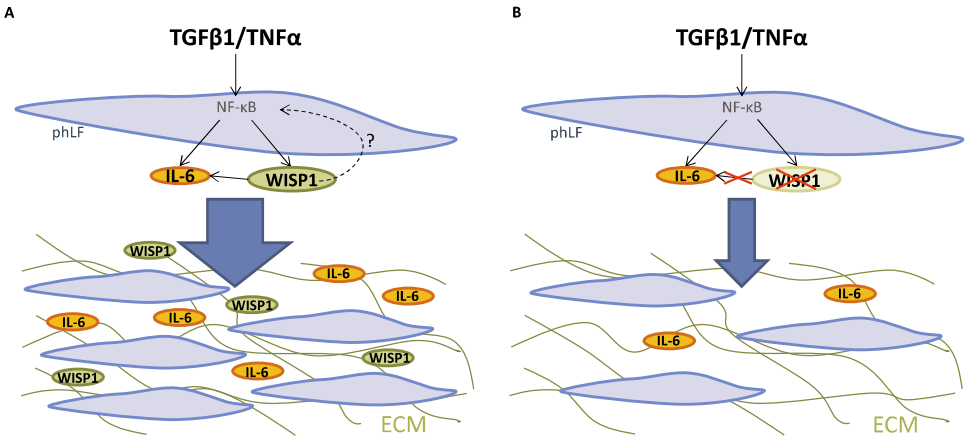

Figure 7. Proposed schematic model. (**A**) The profibrotic cytokines TGFβ1 and TNFα can induce WISP1 presumably via NF-κB in phLFs, which results in WISP1-dependent IL-6 production and increased proliferation of phLFs. (**B**) In the absence of WISP1, decreased IL-6 levels lead to reduced fibroblast proliferation. Our working hypothesis is that WISP1 controls IL-6 expression via a positive feedback on NF-κB.

contributes to profibrotic cellular fibroblast function and thus underline the potential of WISP1 as a therapeutic target for IPF.

IPF is characterized by increased fibroblast proliferation and accumulation along with ECM production. Thus, altering fibroblast function is of main interest as a potential therapeutic strategy in IPF. Pulmonary WISP1 has been shown to be highly expressed by alveolar epithelial type II (ATII) cells. Immunohistochemical analysis of WISP1 in IPF tissue specimen revealed only a weak staining in lung fibroblasts/interstitium of the lung[13]. Our *in vitro* data using primary human lung fibroblasts indicate that fibroblasts are an additional source for WISP1. While comparative analysis of human alveolar epithelial cells and phLFs revealed that the amount of WISP1 secreted by ATII cells *in vitro* exceeds the secretion by fibroblasts by about 25 fold (data not shown), it is likely that fibroblast-derived WISP1 might directly act on surrounding cells in the microenvironment of a fibroblast focus.

IL-6 is a well-described inducer of lung fibroblast proliferation[28]. Here, we found that WISP1 is required for TGFβ1- and TNFα-dependent induction of IL-6 in lung fibroblasts. We have recently described a link between Wnt/β-catenin signaling and interleukin secretion in pulmonary fibrosis[18]. Induction of Wnt/β-catenin signaling by Wnt3a in alveolar epithelial cells *in vitro* and *in vivo* resulted in a significant increase in IL-6. Moreover, IL-6 was shown to be upregulated in the bronchial alveolar lavage fluid (BALF) of IPF patients and in experimental lung fibrosis[18,25]. In this context, it has also been shown that a mutation of the IL-6 receptor subunit gp130, by which signal transduction downstream of gp130 is solely directed through signal transducer and activator of transcription 3 (STAT3) but no longer via ERK/MAPK signaling, led to a worsened fibrotic lung phenotype in

mice upon bleomycin challenge. Interestingly, loss of IL-6 in gp130 mutated mice resulted in protection against bleomycin-induced lung fibrosis, indicating that IL-6 itself is necessary for fibrosis development in this model[34]. Altogether these data support the notion that WISP1 and IL-6 are inter-connected critical mediators contributing to IPF pathogenesis.

We further analysed functional effects of WISP1 and found that depletion of WISP1 by two independent approaches resulted in decreased fibroblast proliferation, thus further corroborating a pro-proliferative role of WISP1[35–38]. In support of these findings, we demonstrate that treatment of WISP1-deficient or WISP1-neutralized phLFs with IL-6, in part, rescued the effect on fibroblast proliferation. These data suggest that WISP1 increases proliferation of human lung fibroblasts via IL-6 induction (Fig. 7). It is also important to note that in our experimental setup IL-6 was not able to fully rescue fibroblast proliferation upon loss of WISP1 by siRNA mediated knockdown *in vitro*. Thus, it is likely that WISP1 potentially affects additional pro-proliferative factors. Some of these, such as IL-8, were included in our multiplex ELISA and were not affected by the loss of WISP1. Other cytokines belonging to the IL-6 interleukin subfamily, which includes IL-11, IL-31, Oncostatin M and Leukemia inhibitory factor (LIF) have been described as pro-proliferative[39], however, they were not tested in our multiplex ELISA approach. Though there are no reports yet, linking WISP1 to any of these latter cytokines, one of these cytokines or a combination thereof might be responsible for the additional effect of WISP1 knockdown on proliferation. Future studies are needed to address these open questions.

We found that TGFβ1 and TNFα mainly induced WISP1 expression in phLFs via NF-κB pathway. Notably, the presence of WISP1 is required for *IL6* expression, which is also an NF-κB-driven target gene and induced by both TGFβ1 and TNFα. As such, we hypothesize that WISP1 has a positive feedback function on NF-κB-driven genes. WISP1 is a member of the CCN family, which also includes Cysteine-rich angiogenic inducer 61 (Cyr61), Connective tissue growth factor (CTGF) and Nephroblastoma overexpressed protein (NOV). These family members have been shown to induce downstream effects via NF-κB in different cell types[40–42]. In line with these findings, WISP1 was previously described to activate NF-κB in synovial fibroblasts[43]. These data strongly support our findings and suggest that WISP1 has a potential positive feedback function on IL-6 through the activation of NF-κB in primary human lung fibroblasts.

Currently, it is unknown, which cell-surface molecules are required for WISP1-mediated signaling in phLFs. However, CCN family members are known to signal through integrins[44]. Integrins are versatile cell surface receptors that, through various combinations of their alpha and beta subunits, can regulate a variety of different responses in a cell-specific manner[45]. Importantly, integrins play a role in the pathogenesis of IPF, both by functioning as receptors and by activating molecules like TGFβ1[45]. For example, integrin $\alpha_v\beta_6$ has been shown to be important for TGFβ1 activation on lung epithelial cells, and β_6 knockout animals or $\alpha_v\beta_6$ neutralization attenuated pulmonary fibrosis development[45]. WISP1 has been reported to signal through $\alpha_v\beta_5$ in synovial fibroblasts[43], an integrin heterodimer that is also expressed on lung fibroblasts[46] and thus represents a potential integrin involved in WISP1-induced IL-6 production in lung fibroblasts.

Taken together, our data show that WISP1 is a common downstream target of major pro-fibrotic factors, TGFβ1 and TNFα, in primary human lung fibroblasts. Moreover, WISP1 exerts its profibrotic functions through IL-6-dependent induction of fibroblast proliferation. These data further underline the importance of WISP1 in the progression of lung fibrosis and strengthen the potential benefit of an anti-WISP1 therapy in IPF patients.

Materials and Methods

Reagents. Recombinant TGFβ1 (human; 240-B/CF), recombinant TNFα (human, 210-TA/CF) and recombinant IL-6 (206-IL/CF) were purchased at R&D systems (Abingdon, UK).

Cell culture. Primary human lung fibroblasts (phLFs) isolation was performed as previously described[47]. The phLFs were cultured in Dulbecco's Modified Eagle's medium/Nutrient mixture F12 medium (DMEM/F12) containing 20% (v/v) fetal calf serum (FCS), 100 mg/l streptomycin and 100 U/ml penicillin. Cells were synchronized before stimulation by culturing them for 24 hours in corresponding starvation medium supplemented with 0.1% (v/v) FCS and antibiotics. Cell stimulations were performed in fresh medium with identical composition as medium for cell synchronization. Cells were incubated at 37 °C, 5% CO_2. For inhibitor studies, phLFs were seeded in 6 well-plates with a total of 2×10^5 cells/well. 24 hours after seeding, cells were synchronized for 24 hours. Cells were pre-treated with different inhibitors for 1 hour (SB431542 – 10 μM; SC-514 – 50 μM; U0126 – 3 μM; SP600125 – 10 μM; 7-Z-Oxozeaneol – 500 nM) and subsequently treated with TGFβ1 (2 ng/ml) or TNFα (10 ng/ml) for 24 hours. For analysis of the time-dependent induction of WISP1, phLFs were seeded in 6 well-plates in a total of 2×10^5 cells/well. 24 hours after seeding, cells were synchronized for 24 hours. Cells were subsequently treated with TGFβ1 (2 ng/ml) or TNFα (10 ng/ml) and treated for 8 to 48 hours. Supernatants were taken at 24 and 48 hours for WISP1 ELISA measurements. Cells were washed with cold PBS and thereafter taken for RNA isolation. To analyse the concentration-dependent induction of WISP1, phLFs were seeded in 6 well-plates with a total of 2×10^5 cells/well. 24 hours after seeding, cells were synchronized for 24 hours. Cells were subsequently treated with TGFβ1 (0.5–10 ng/ml) or TNFα (10–100 ng/ml) and treated for 24 hours. Cells were washed with cold PBS and thereafter taken for RNA purification. Supernatants were stored at −80 °C until further use.

WISP1 Luciferase promoter studies. The sequence of the WISP1 promoter region (2.5 kb from the transcription start site) was obtained from the USCS Genome Bioinformatics database (genome assembly: GRChg38; location: 133,188,539–133,191,039) and analysed using the Genomatix software version 3.4. The 2.5 kb element of the WISP1 promoter was cloned into the pGL4.10 vector. The phLFs were seeded in a 48 well plate at a density of 2.5×10^4 cells/well in DMEM/F12 containing 20% (v/v) (FCS), 100 mg/l streptomycin and 100 U/ml penicillin. Cells were transfected 24 hours after seeding in serum-free Opti-MEM medium (Life Technologies, Darmstadt, Germany) plus Dulbecco's Modified Eagle's medium/Nutrient mixture F12 medium (DMEM/F12)

containing 20% (v/v) fetal calf serum (FCS), 100 mg/l streptomycin and 100 U/ml penicillin (ratio 1:3) using 250 ng/ml of the vector construct including the 2.5 kb element of the WISP1 promoter region in combination with Lipofectamine LTX transfection reagent and PLUS reagent (Life Technologies, Darmstadt, Germany). Control transfections were performed using 250 ng of the pGL4.10 vector construct. The transfection mix was incubated in Opti-MEM medium for 30 minutes at room temperature. The transfection mix was then added to the wells on top of the refreshed starvation medium. Cells were transfected for 6 hours and thereafter cells were incubated in starvation medium overnight. Cells were subsequently stimulated for 24 hours with either Wnt3a (100 ng/ml), TGFβ1 (2 ng/ml) or TNFα (10 ng/ml) in DMEM/F12 medium supplemented with 0.1% FCS (v/v) and antibiotics. After 24 hours, cells were lysed and cell lysate suspension was used to determine the luciferase activity using the Berthold Tristar LB941 (luciferase reagent: Bright-Glo™ Luciferase Assay System, Promega, Mannheim, Germany). Measurements were performed in quadruplicates.

WISP1 siRNA transfection. Primary human fibroblasts were seeded in different well plate formats (6 well: 2×10^5 cells/well; 24 well: 5×10^4 cells/well; 96 well: 5×10^3 cells/well) and transiently transfected with a pool of specific double-stranded siRNAs targeted against the WISP1 transcript (On-Targetplus siRNA, J-010555-05, -07, -08; Dharmacon, Lafayette, Colorado, USA). Cells were transfected in serum-free Opti-MEM medium (Life Technologies, Darmstadt, Germany) plus DMEM/F12 containing 20% (v/v) FCS, 100 mg/l streptomycin and 100 U/ml penicillin (ratio 1:3) using 10 nM of siRNA in combination with Lipofectamine RNAiMax transfection reagent (Life Technologies, Darmstadt, Germany). Control transfections were performed using 10 nM ON-TARGETplus Non-targeting siRNAs (D-001810-10, Dharmacon, Lafayette, Colorado, USA). The siRNAs were incubated in Opti-MEM medium for 30 minutes at room temperature. The siRNA mix was then added to the wells and cell suspension was added on top. Cells were transfected overnight. Cells were subsequently stimulated for the indicated time-points with either TGFβ1 (2 ng/ml) or TNFα (10 ng/ml) in DMEM/F12 medium supplemented with 0.1% FCS and antibiotics. Supernatants were collected and cells were washed with cold PBS and thereafter taken for RNA purification. Supernatants were stored at −80 °C until further use.

Treatment of phLFs with neutralizing αWISP1 antibody. Cells were seeded in 96 well format (5×10^3 cells/well) in Dulbecco's Modified Eagle's medium/Nutrient mixture F12 medium (DMEM/F12) containing 20% (v/v) fetal calf serum (FCS), 100 mg/l streptomycin and 100 U/ml penicillin. After 24 hours cells were serum-starved (DMEM/F12 medium containing 0.1% FCS and antibiotics) for 24 hours. Prior to treatment, cells were pre-incubated with the neutralizing αWISP1 antibody (10μg/ml; R&D, AF1627) for 1 hour. Subsequently, cells were treated with or without 10 ng/ml IL-6 for 48 hours.

Immunofluorescence staining. The phLFs were transfected as described above and cultured for 72 hours on poly-l-lysine-coated cover slips. Cells were fixed with acetone/methanol (1:1), permeabilized with 0.1% Triton X-100 in 1xPBS for 20 minutes and blocked with 5% (w/vol) bovine serum albumin (Sigma Aldrich) for 30 minutes. Cells were subsequently incubated with the respective primary antibody (PCNA, Zymed 18-0110, Vienna, Austria) at room temperature (RT) for 1 hour in PBS containing 0.1% (w/vol) BSA, followed by incubation with a fluorescently labeled secondary antibody (anti-mouse Alexa 488, Life Technologies). DAPI staining (Roche) was used to visualize cell nuclei.

Immunoblotting. Cells were washed twice with phosphate-buffered saline (PBS; PAA Laboratories), lysed in T-PER lysis buffer (Thermo Fisher Scientific, Waltham, MA, US) supplemented with proteinase inhibitor cocktail tablets and PhosSTOP™ (Roche), and lysates were centrifuged at 13 000 rpm at 4 °C. Supernatant was collected and protein concentration was determined using the Quick Start Bradford Dye Reagent according to the manufacturer's instructions. 15 μg of total protein was separated on SDS-polyacrylamide gels and transferred to PVDF (Biorad, Hercules, CA, US). Membranes were blocked in 1x Roti®-Block (Roth, Karlsruhe, Germany) in TRIS-buffered saline containing 0.05% (v/v) Tween (TBST) (Applichem) and incubated with the primary antibody (Cyclin D1, 2978 P, New England Biolabs; Ipswich, MA, USA) at 4 °C overnight. The HRP-labeled secondary antibody (anti-rabbit-HRP antibody; GE Healthcare, Chalfont St Giles, UK) was applied after washing of the membrane in TBST. Proteins were visualized by autoradiography following incubation with SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific). β-actin served as loading control and was detected using a HRP-conjugated β-actin antibody (Sigma Aldrich).

RNA isolation and reverse transcription real-time polymerase chain reaction. Total RNA was isolated from cells using the Peqlab Total RNA Kit (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. An amount of 1000 ng of RNA was used for cDNA synthesis as previously described[18]. The following primers were used: *Wnt1-inducible signaling protein 1* (forward: GGCATGAGGTGGTTCCTG; reverse: GGAGCTGGGGTAAAGTCCAT), *Interleukin 6* (forward: TTCCTGCAGAAAAAGGCAAAGA; reverse: CTGCGCAGAATGAGATGAGT), *Interleukin 8* (forward: CAGGAAGAAACCACCGGAAG; reverse: AACTGCACCTTCACACAGAG), *Serpine 1* (forward: GACATCCTGGAACTGCCCTA; reverse: GGTCATGTTGCCTTTCCAGT).

WISP1 and Interleukin-6 enzyme-linked immunosorbent assay. Supernatants were taken from time-dependent TGFβ1 and TNFα WISP1-inductions or siRNA transfection assays and concentrated for WISP1 measurements by a factor of 5 using Amicon Ultra-0.5 centrifugal filter devices according to the manufacturer's instructions (Merck Millipore, Amsterdam, The Netherlands) and the assay was performed according to the manufacturer's instructions. Samples were then transferred to the WISP1 ELISA plate (DY1627; R&D, Minneapolis, Minnesota, USA). Samples for IL-6 measurements were diluted 1:10 in dilution buffer prior to transfer to the

IL-6 ELISA plate (DY206; R&D, Minneapolis, Minnesota, USA) and the assay was performed according to the manufacturer's instructions.

WST1-Proliferation assay. Primary human lung fibroblasts were plated at a density of 5×10^3 cells per well in a 96 wells plate. The next day cells were synchronized for 24 hours using DMEM/F12 medium supplemented with 0.1% FCS and antibiotics. Cells were stimulated with DMEM/F12 medium with 0.1% (v/v) FCS, DMEM/F12 medium with 10% (v/v) FCS or, or DMEM/F12 with 10% (v/v) FCS plus IL-6 (0.1–20 ng/ml) for 48 hours. Subsequently, 10 μl of WST-1 per 100 μl medium (10% v/v; Cat. No. 11 644 807 001, Roche Diagnostics GmbH, Mannheim, Germany) was added to each well and incubated for 2 hours. Plates were then measured using the Tecan Sunrise ELISA Reader at a wave length of 440 nm (reference wave length: 620 nm). Each condition was measured in triplicates.

Cell counting. Primary human lung fibroblasts were plated at a density of 5×10^4 cells per well in a 24 well plate. The next day cells were synchronized for 24 hours using DMEM/F12 medium supplemented with 0.1% FCS and antibiotics. Cells were stimulated with DMEM/F12 medium with 10% (v/v) for 48 hours. Subsequently, cells were washed with PBS, trypsinized and counted using a Neubauer chamber. Experiments were performed in duplicates.

Correlation analysis. Data for the analysis were extracted from Lung Genomics Research Consortium (GSE47460 GPL4680) and correlated to diffusion capacity of the lung for carbon monoxide (DLCO) and the forced vital capacity (FVC) in human patients as a measure of disease severity. Only normal control patients and patients with confirmed IPF were used from the dataset.

Statistical analysis. Data represent means ± SEM, from *n* independent experiments. Statistical significance of differences was evaluated by Student's *t*-test or one-way ANOVA followed by a Newman-Keuls multiple comparison test, where appropriate. Correlation was evaluated by using the Pearson test. Differences were considered to be statistically significant when $p < 0.05$.

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Author Contributions

S.K., M.L, D.E.W. and H.B. designed and performed experiments and data analysis; M.K. oversaw all experimental design and data analysis; S.K., M.L. and M.K. wrote the manuscript.

Additional Information

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WISP1 mediates IL-6-dependent proliferation in primary human lung fibroblasts

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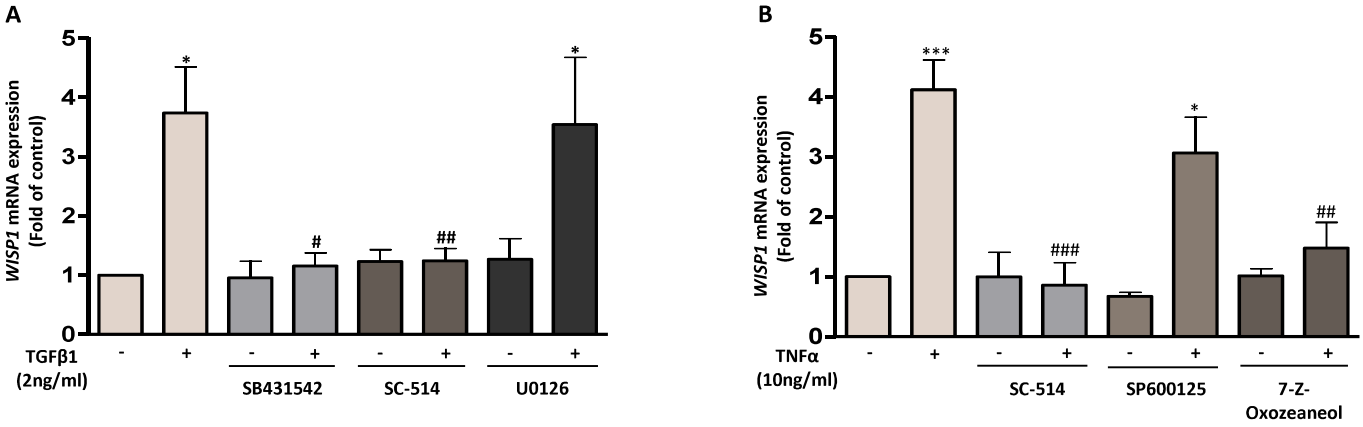
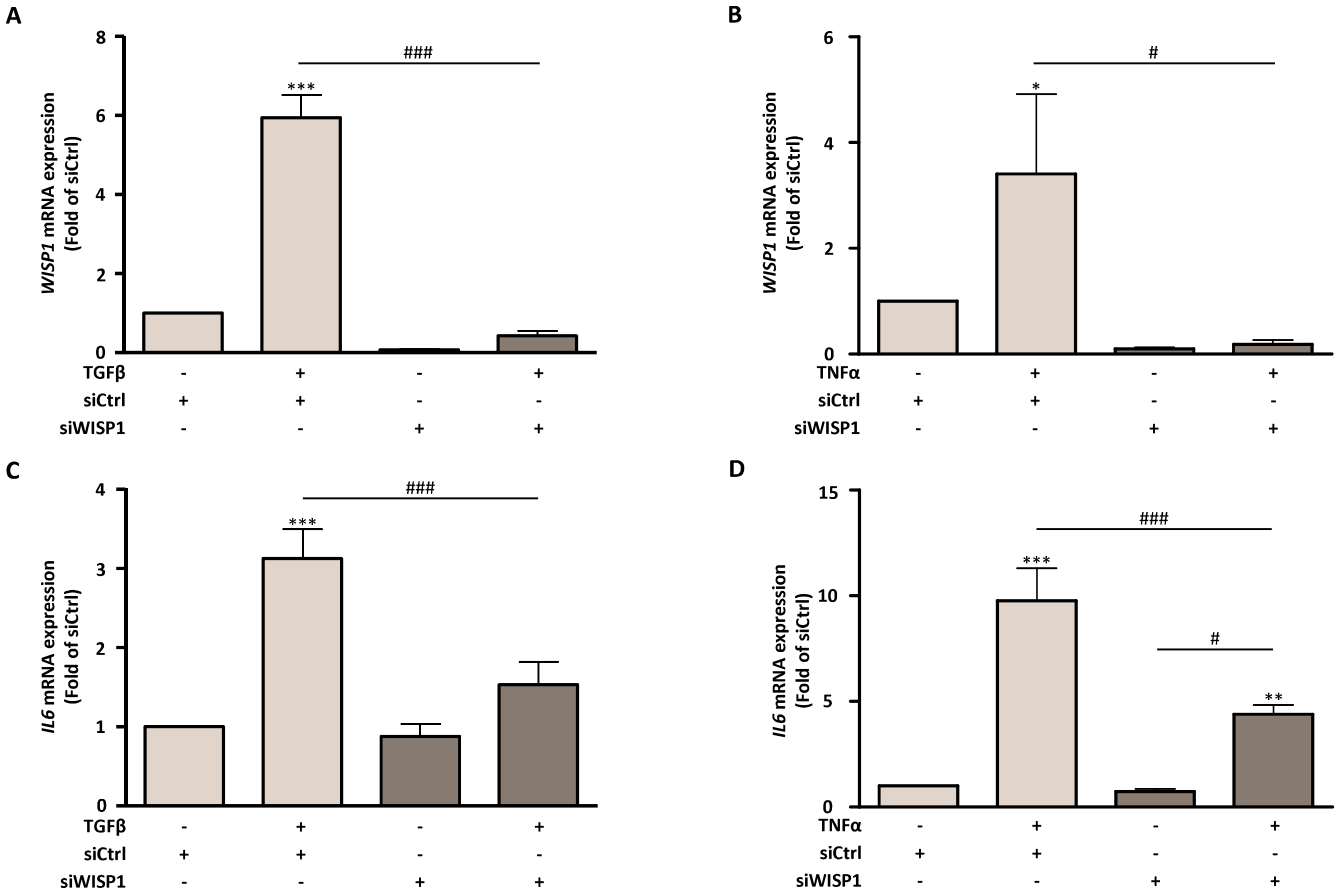
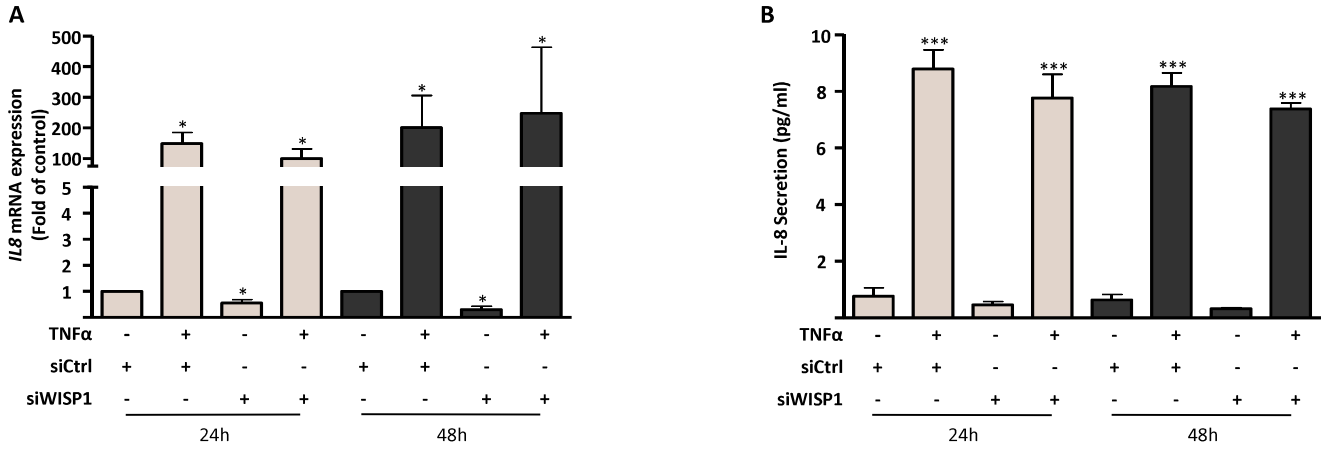


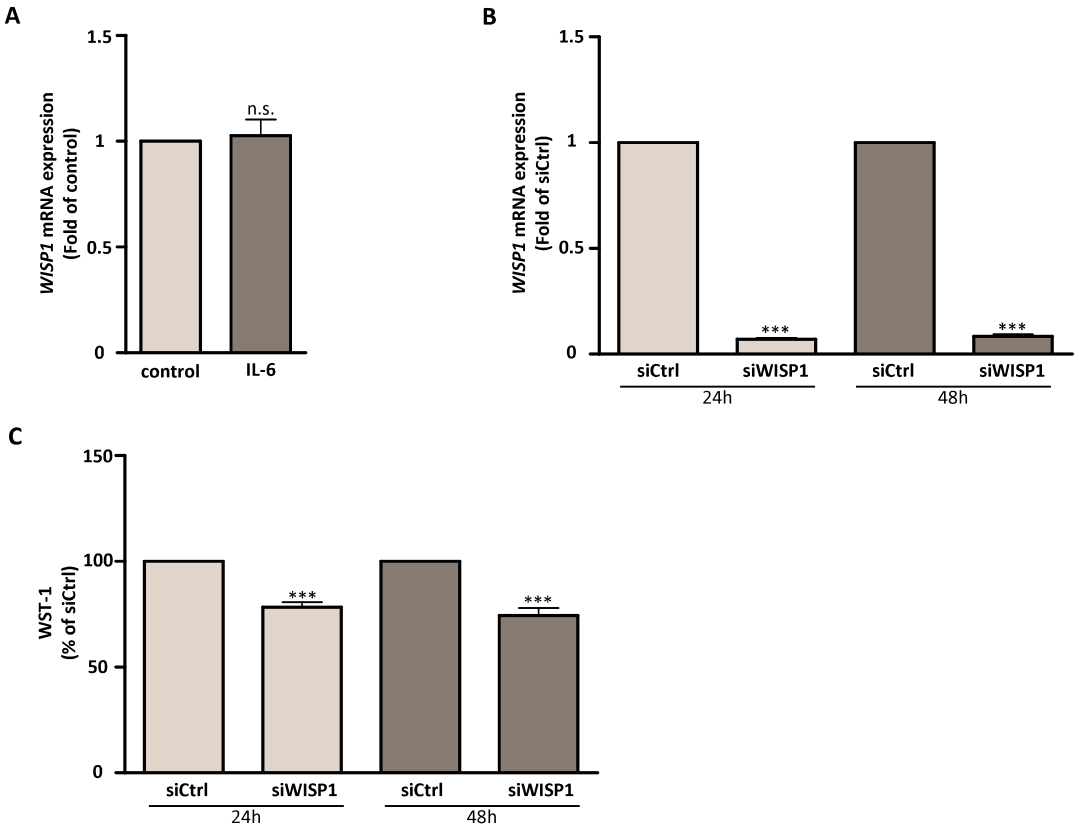
Supplementary Figure 1: TGFβ1- and TNFα induce *WISP1* in phLFs via NF-κB. The phLFs were treated for 24 hours with either (A) 2 ng/ml TGFβ1 in the absence or presence of the ALK5 inhibitor SB 431542 (10 µM), the IKKβ inhibitor SC-514 (10 µM) or the MEK1/2 inhibitor U126 (3 µM) or (B) with 10 ng/ml TNFα in the absence or presence of the IKKβ inhibitor SC-514 (10 µM), the JNK inhibitor SP 600125 (10 µM) or the TAK1 inhibitor 7-Z-Oxozeanol (500 nM). (n=3; *,# p<0.05; student´s t-test)



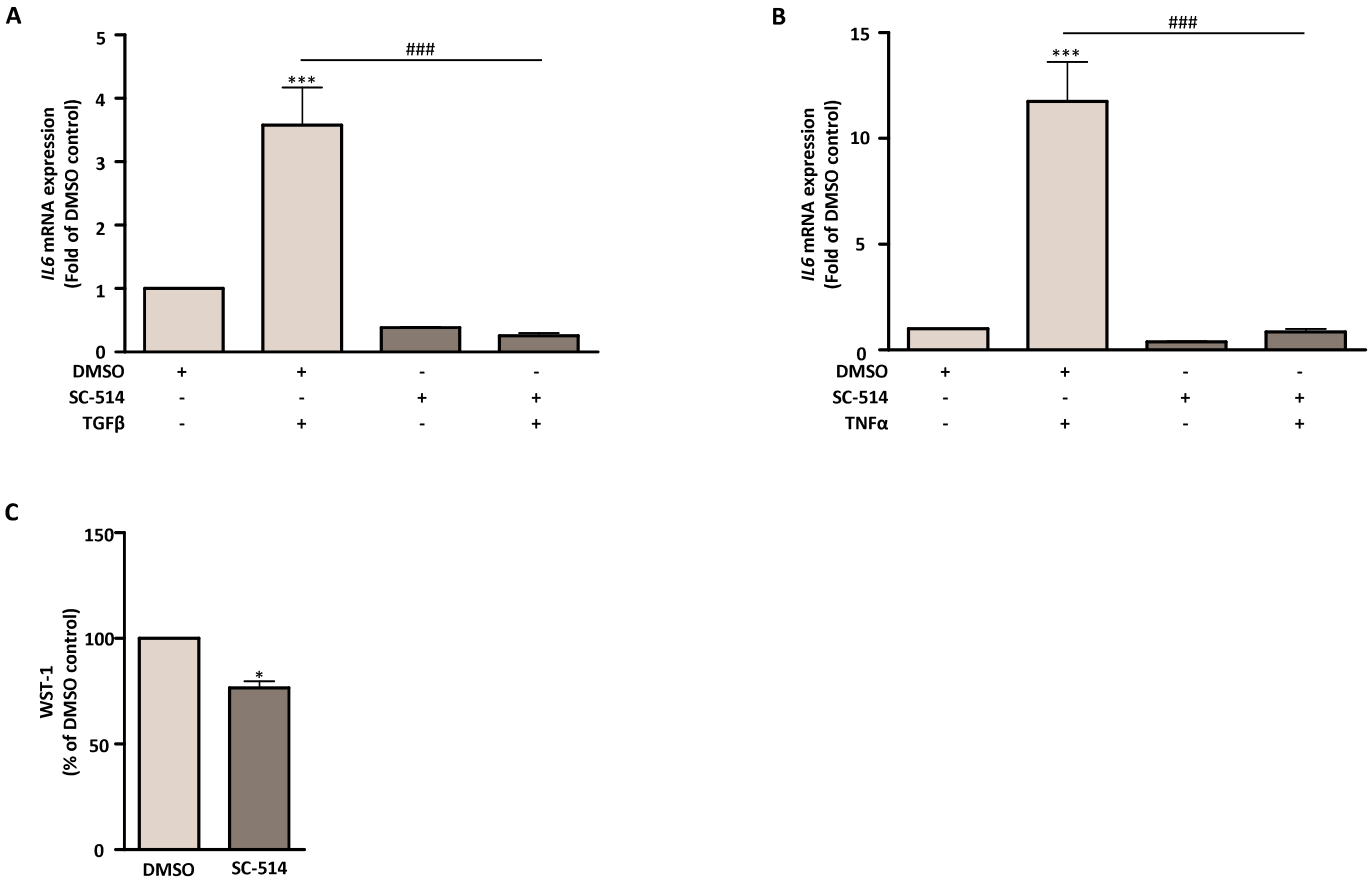
Supplementary Figure 2: WISP1 is required for *IL6* induction in phLFs derived from IPF patients. The phLFs were treated for 24 hours with either (A,C) 2 ng/ml TGFβ1 or (B,D) with 10 ng/ml TNFα in the absence or presence of the siRNA targeting WISP1 (siWISP1) or a non-targeting control siRNA (siCtrl). Effects on the expression of (A,C) *WISP1* and (B,D) *IL6* were analyzed using qPCR (n=3; *,# p<0.05; ** p<0.01; ***, ### p<0.001; 1-way Anova with Newman-Keuls Multiple comparison testing).



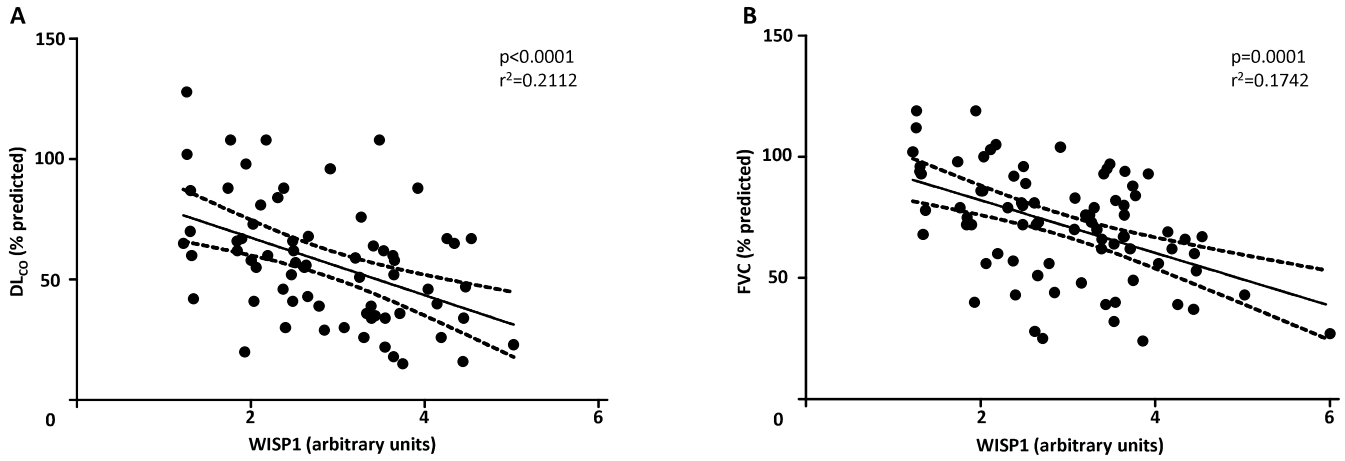
Supplementary Figure 3: Production of IL-8 upon TNFα stimulation is not affected by the absence of WISP1 in phLFs. The phLFs were treated for 24 and 48 hours with with 10 ng/ml TNFα in the absence or presence of the siRNA targeting WISP1 (siWISP1) or a non-targeting control siRNA (siCtrl). (A) The expression of *IL8* was measured by qPCR and (B) the secretion of IL-8 was analyzed using a multiplex ELISA (n=4; * $p<0.05$; *** $p<0.001$; 1-way Anova with Newman-Keuls Multiple comparison testing).



Supplementary Figure 4: *WISP1* is not induced by IL-6 stimulation in phLFs. (A) The phLFs were treated for 24 hours with 10 ng/ml IL-6 or (B,C) transfected with a WISP1-silencing siRNA (siWISP1) or a control siRNA (siRNA) and kept in 0.1% FCS-containing medium according to the stimulation protocol for 24 and 48h. (A,B) The expression of *WISP1* was measured by qPCR and (C) the metabolic activity of the phLFs was measured by WST-1 assay (n=7; *** $p<0.001$; 1-way Anova with Newman-Keuls Multiple comparison testing).



Supplementary Figure 5: The IKKβ inhibitor SC-514 abrogates the expression of *IL6* and reduces proliferation in phLF. The phLFs were treated for 24 hours with (A) 2 ng/ml TGFβ or (B) 10 ng/ml TNFα in the absence or presence of the IKKβ inhibitor SC-514 (50µM) and the expression of *IL6* was measured by qPCR. (C) Moreover, treatment of phLFs with SC-514 (50µM) for 48 hours and proliferation was assessed by WST-1 assay (n=3; ***,### $p<0.001$, 1-way Anova with Newman-Keuls Multiple comparison testing for (A) and (B); * $p<0.05$, students t-test for (C))



Supplementary Figure 6: WISP1 mRNA levels negatively correlate with the diffusion capacity of the lung for carbon monoxide (DL_{CO}) and the forced vital capacity (FVC) in human patients. The correlation of WISP1 and (A) the predicted DL_{CO} (N=68) or (B) the FVC (N=80) showed a significantly negative linear correlation. (dashed line = 95% CI; Data extracted from the LGRC GSE47460 GPL4680)

TGFβ treatment

	siCtrl (pg/ml)	siWISP1 (pg/ml)	siCtrl + TGFβ (pg/ml)	siWISP1 + TGFβ (pg/ml)
IL-6	0.76 ± 0.10	0.24 ± 0.05*	2.33 ± 0.10*	0.9 ± 0.17#
IL-8	0.75 ± 0.42	0.39 ± 0.09	0.53 ± 0.25	0.33 ± 0.09
MCP1	1.57 ± 0.44	0.83 ± 0.31	0.5 ± 0.14*	0.31 ± 0.08*
IFNγ	0.07 ± 0.03	0.01 ± 0.01*	0.11 ± 0.02	0.02 ± 0.01*
GM-CSF	n.d.	n.d.	n.d.	n.d.
IL-7	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
TNFα	0.01 ± 0.00	n. d.	0.01 ± 0.00	n. d.
GCSF	0.01 ± 0.00	n. d.	0.01 ± 0.00	n. d.
IL-4	0.01 ± 0.00	n. d.	0.01 ± 0.00	n. d.
MIP1b	n.d.	n.d.	n.d.	n.d.
IL-2	n.d.	n.d.	n.d.	n.d.
IL-17	n.d.	n.d.	n.d.	n.d.
IL-10	n.d.	n.d.	n.d.	n.d.
IL-12	n.d.	n.d.	n.d.	n.d.
IL-13	n.d.	n.d.	n.d.	n.d.
IL-5	n.d.	n.d.	n.d.	n.d.
IL-1b	n.d.	n.d.	n.d.	n.d.

Supplementary Table 1: The secretion of different cytokines by phLFs was measured after TGFβ1 (2 ng/ml) stimulation *in vitro* for 24 hours using a multiplex ELISA kit (Bio-Plex Pro™ Human Cytokine 17-plex). (n=4; *,# p<0.05; 1-way ANOVA followed by Neuman-Keuls multiple comparison test)

TNFα treatment

	siCtrl (pg/ml)	siWISP1 (pg/ml)	siCtrl + TNFα (pg/ml)	siWISP1 + TNFα (pg/ml)
IL-6	0.77 ± 0.16	0.26 ± 0.05*	13.86 ± 3.97*	5.61 ± 0.92*,#
IL-8	0.77 ± 0.52	0.47 ± 0.19	8.79 ± 1.17*	7.77 ± 1.45*
MCP1	1.52 ± 0.41	0.90 ± 0.38	2.85 ± 0.60*	2.35 ± 0.23*
IFNγ	0.08 ± 0.03	0.03 ± 0.02*	0.39 ± 0.02*	0.28 ± 0.02*,#
GM-CSF	n.d.	n.d.	0.24 ± 0.08*	0.06 ± 0.07#
IL-7	0.01 ± 0.00	n.d.	0.01 ± 0.01	0.01 ± 0.00
TNFα	0.01 ± 0.00	n.d.	0.04 ± 0.00	0.03 ± 0.00
GCSF	0.01 ± 0.00	0.05 ± 0.01	n.d.	0.05 ± 0.01
IL-4	0.01 ± 0.00	n.d.	0.02 ± 0.01	0.02 ± 0.00
MIP1b	n.d.	n.d.	0.05 ± 0.01	0.04 ± 0.01
IL-2	n.d.	n.d.	0.04 ± 0.00	0.02 ± 0.00
IL-17	n.d.	n.d.	0.05 ± 0.01	0.03 ± 0.02
IL-10	n.d.	n.d.	n.d.	n.d.
IL-12	n.d.	n.d.	n.d.	n.d.
IL-13	n.d.	n.d.	n.d.	n.d.
IL-5	n.d.	n.d.	n.d.	n.d.
IL-1b	n.d.	n.d.	n.d.	n.d.

Supplementary Table 2: The phLFs were treated with 10ng/ml TNFα for 24 hours in vitro and different cytokines were measured using a multiplex ELISA kit (Bio-Plex Pro™ Human Cytokine 17-plex) (n=4; *,# $p<0.05$; 1-way ANOVA followed by Neuman-Keuls multiple comparison test)

8. Publication II

Senolytic drugs target alveolar epithelial cell function and attenuate experimental lung fibrosis *ex vivo*

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Alveolar epithelial cell senescence occurs in IPF and senolytic treatment attenuates experimental lung fibrosis http://ow.ly/nFlz30bsmNm

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ABSTRACT Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease with poor prognosis and limited therapeutic options. The incidence of IPF increases with age, and ageing-related mechanisms such as cellular senescence have been proposed as pathogenic drivers. The lung alveolar epithelium represents a major site of tissue injury in IPF and senescence of this cell population is probably detrimental to lung repair. However, the potential pathomechanisms of alveolar epithelial cell senescence and the impact of senolytic drugs on senescent lung cells and fibrosis remain unknown. Here we demonstrate that lung epithelial cells exhibit increased *P16* and *P21* expression as well as senescence-associated β-galactosidase activity in experimental and human lung fibrosis tissue and primary cells.

Primary fibrotic mouse alveolar epithelial type (AT)II cells secreted increased amounts of senescence-associated secretory phenotype (SASP) factors *in vitro*, as analysed using quantitative PCR, mass spectrometry and ELISA. Importantly, pharmacological clearance of senescent cells by induction of apoptosis in fibrotic ATII cells or *ex vivo* three-dimensional lung tissue cultures reduced SASP factors and extracellular matrix markers, while increasing alveolar epithelial markers.

These data indicate that alveolar epithelial cell senescence contributes to lung fibrosis development and that senolytic drugs may be a viable therapeutic option for IPF.

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease with a median survival of 2–4 years [1]. Mechanisms involved in disease development and progression include repetitive injury to the lung epithelium, activation and proliferation of (myo)fibroblasts and altered production of extracellular matrix, together resulting in the destruction of lung architecture and function [1, 2]. Two drugs (pirfenidone and nintedanib) have been approved for the treatment of mild/moderate IPF [3, 4]; however, therapies halting or reversing disease progression are still lacking. Thus, there is a tremendous interest in deepening our understanding of the pathomechanism(s) underlying IPF in order to identify novel therapies.

The incidence of IPF increases with age and accumulating evidence strongly suggests ageing as a crucial contributor to IPF initiation and progression [5]. In support of ageing as one proposed driver of disease pathogenesis, normal and accelerated-aged mice are more susceptible to experimentally induced fibrosis [6, 7]. A landmark paper in 2013 described nine hallmarks of ageing [8], and importantly, all nine hallmarks have been found to contribute to IPF pathogenesis, albeit to a variable degree [5]. Cellular senescence, representing one of these hallmarks, is characterised by stable cell cycle arrest accompanied by secretion of mediators, including pro-inflammatory cytokines and metalloproteinases, collectively termed the "senescence-associated secretory phenotype" (SASP) [9, 10]. While the detrimental effects of senescence are thought to be a result of stem or progenitor cell depletion or of the SASP components, senescence has also been described to be beneficial in tumour suppression and wound healing [10–12].

In the lung, as in other organs, the number of senescent cells increases with age [13] and cellular senescence has been linked to the pathogenesis of chronic lung diseases such as chronic obstructive pulmonary disease [14, 15] or IPF [16–20]. The contribution of senescent cells to disease onset and progression remain unclear. Some studies have suggested a link between increased senescence and fibrotic burden [17, 21, 22], while others report that attenuation of lung fibrosis correlates with lung fibroblast senescence [23]. In addition to lung fibroblasts, evidence has emerged that alveolar epithelial cells can become senescent in IPF [16, 20, 24]. However, lung epithelial cell senescence and its potential pathogenic role in IPF remains largely unexplored. Here, we aimed to investigate whether senescence of this cell population is detrimental or beneficial to lung repair. We analysed cell senescence in lung tissue and in primary alveolar epithelial type (AT)II cells derived from human IPF and an experimental model of murine lung fibrosis. We demonstrate that depletion of senescent epithelial cells *in vitro* and *ex vivo* stabilises the epithelial cell phenotype and decreases fibrotic markers, indicating that senescence of alveolar epithelial cells may contribute to disease pathogenesis.

Materials and methods

Senescence-associated β-galactosidase staining

Primary mouse (pm) ATII cells or three-dimensional lung tissue cultures (3D-LTCs) were prepared from PBS- or bleomycin-treated mice, as described previously [25] (online supplementary material) and cultured in multiwell plates. pmATII cells from PBS- and bleomycin-treated mice express high levels of prosurfactant protein (proSP)-C as well as the epithelial cell markers E-cadherin, cytokeratin (CK) and zona occludens (ZO)-1. Fibrotic ATII cells further exhibit co-staining of ZO-1 and proSP-C with α-smooth muscle actin (figure 3a, online supplementary figure S4B and [26, 27]). Cytochemical staining for senescence-associated (SA) β-galactosidase was performed using a staining kit (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's instructions. Images were acquired using a Zeiss Axiovert40C microscope (Jena, Germany). The percentage of senescent cells was determined by counting of total and SA-β-galactosidase-positive cells in three random microscopic fields per condition (100× magnification).

Flow cytometry-based detection of SA-β-galactosidase

Flow cytometry-based detection of SA-β-galactosidase was performed as described previously [28]. Briefly, pmATII cells from PBS- and bleomycin-treated animals were incubated with bafilomycin A1 (100 nM;

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Enzo Life Sciences, Farmingdale, NY, USA) and C_{12}FDG (20 nM; Life Technologies, Carlsbad, CA, USA) for 1 and 2 h, respectively, directly after isolation or at day 2 of culture. Cells were washed once and stained for allophycocyanin-conjugated epithelial cell adhesion molecule (EpCAM) antibody (118214; BioLegend, San Diego, CA, USA) for 20 min at room temperature, washed once and analysed using a fluorescence-activated cell sorter (LSRII; BD Bioscience, San Jose, CA, USA). Additional information can be found in the online supplementary material.

Statistical analysis

Data are presented as mean±SEM, from n separate experiments. Statistical significance of differences was evaluated using t-tests, paired t-tests or one-way ANOVA followed by a Newman–Keuls multiple comparison test, where appropriate. Correlation was evaluated using Pearson's test. Differences were considered to be statistically significant when $p<0.05$. Additional information can be found in the online supplementary material.

Results

Senescence marker expression is upregulated in the lung epithelium in IPF

First, we aimed to investigate the occurrence of senescence in our IPF patient cohort. To this end, we analysed the gene expression of the senescence effector proteins cyclin dependent kinase inhibitor (CDKN) 2A (*P16*) and CDKN1A (*P21*) in explanted lung tissue specimens of IPF or donor patients. *P16* levels were significantly increased in lung homogenates of IPF patients as compared to donor lung homogenates (figure 1a; mean±SD change in threshold cycle (ΔCt) donor −1.91±0.74 *versus* IPF 0.74±0.40, $p<0.01$), whereas *P21* levels remained unchanged. Our cohort matches results extracted from the Lung Genomics Research Consortium microarray data (GSE47460 and GPL4680) (online supplementary figure S1A). Furthermore, we found that *P16* expression levels in IPF tissue negatively correlated with diffusing capacity of the lung for carbon monoxide (online supplementary figure S1B), indicating that patients with higher *P16* levels had more severe disease. Furthermore, we observed increased P16 as well as P21 protein in whole-lung homogenates from IPF patients compared to donor lung tissue, as assessed using Western blotting (figure 1b).

To identify which cell types express phenotypic markers of senescence in IPF, we next performed immunohistochemical staining of P16 and P21 on IPF and donor lungs and found that IPF lungs exhibited intense nuclear and cytoplasmic staining for both P16 and P21 compared to age-matched donor lungs (figure 1c and d). Co-staining with epithelial cell marker proSP-C, KRT5 or KRT7 revealed that P16- and P21-positive cells were found in the alveolar epithelium of IPF lungs, largely in proSP-C^+ $KRT7^+$ ATII cells (figure 1c; arrows), while no or only sporadic staining for P16 and P21 was observed in donor lungs (figure 1d; arrows). Furthermore, $KRT5^+$ $KRT7^+$ abnormal basal cells in areas of bronchiolisation exhibited positive staining for P16 and P21 (online supplementary figure S2A–C), while no or only weak staining was observed in mesenchymal cells (online supplementary figure S2A–D). In addition, P16 and P21 staining was observed in proSP-C^- $KRT5^-$ $KRT7^+$ epithelial cells of IPF lungs (figure 1c; arrowheads). Notably, P21 (and P16) staining was also found in proSP-C^+ $KRT5^+$ double-positive cells (figure 1c; dashed arrow; and figure S2B). We further detected increased amounts of γH2A.X (phospho S139), a marker for DNA double strand breaks and activated DNA damage response, which has been implicated in cellular senescence, in epithelial cells of IPF patients co-localising with P16 staining (online supplementary figure S3). In addition, an increase in *P16*, but not *P21* expression was detectable on the mRNA level in primary human ATII cells isolated from IPF patients compared to non-IPF donors (figure 1e). This discrepancy between changes in the P21 protein and gene expression level might be due to differential post-transcriptional control of P21 protein expression [29, 30]. Collectively, these data suggest that senescence occurs in the lung epithelium in IPF.

Senescence markers are upregulated in experimental lung fibrosis

Next, we analysed cellular senescence in mice subjected to bleomycin (Bleo)-induced lung fibrosis (2 $U \cdot kg^{-1}$ body weight, sacrificed at day 7, 14 or 21 after instillation). Both *P16* and *P21* were significantly upregulated on the gene expression level in fibrotic mouse lungs (figure 2a; mean±SD ΔCt *P16*: day 14 PBS −5.66±0.34 *versus* Bleo −4.33±0.21; *P21*: day 14 PBS −0.64±0.22 *versus* Bleo 1.42±0.22; $p<0.001$). While *P16* was upregulated as early as day 14 post-Bleo instillation, *P21* was upregulated earlier at day 7 and decreased back to baseline by day 21. The different kinetics of *P16* and *P21* expression were also observed in a previously published microarray dataset (online supplementary figure S4A) [34], and probably represent the different kinetics of *P16*/*P21* induction upon DNA damage [35, 36]. P21 protein expression was significantly increased at day 7 as analysed using Western blotting (figure 2b). Unfortunately, due to the lack of reliable and specific mouse P16 antibodies, we could not analyse P16 on protein level [11]. Next, we assessed SA-β-galactosidase activity, a widely used surrogate marker for the detection of

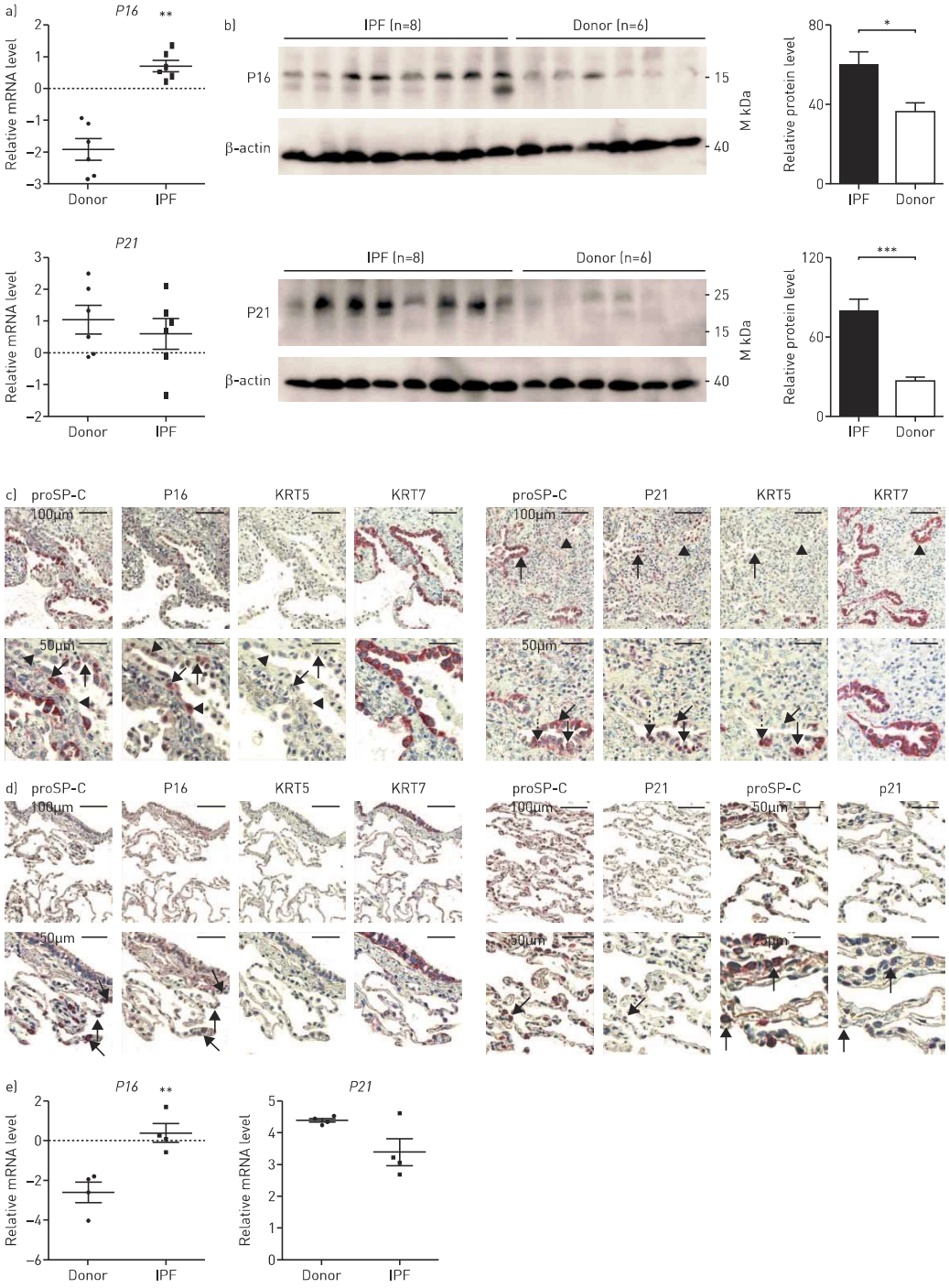

FIGURE 1 Senescence marker expression is upregulated in idiopathic pulmonary fibrosis (IPF) patients. a) Gene expression of *P16* and *P21* in lung homogenates of IPF and donor tissue was measured by quantitative (q)PCR and normalised to *HPRT*. Data are presented as mean±SEM. n=6. Means were compared using unpaired t-tests. b) Representative and quantitative immunoblot analyses of subpleural lung tissue from patients with sporadic IPF (n=16) and human donor lungs (n=11) using specific antibodies against P16 and P21, and β-actin as loading control. Densitometric ratios of the respective protein to β-actin are given as mean±SEM. Means were compared using unpaired t-tests. Immunohistochemical staining of serial sections of c) IPF or d) donor lung tissue for prosurfactant protein-C (proSP-C; marker for alveolar epithelial type (AT)II cells), cytokeratin 5 (KRT5, marker for bronchiolar basal cells), cytokeratin 7 (KRT7, marker for simple epithelial cells) and P16 and P21 protein. ProSP-C$^+$ KRT7$^+$ ATII cells expressing P16 or P21 are indicated by arrows; proSP-C$^-$ KRT5$^-$ KRT7$^+$ epithelial cells expressing P16 or P21 are indicated by arrowheads; proSP-C$^+$ KRT5$^+$ KRT7$^+$ epithelial cells expressing P21 are indicated by dashed arrows. e) Gene expression of *P16* and *P21* in primary human ATII cells isolated from IPF and donor tissue was measured using qPCR and normalised to *HPRT*. Data are presented as mean±SEM. n=4. Means were compared using unpaired t-tests. *: p<0.05; **: p<0.01; ***: p<0.001.

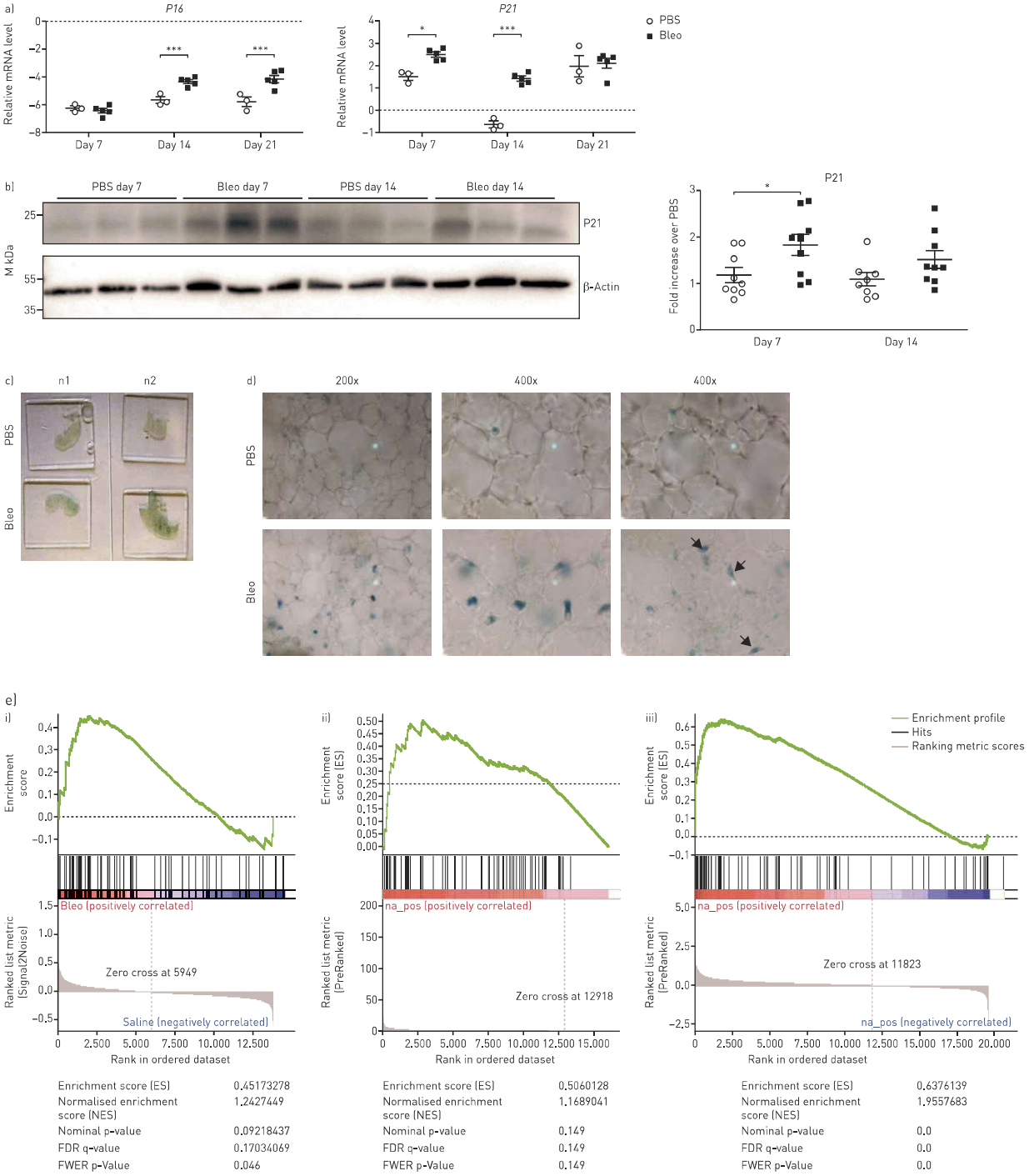

FIGURE 2 Senescence markers are upregulated in experimental lung fibrosis. Mice were instilled with PBS or bleomycin (Bleo) and sacrificed at the timepoints indicated. a) Gene expression of *P16* and *P21* in lung homogenates of mice sacrificed at day 7, 14 or 21 was measured using quantitative PCR and normalised to *Hprt*. Change in cycle threshold (ΔCt) is presented as mean±SEM; n=3 for PBS and n=5 for Bleo. Statistical significance was tested using one-way ANOVA followed by Newman–Keuls' multiple comparison test. b) Immunoblot of P21 protein in mouse whole-lung homogenates of mice treated with PBS or Bleo and sacrificed after 7 or 14 days. β-Actin was used as a loading control. Respective sizes of marker are indicated. Data were quantified and normalised to loading control. Data are presented as mean±SEM; n=9. Statistical significance was tested using one-way ANOVA followed by Newman-Keuls' multiple comparison test. c, d) Three-dimensional lung tissue cultures (3D-LTCs) were obtained from mice instilled with PBS or Bleo and sacrificed at day 14. 3D-LTCs were stained for senescence-associated β-galactosidase activity and c) macroscopic images and d) microscopic (magnification of 200× or 400×) images were taken. Epithelial cells are marked by arrows. e) Enrichment of senescence-associated genes [31] in microarray data of i) whole lung [32] (GSE16846), ii) mouse fibroblasts [33] (GSE42564) or iii) primary mouse (pm) alveolar epithelial type (AT)II cells [26] of mice with experimental lung fibrosis induced by Bleo. FDR: false discovery rate; FWER: family-wise error rate. *: p<0.05; ***: p<0.001.

senescent cells [28], in three-dimensional lung tissue cultures (3D-LTCs) from fibrotic mouse lungs (day 14 post-Bleo). Importantly, increased blue staining, indicating a higher number of senescent cells, was observed in fibrotic lungs as compared to healthy lungs (figure 2c). The SA-β-galactosidase activity was predominantly observed in structural cells that morphologically resembled lung epithelial cells (figure 2d; arrows). To further explore whether senescence is increased and in which cell types senescence occurs in experimental lung fibrosis, we utilised gene set enrichment analysis [37] of previously published microarray data sets obtained from whole murine lungs [32], primary murine fibroblasts (pmFb) [33] or pmATII cells [26] of Bleo- *versus* PBS-treated mice and compared those to a previously published gene signature list for senescence [31] (figure 2e). We found a significant enrichment of senescence-associated genes in fibrotic pmATII cells, but not in whole lung or pmFb from Bleo-treated mouse lungs (figure 2e). These data demonstrate that senescence-associated genes are enriched in experimental lung fibrosis and indicate that the lung epithelium is a potential source of senescent cells in the fibrotic lung.

Increased senescence of ATII cells in experimental lung fibrosis

To further analyse whether ATII cells are a major senescent cell type in lung fibrosis, we isolated pmATII cells from Bleo (fibrotic) and PBS (healthy) mouse lungs (figure 3a and online supplementary figure S4B). Notably, freshly isolated fibrotic EpCAM$^+$ pmATII cells exhibited increased staining for SA-β-galactosidase activity over control EpCAM$^+$ pmATII cells, as analysed using flow cytometry (figure 3b). After 48 h of culture, fibrotic ATII cells maintained an increase of 2.45±0.45-fold in SA-β-galactosidase staining over healthy cells (figure 3c). This was further confirmed by conventional light microscopy following *in vitro* culture and SA-β-galactosidase staining (figure 3d). Accordingly, freshly isolated fibrotic pmATII cells showed increased *P16* and *P21* transcript levels as well as senescence-associated heterochromatic foci marked by foci of histone H3 lysine 9 trimethylation (H3K9me3) (figure 3e and online supplementary figure S4C). Additionally, upregulation of *secreted phosphoprotein* (*Spp*) *1* and *matrix metalloproteinase* (*Mmp*) *2*, two well-known components of the SASP, was observed (figure 3e). In order to examine whether *P16* and *P21* activation translated to increased SASP activity, we performed a proteomic analysis of the secretome of fibrotic and normal pmATII cells. We identified several SASP components in our screen and found that 52% of those identified were upregulated >1.5-fold in the fibrotic secretome, whereas only 10% of detected SASP components were downregulated (figure 3f and g). Among the most upregulated SASP components were insulin growth factor binding proteins (Igfbp) 3, 4 and 7 and MMP 3, 12 and 14 (figure 3h). Together, these data strongly suggest increased senescence of fibrotic ATII cells along with increased secretion of SASP factors.

Depletion of senescent cells by senolytic drugs decreases fibrotic markers and increases epithelial cell marker expression

Whether senescence contributes to or limits pulmonary fibrosis is still an area of active discussion. To address this point, we used a recently described combination of senolytic drugs, dasatinib (D) and quercetin (Q) (a tyrosine kinase inhibitor and flavonol combination) to deplete senescent cells from culture [39–41]. Fibrotic pmATII cells exhibited stable expression of profibrotic markers during culture (online supplementary figure S5A). Treatment with senolytic compounds reduced total cell numbers and the percentage of senescent cells (figure 4a and b) and *P16* expression level dropped significantly (figure 4c). Importantly, an increase in apoptotic cleaved caspase 3 and annexin V staining was observed in ATII cells upon senolytic treatment (figure 4d and e). Apoptosis was predominantly induced in senescent cells (figure 4f), consistent with a depletion of senescent cells induced by senolytic drugs [41].

We next analysed whether senolytic treatment affected SASP factors in pmATII cells. Notably, treatment with DQ led to a reduction of SASP factors such as *Mmp12*, *Serpine1* and *Spp1* (figure 4g). Senescent cell depletion further correlated with reduced extracellular matrix components *Collagen1a1*, *Collagen5a3* and *Fibronectin* (figure 5a and online supplementary figure S5C), which have been suggested to be part of the SASP [22, 35]. Notably, we found significantly increased mRNA expression of the epithelial cell marker *E-cadherin* (*Cdh1*) as well as functional ATII cell markers, such as *Sftpc* and *Sftpa*, while the ATI cell marker *T1α* was unaffected (figure 5b and online supplementary figure S5D). In addition, protein secretion of SP-C was increased upon senolytic treatment (figure 5e) along with increased E-cadherin protein level (figure 5f). We further analysed interleukin (IL)-6 protein secretion (a major component of the SASP), and found significantly decreased amounts upon senolytic treatment (figure 5c; DMSO 3.95±0.81-fold over control *versus* DQ 1.34±0.65-fold over control). Moreover, transcript level as well as secretion of the ATII cell-derived fibrotic mediator *Wnt-inducible signalling protein* (*Wisp) 1* [26] were significantly reduced upon senolytic treatment (online supplementary figure S5C and figure 5d; DMSO 2.10±0.91-fold over control *versus* DQ 0.71±0.26-fold over control). Together, these data suggest effective depletion of senescent cells and modulation of their associated SASP. Notably, when treating ATII cells isolated from PBS-treated lungs, which show reduced senescence as compared to fibrotic ATII cells, with

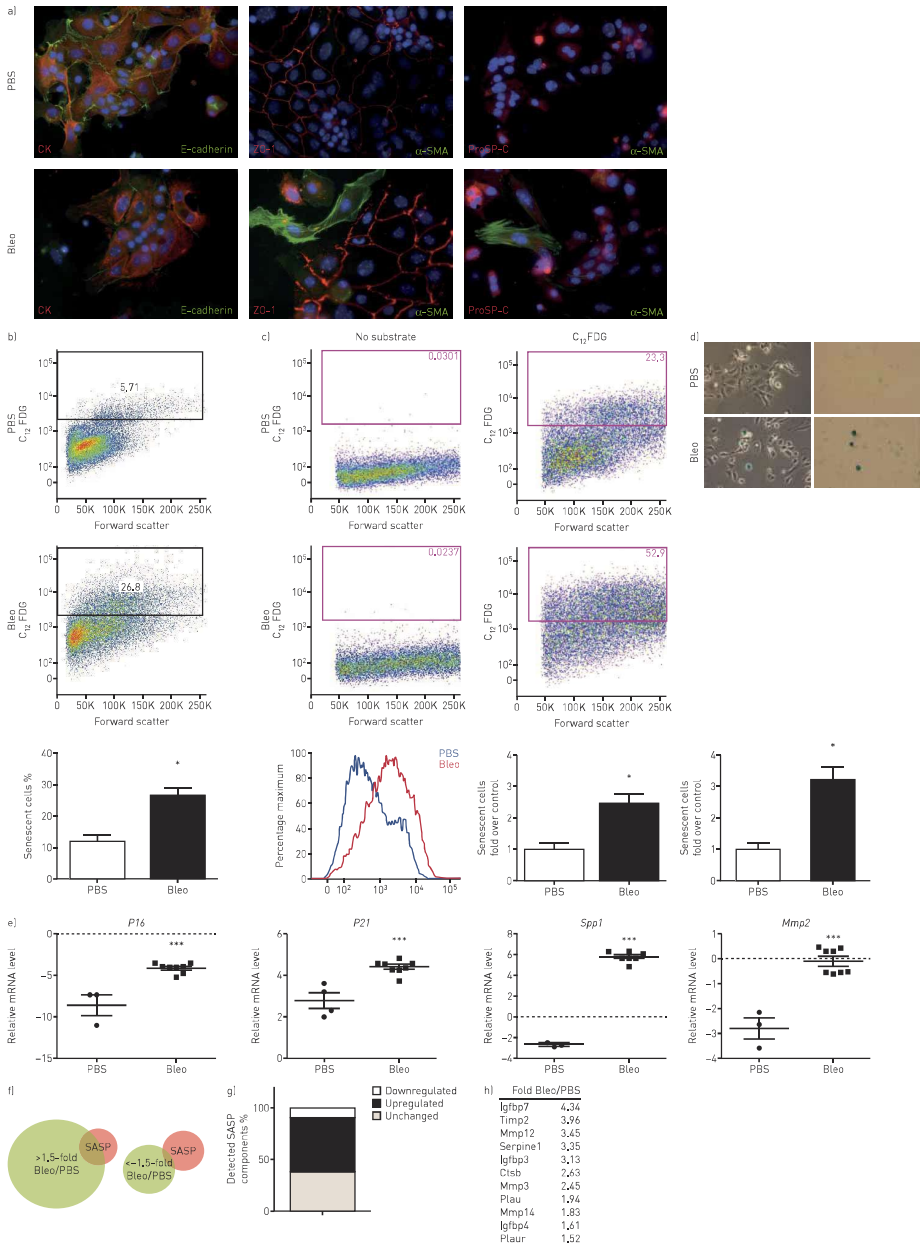

FIGURE 3 Senescence markers are upregulated in alveolar epithelial type (AT)II cells in experimental lung fibrosis. Mice were instilled with either PBS or bleomycin (Bleo). At day 14 after instillation, mice were sacrificed and primary mouse (pm)ATII cells were isolated. a) Immunofluorescence staining of fibrotic or nonfibrotic pmATII cells on cover slips for epithelial cell marker expression at day 2 after isolation. Fluorescent images represent a 400× magnification. b) pmATII cells were analysed for epithelial cell adhesion molecule (EpCAM) positivity and senescence-associated (SA)-β-galactosidase activity by fluorescence-activated cell sorting (FACS) directly after isolation. Representative dot blots of the EpCAM$^+$ population are shown for PBS and Bleo, as well as quantifications of percentages of senescent cells of the EpCAM$^+$ population. Means were compared to time-matched PBS controls using unpaired t-tests; n=3. c) pmATII cells (day 2) were analysed for SA-β-galactosidase activity using FACS. Representative dot blots are shown for PBS and Bleo pmATII cells incubated with C_{12}FDG or respective controls, a representative histogram comparing PBS and Bleo pmATII cells incubated with C_{12}FDG as well as quantifications of percentages of senescent cells normalised to respective PBS control. Means were compared to time-matched PBS controls using unpaired t-tests; n=3. d) pmATII cells (day 2) were stained for SA-β-galactosidase activity and blue cells and total cells were counted. Representative images and quantitative data normalised to respective PBS controls are shown. Data represent mean±SEM. Means were compared to time-matched PBS controls using unpaired t-tests; n=3. e) Gene expression of senescence-associated genes in freshly isolated pmATII cells from PBS- or Bleo-treated mice was measured using quantitative PCR. Data were normalised to *Hprt*. Change in threshold cycle (ΔCt) is presented as mean±SEM; n=3–4 for PBS and n=8 for Bleo. Means were compared to time-matched PBS controls using unpaired t-tests. f–h) pmATII cells isolated from PBS- or Bleo-treated mice were plated onto plastic tissue culture plates. After 48 h of culture, the supernatant was collected and analysed using mass spectrometry proteomics. The senescence-associated secretory phenotype (SASP) list [9] was compared to the list of secreted proteins (1.5-fold upregulated or downregulated). f) A Venn diagram (BioVenn; [38]) showing the overlap of SASP proteins with the up-/downregulated proteins in the pmATII supernatant. g) Percentage of detected SASP factors that are upregulated (>1.5-fold) or downregulated (<-1.5-fold) or not changed. h) List of upregulated SASP components >1.5-fold Bleo/PBS. CK: cytokeratin; ZO: zona occludens; SMA: smooth muscle actin. *: p<0.05; ***: p<0.001.

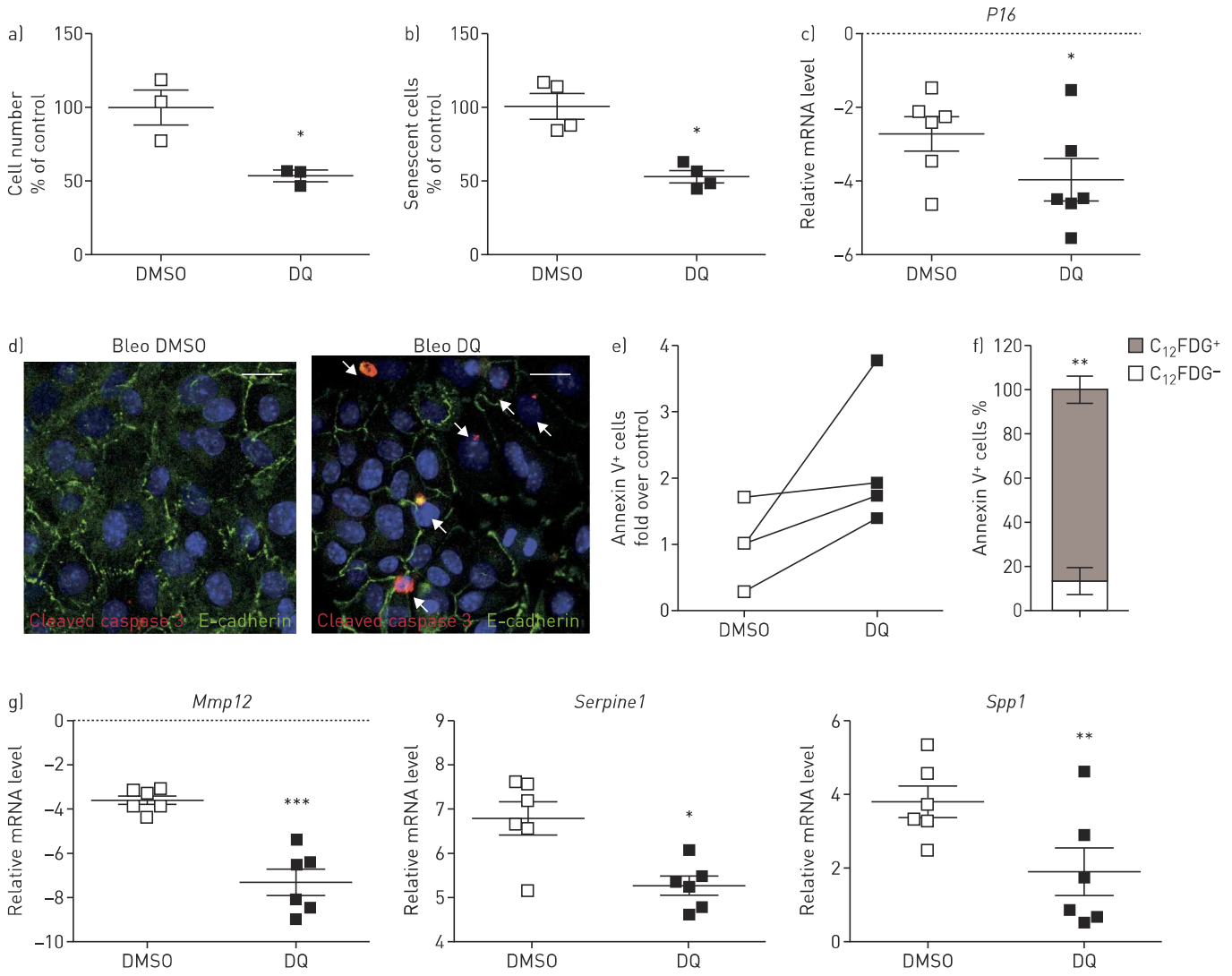

FIGURE 4 Treatment of fibrotic primary mouse (pm) alveolar epithelial type (AT)II cells with senolytic drugs decreases senescent markers and increases apoptosis. Mice were instilled with either PBS or bleomycin (Bleo). At day 14 after instillation mice were sacrificed and pmATII cells were isolated. Fibrotic pmATII cells were cultured for 48 h in the presence of the senolytic drugs dasatinib (D; 200 nM) and quercetin (Q; 50 µM). a) The senolytic activity was assessed by cell numbers. Data are presented as normalised to dimethylsulfoxide (DMSO) control and as mean±SEM. Significance was assessed using paired t-tests; n=3. b) Senescence-associated (SA)-β-galactosidase activity. pmATII cells were stained for SA-β-galactosidase activity and blue cells and total cells were counted. Quantitative data are normalised to respective DMSO control. Data are presented as mean±SEM. Means were compared to time-matched controls using paired t-tests; n=4. c) Gene expression analysis for the senescence marker *P16*. Data were normalised to *Hprt* level. Data are presented as normalised to DMSO control and as mean±SEM. Significance was assessed with paired t-tests; n=6. d) Representative images of immunofluorescence staining for apoptotic marker cleaved caspase 3 and E-cadherin in fibrotic pmATII cells exposed to DMSO or DQ. Fluorescent images represent a 630× magnification. Scale bars=20 µm. e) Fibrotic ATII cells were exposed to DMSO or DQ and stained for annexin V level and analysed using fluorescence-activated cell sorting (FACS); n=4. f) Fibrotic ATII cells were exposed to DQ and stained for senescence (C_{12}FDG), co-stained for annexin V level and analysed using FACS. Data are presented as mean±SEM percentage of total apoptotic cells in the senescent ($C_{12}FDG^+$) and nonsenescent ($C_{12}FDG^-$) population. Significance was assessed using unpaired t-tests; n=3. g) Expression of senescence-associated secretory phenotype (SASP) markers in pmATII cells treated with senolytic drugs was analysed using quantitative PCR. Data were normalised to *Hprt* level. Change in threshold cycle (ΔCt) is presented as mean ±SEM. Significance was assessed using paired t-tests; n=6. Spp: secreted phosphoprotein; Mmp: matrix metalloproteinase. *: p<0.05; **: p<0.01; ***: p<0.001.

senolytic drugs, we only observed a slight reduction in cell number, *P16* expression and SASP components accompanied by an increase in epithelial cell markers as compared to control (online supplementary figure S6).

Finally, we aimed to elucidate whether depletion of senescent cells further modulates fibrotic burden in an *ex vivo* model using native lung tissue slice cultures. 3D-LTCs derived from Bleo-treated mouse lungs exhibit increased expression of fibrotic marker (online supplementary figure S7A), as well as senescence-associated *P16* and *P21* expression (figure 6a). Treatment of fibrotic 3D-LTCs with senolytic

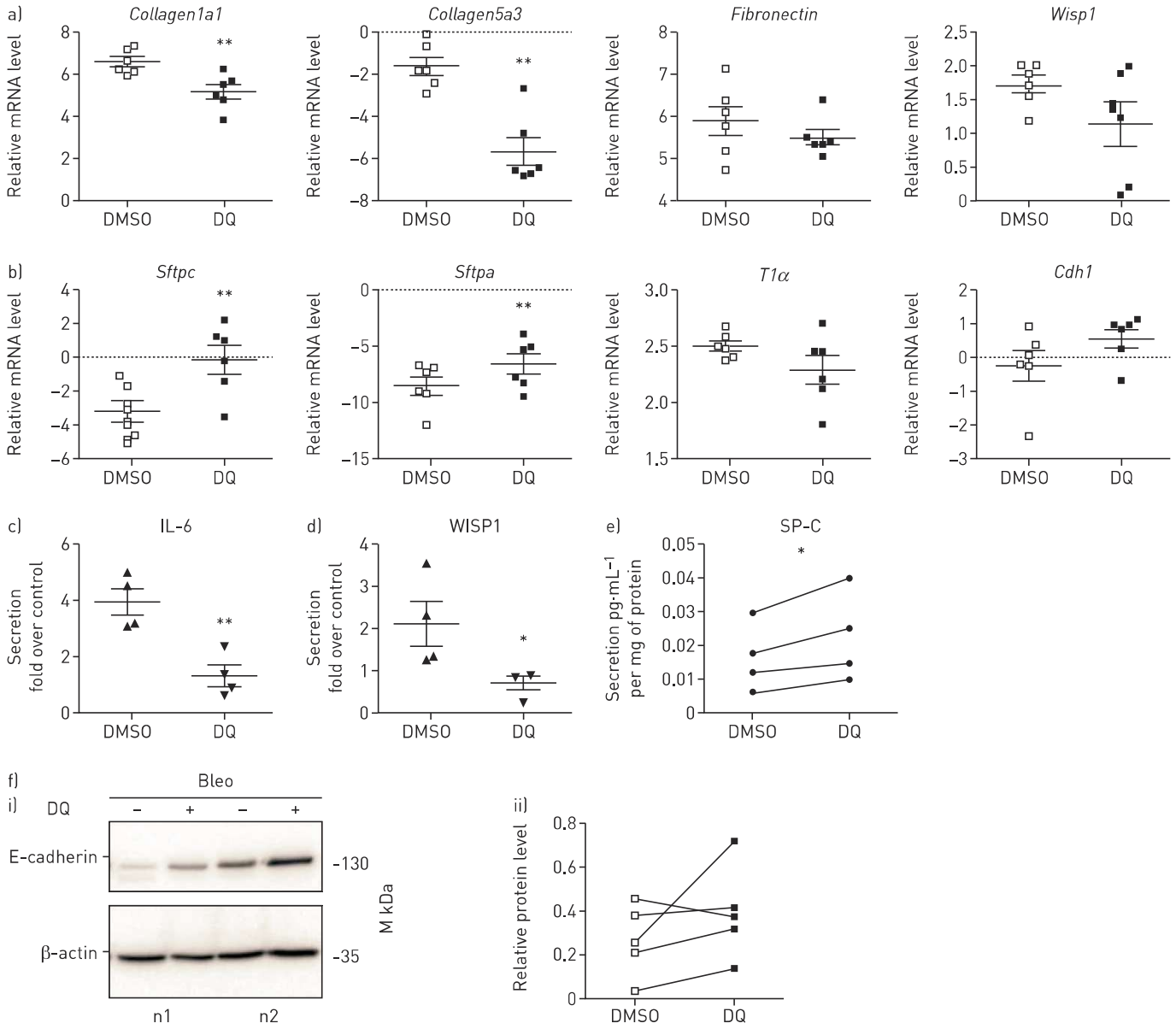

FIGURE 5 Treatment of fibrotic primary mouse (pm) alveolar epithelial type (AT)II cells with senolytic drugs decreases fibrotic and increases epithelial cell markers. Mice were instilled with either PBS or bleomycin (Bleo). At day 14 after instillation, mice were sacrificed and pmATII cells were isolated. Fibrotic pmATII cells were cultured for 48 h in the presence of senolytic drugs dasatinib (D; 200 nM) and quercetin (Q; 50 μM). a) Expression of fibrotic markers was analysed using quantitative (q)PCR. Data were normalised to *Hprt* level. Change in threshold cycle (ΔCt) is presented as mean±SEM. Significance was assessed using paired t-tests; n=6. b) Expression of epithelial markers was analysed by qPCR. Data were normalised to *Hprt* level. ΔCt is presented as mean±SEM. Significance was assessed using paired t-tests; n=6. c) Secretion of interleukin (IL)-6 was analysed using ELISA. Data are presented as normalised to PBS control treated with dimethylsulfoxide (DMSO) (mean±SEM). Significance was assessed using paired t-tests; n=4. d) Secretion of Wnt-inducible signalling protein (WISP)1 in pmATII cells treated with senolytic drugs was analysed using ELISA. Data are presented as normalised to PBS control treated with DMSO (mean±SEM). Significance was assessed using t-tests; n=4. e) Secretion of surfactant protein-C (SP-C) was analysed using ELISA. Data are presented as normalised to total cell protein amount. Significance was assessed using paired t-tests; n=4. f) i) E-cadherin expression was assessed using Western blotting. β-actin was used as a loading control; ii) quantification of E-cadherin Western blot; n=5. Data were normalised to β-actin. *: p<0.05; **: p<0.01; ***: p<0.001.

drugs reduced SA-β-galactosidase staining and *P16* expression (figure 6b and c) and increased cleaved caspase 3 staining (figure 6d). In addition, senolytic treatment reduced expression of the SASP components *Mmp12*, *Serpine1* and *Spp1* (figure 6e). Importantly, DQ treatment further reduced fibrotic markers, such as Collagen1a1 and Wisp1 transcript and protein levels (figure 7a, c and e), whereas Sftpc transcript and protein expression increased compared to time-matched control (figure 7b and d). Notably, we observed similar trends when treating healthy 3D-LTCs with senolytic drugs, albeit to a lower extent than in the fibrotic 3D-LTCs (online supplementary figure S8). In summary, senescent cell depletion by

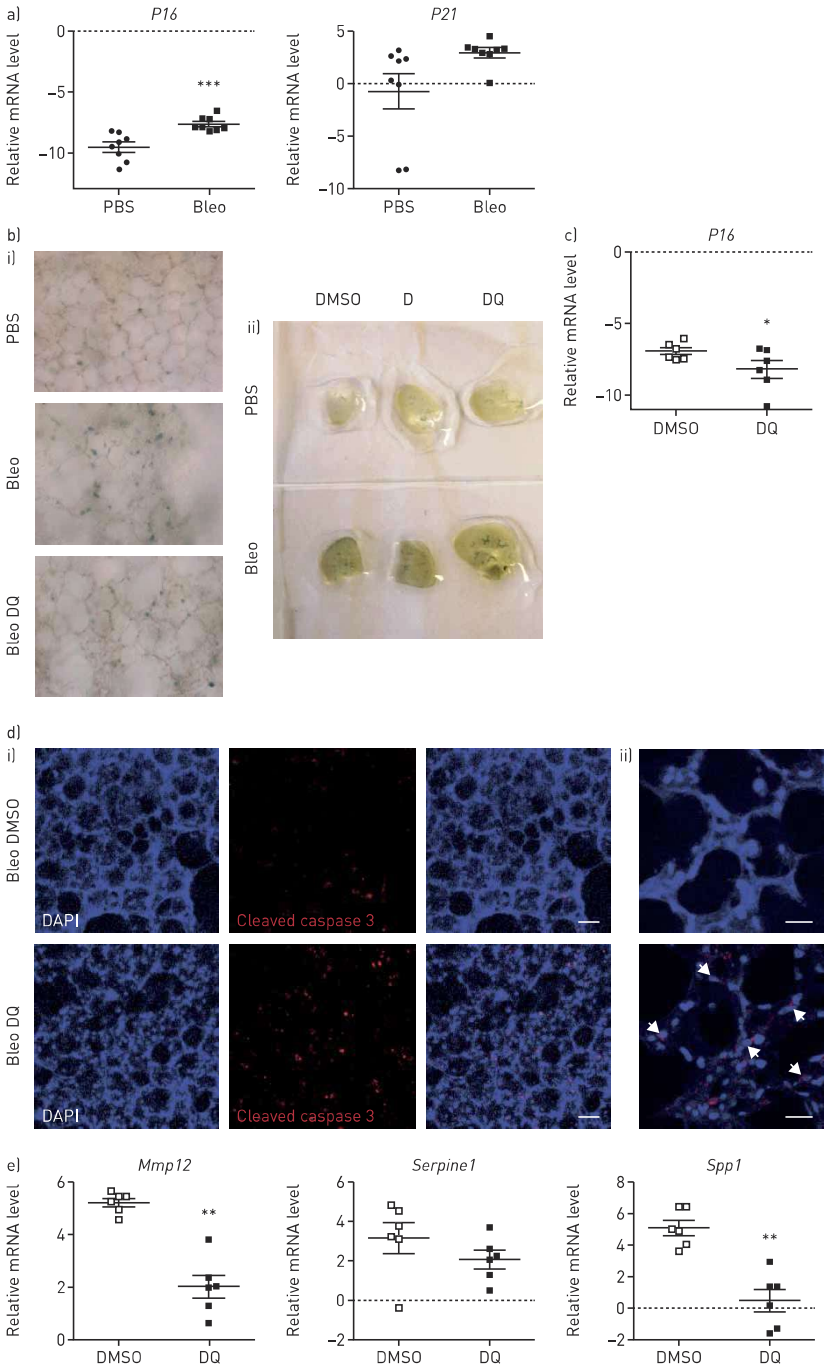

FIGURE 6 Treatment of fibrotic three-dimensional lung tissue cultures (3D-LTCs) with senolytic drugs decreases senescence markers and increases apoptosis markers. Mice were instilled with either PBS or bleomycin (Bleo). At day 14 after instillation mice were sacrificed and 3D-LTCs were generated. a) Gene expression of senescence markers in 3D-LTCs after 48 h of culture was analysed using quantitative (q)PCR. Data were normalised to *Hprt*. Change in threshold cycle (ΔCt) is presented as mean±SEM. Significance was assessed using unpaired t-tests; n=8. b–e) Fibrotic 3D-LTCs were cultured for 48 h in the presence of senolytic drugs dasatinib (D; 200 nM) and quercetin (Q; 50 µM). The senolytic activity was assessed using b) senescence-associated β-galactosidase staining (i) 200×), as well as by c) gene expression analysis for the senescence marker *P16*. Data were normalised to *Hprt*. ΔCt is presented as mean±SEM. Significance was assessed using paired t-tests; n=6. d) Representative images of immunofluorescence staining for the apoptotic marker cleaved caspase 3. i) Scale bars=50 µm; ii) scale bars=20 µm. Epithelial cells are marked by arrows. e) Gene expression of SASP markers in 3D-LTCs treated with senolytic drugs was analysed using qPCR. Data were normalised to *Hprt*. ΔCt is presented as mean±SEM. Significance was assessed using paired t-tests; n=6. *: p<0.05; **: p< 0.01; ***: p<0.001.

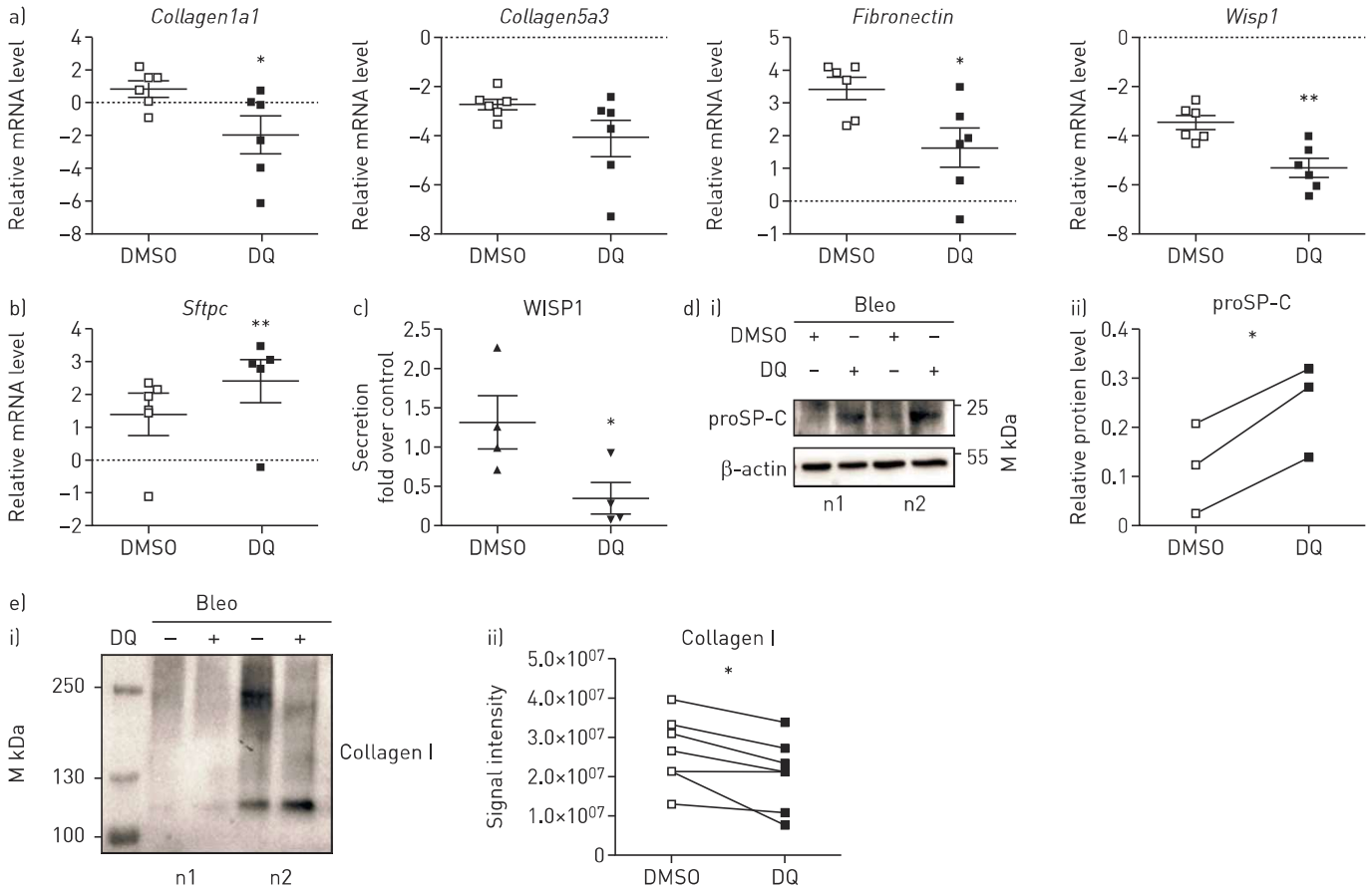

FIGURE 7 Treatment of fibrotic three-dimensional lung tissue cultures (3D-LTCs) with senolytic drugs decreases fibrotic and increases epithelial cell markers. Mice were instilled with either PBS or bleomycin (Bleo). At day 14 after instillation mice were sacrificed and 3D-LTCs were generated. Fibrotic 3D-LTCs were cultured for 48 h in the presence of senolytic drugs dasatinib (D; 200 nM) and quercetin (Q; 50 µM). a) Gene expression of fibrotic markers in 3D-LTCs treated with senolytic drugs was analysed using quantitative (q)PCR. Data were normalised to *Hprt*. Change in threshold cycle (ΔCt) is presented as mean±SEM. n=6. b) Gene expression of *Sftpc* in 3D-LTCs treated with senolytic drugs was analysed using qPCR. Data were normalised to *Hprt*. ΔCt is presented as mean±SEM. n=5. c) Secretion of Wnt-inducible signalling protein (WISP)1 from 3D-LTCs treated with senolytic drugs was analysed using ELISA. Data are presented as normalised to PBS dimethylsulfoxide (DMSO) control (mean±SEM). n=4. d) i) Prosurfactant protein-C (proSP-C) expression was assessed using Western blotting in fibrotic 3D-LTCs. β-actin was used as a loading control; ii) quantification of proSP-C protein relative to β-actin. n=3. e) i) Secreted collagen I was assessed using Western blotting in fibrotic 3D-LTCs; ii) quantification of secreted collagen I normalised to supernatant volume. n=7. Significance was assessed using paired t-tests. *: p<0.05; **: p<0.01; ***: p<0.001.

senolytic drugs reduced fibrotic burden and increased ATII cell markers in primary ATII cells as well as in *ex vivo* 3D-LTCs.

Discussion

IPF is a disease of the elderly, and several hallmarks of ageing such as cellular senescence have been linked to this disease [5]. Recently, Baker *et al.* [42] were able to demonstrate that depletion of naturally occurring senescent cells extends healthy lifespan and decreases age-induced pathologies in mice. Nevertheless, there is evidence that senescence might also limit diseases, such as cancer or fibrotic disorders of the skin or heart [10–12]. Recent reports in lung fibrosis are conflicting, since both a detrimental role [17, 21, 22, 43] as well as an antifibrotic role [23] have been reported. In this study, we utilised senolytic drugs on fibrotic lung epithelial cells *in vitro* and *ex vivo* in 3D-LTCs and demonstrated that senolytic treatment attenuates fibrotic mediator expression, while stabilising epithelial cell marker expression and function. These findings suggest that senescence contributes to development of lung fibrosis and that treatment of pulmonary fibrosis with senolytic drugs might be beneficial.

Increased senescence has been described for IPF as well as in mouse models of pulmonary fibrosis in both epithelial cells and (myo)fibroblasts [16–18, 21, 23, 44–46]. Here, we confirm that lung epithelial cells from experimental and human IPF exhibit increased cellular senescence. Interestingly, we observed cellular senescence in different subpopulations of human lung epithelial cells, including a population of proSP-C$^+$ KRT5$^+$ double-positive cells. These double-positive cells have been described in the mouse as derived from

a rare undifferentiated epithelial cell population, which is activated upon influenza infection or bleomycin challenge [47]; however, the origin of these cells as well as the contribution of single cell subpopulations in the human lung to disease pathogenesis requires further investigation.

Senescent cells secrete several mediators in the SASP that have been shown to directly influence their surrounding microenvironment. Importantly, we identified fibrotic ATII cells as a potent source of profibrotic SASP components. In line with this, increased expression of SASP components has been found in bleomycin-induced lung fibrosis *in vivo* [22, 44]. Different components of the SASP such as IL-6, MMP-12 [48], IL-1β [49] or keratinocyte growth factor [50] have been described to induce alveolar epithelial cell reprogramming, a prominent feature of IPF pathogenesis [2, 51]. Notably, several different epithelial cell phenotypes can be observed in the fibrotic lung, including cellular senescence [51]. The distinct phenotypes are most likely determined by 1) cell intrinsic properties that differ in specific (and to be characterised) subpopulations and 2) extrinsic factors, such as the direct microenvironment. Components of the SASP have been described to influence cell proliferation [13]. Thus, it may be that the SASP of senescent fibrotic cells contributes to the hyperproliferative phenotype of some epithelial cells or to neighbouring fibroblasts. By depleting senescent epithelial cells, we were able to reduce their associated SASP, which had potent antifibrotic effects and partly restored the normal epithelial cell phenotype. While our results strongly support the hypothesis that senescent epithelial cells and their SASP contribute to fibrosis pathogenesis, it will be important to further elucidate specific SASP compositions of different cell types which might account for distinct outcomes in fibrotic diseases in further studies.

Different mechanisms can lead to the induction of senescence [10]. DNA damage, as well as telomere shortening, can trigger senescence. Notably, telomere attrition is a driving force in IPF and mutations in telomerase genes have been found in familial and sporadic cases of IPF [52, 53]. Naikawadi *et al.* [24] reported that telomere dysfunction in ATII cells, but not mesenchymal cells, led to increased cellular senescence and lung fibrosis. The same group previously found that alveolar epithelial cell senescence is regulated by microRNA-34a [16], which has also been reported to regulate senescence of lung fibroblasts [23]. In addition to DNA damage- or telomere shortening-induced senescence, overexpression of the canonical WNT mediator β-catenin can result in oncogene-induced senescence and WNT signalling has further been demonstrated in senescence occurring during embryonic development [54, 55]. Several WNT ligands, such as WNT3A, have been shown to induce senescence upon prolonged cellular exposure [56]. In line with this, increased WNT/β-catenin activity has been reported in human and experimental lung fibrosis [26, 56–59]. Here we found that the expression of the WNT target WISP1 was reduced by senolytic treatment of fibrotic ATII cells, thus suggesting that WISP1 might contribute to the profibrotic SASP.

Depletion of senescent cells presents a potential therapeutic option for the treatment of several chronic diseases, including those of the ageing lung. Pharmacological targeting of senescent cells has been recently developed [39, 41]. Both pharmacological approaches target antiapoptotic pathways that senescent cells are highly reliant on. Inhibiting these antiapoptotic pathways induces apoptosis in the senescent cells, however, while the drugs target predominantly senescent cells, other mechanisms cannot be excluded [39, 41, 60]. Here, we provide evidence that senolytic treatment induces apoptosis in senescent alveolar epithelial cells, which subsequently led to an attenuation of profibrotic marker expression and increased epithelial cell function. Concerns that antisenescent therapies might increase the risk of cancer have been addressed by a study showing that depletion of senescent cells actually reduced tumour burden in naturally aged animals, rather than increasing it [42].

Why senescent cells exhibit antifibrotic properties in some fibrotic disorders, while appearing to be detrimental in pulmonary fibrosis is an intriguing question. One explanation might be that distinct cell types are affected by senescence [10, 20, 35]. In the case of liver, heart and skin fibrosis, where senescence is thought to be beneficial, myofibroblasts are the major senescent cell type [10–12, 20, 61]. However, in IPF we and others demonstrate that epithelial cells represent a major cell type that is affected by senescence [16, 18] and while we did not find fibroblasts to be affected to a large extent, other recent publications report senescence in fibroblasts in IPF as well [17, 22]. Interestingly, senescence induced by microRNA-34a in epithelial cells seems to promote fibrosis in aged animals, while it reduces fibrotic burden when lung fibroblast senescence is induced [23, 43], indicating that epithelial cell senescence is indeed detrimental, whereas fibroblast senescence is protective. A recent study employed a combination of senolytic drugs in experimental lung fibrosis *in vivo* and report a reduction of senescent cells as well as fibrosis development [22], similar to our findings. However, their work focused primarily on examining the contribution of senescent fibroblasts using *in vitro* approaches and they did not examine epithelial cell behaviour in their model. We demonstrated that both senolytic drugs reduce the senescent cell burden and attenuated fibrotic marker using an *ex vivo* model of lung fibrosis in 3D-LTCs. This technique allows the analysis of tissue-level responses to senolytic drugs in living tissue *ex vivo*. Moreover, murine 3D-LTCs can

be applied to extend mechanistic studies, while reducing overall animal experimentation. Notably, we were able to confirm our *in vitro* findings with respect to epithelial cell marker expression as well as fibrosis markers and provide evidence that epithelial cells are also targeted in 3D-LTCs. However, it is most likely that also other cell types, such as fibroblasts [22] are affected by senolytic treatment in this system. Future studies using *in vivo* models targeting specific senescent cell populations are needed to further delineate senescent cell-specific contribution to the development of pulmonary fibrosis. In addition, it will be important to further confirm the role of senescent cell types in human lung tissue. To this end, we have recently developed a model that induces early fibrotic-like changes in human 3D-LTCs from non-IPF patients [62], which may help us define the potential for as well as the limitations of antisenescent therapy in the context of lung fibrosis.

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Author contributions were as follows. M. Lehmann, K. Mutze, D.E. Wagner and M. Königshoff conceived and designed the research; M. Lehmann, K. Mutze, M. Korfei, S. Klee, D.E. Wagner, W. Skronska-Wasek, H.N. Alsafadi, C. Ota, R. Costa and H.B. Schiller planned and performed experiments and analysed the data; M. Korfei performed immunohistochemistry; H.B. Schiller performed mass spectrometry; A. Guenther and M. Lindner contributed tissue specimens and clinical expertise; M. Lehmann and M. Königshoff wrote the manuscript; K. Mutze, M. Korfei, D.E. Wagner and C. Ota edited the manuscript; and all authors approved the final version of the manuscript.

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Supplementary information

Supplemental Methods

Human samples

Primary human (ph) ATII cells were isolated from non-IPF (N=4) or IPF (N=4) lung tissue biopsies from the Comprehensive Pneumology Center cohort of the BioArchive CPC-M at the University Hospital Grosshadern of the Ludwig Maximilian University. Participants provided written informed consent to participate in this study, in accordance with approval by the local ethics committee of the LMU, Germany (Project 333-10, 455-12). Additionally, formalin-fixed, paraffin-embedded lung tissue samples from 14 patients with sporadic IPF (mean age ± sd: 55.82 ± 9.74 years; 5 females, 9 males) and 5 non-diseased control subjects (organ donors; mean age ± sd: 56.60 ± 11.46 years; 4 females, 1 male) were immunohistochemically investigated. All lung tissue samples were collected in frame of the European IPF registry (eurIPFreg) and provided by the UGMLC Giessen Biobank (member of the DZL Platform Biobanking). The study protocol was approved by the Ethics Committee of the Justus-Liebig-University Giessen (No. 111/08 and 58/15).

Animal experiments

Six- to eight-week-old pathogen-free female C57BL/6N mice were obtained from Charles River and housed in rooms with constant humidity and temperature with 12h light cycles and free access to water and rodent chow. For the induction of experimental fibrosis, mice were subjected to intratracheal bleomycin (Bleomycin sulfate, Almirall, Barcelona, Spain, was dissolved in sterile PBS) instillation using the Micro-Sprayer Aerosolizer, Model IA-1C (Penn-Century, Wyndmoor, PA), as a single dose of 2 U/kg body weight in 50 µl PBS. Control mice were treated with 50 µl PBS. Mice were sacrificed at day 7, 14 or day 21 after instillation. Lungs were used for collection of whole lung tissue, ATII cells or 3D-LTCs. All animal studies were conducted under strict governmental and international guidelines and approved by the

local government for the administrative region of Upper Bavaria (Project 55.2-1-54-2532-88-12).

Isolation of primary murine alveolar epithelial cell type II (pmATII) cells

The pmATII cells were isolated from pathogen-free C57BL/6 mice treated with PBS/Bleomycin and sacrificed at day 14 after instillation as previously described [1, 2] with slight modifications. In brief, lungs were filled with dispase (BD Bioscience, San Jose, CA, US) and low gelling temperature agarose (Sigma Aldrich, Saint Louis, MO, USA) before tissue was minced and the cell suspension was filtered through 100-, 20-, and 10-µm nylon meshes (Sefar, Heiden, Switzerland). Negative selection of fibroblasts was performed by adherence on non-coated plastic plates. Macrophages and white blood cells were depleted with CD45 and endothelial cells were depleted with CD31 specific magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Cell purity was assessed routinely by analysis of endothelial (CD31), mesenchymal (α-SMA, CD90), epithelial (EpCAM, panCK and proSP-C), and hematopoietic cell (CD45) markers by immunofluorescence or flow cytometry.

Isolation of primary human alveolar epithelial type II (phATII) cells

Isolation of phATII cells was performed as previously described [1, 3], with some modifications. Briefly, lung tissue was minced and digested with dispase/collagenase (Roche, Basel, Switzerland) at 37°C for 2 h before filtering through nylon meshes and centrifugation at 400 g, 4°C for 10 min. Next, the cells were layered onto a discontinuous Percoll density gradient (1.04–1.09 g/ml) and centrifuged at 300 g for 20 min. The interphase containing macrophages and alveolar epithelial cells was recovered. Macrophages and white blood cells were depleted with CD45 specific magnetic beads (Miltenyi Biotec, Bergisch) according to the manufacturer's instructions.

Cell culture

In experiments using pmATII cells were seeded, cultured for 48h, then treated with senolytic agents Dasatinib (500 nM, Selleck Chemicals, Houston, TX, USA) and Quercetin (50 µM, Sigma Aldrich, St Louis, MO, USA) or respective DMSO control for 24 or 48 h in DMEM (Sigma Aldrich) containing 10% FCS (PAA Laboratories, Pasching, Austria), 2 mM l-glutamine, 100 U·mL−1 penicillin, 100 µg·mL−1 streptomycin (both Life Technologies, Carlsbad, CA), 3.6 mg/ml glucose (Applichem GmbH, Darmstadt, Germany) and 10 mM HEPES (PAA Laboratories). Senolytic treatment with ABT263 (10 µM, Cayman Chemical, Ann Arbor, MI) led to similar results (data not shown). Cells were counted 48 h after treatment with a Neubauer counting chamber. RNA was isolated isolated and reverse transcribed as described previously [2] and fibrotic gene expression was measured by quantitative (q) RT-PCR. Supernatants were collected, centrifuged at 14000 g for 10 min and stored at -80°C before analysis.

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR, qPCR)

Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Hilden, Germany) for human tissue and Peqlab Total RNA extraction Kit (Peqlab, Erlangen, Germany) for mouse samples, according to the manufacturer's instructions. cDNAs were generated by reverse transcription using SuperScriptTM II (Invitrogen, Carlsbad, CA, USA) and for human tissue with iScript Advanced kit (BioRad, Hercules, CA, USA). Quantitative (q)RT-PCR was performed using Light Cycler 480 detection system and SYBR Green (Roche Diagnostics, Mannheim, Germany). Hypoxanthine phosphoribosyltransferase (HPRT) was used as a reference gene.

Relative gene expression is presented as ΔCt value (ΔCt =[Ct Hprt]-[Ct gene of interest]). Relative change in transcript level upon treatment is expressed as fold change 2^ΔΔCt value (ΔΔCt= ΔCt of treated sample-ΔCt of control).

Primers for *Col1a1, Fibronectin, Sftpc, Sftpa, T1a, Cdh1* and *MMP12* were as previously reported with *HPRT* as reference gene [2, 4]. Additional primers were

Gene	forward primer	reverse primer
hp16	ACCAGAGGCAGTAACCATGC	CCTGTAGGACCTTCGGTGAC
hp21	GTCAGTTCCTTGTGGAGCCG	TGGGTTCTGACGGACATCCC
mp16	CGGGGACATCAAGACATCGT	GCCGGATTTAGCTCTGCTCT
mp21	ACATCTCAGGGCCGAAAACG	AAGACACACAGAGTGAGGGC
mSpp1	AGCCAAGGACTAACTACGACC	TGGCTATAGGATCTGGGTGC
mMMP2	ATCCACGGTTTCAGGGTCC	ATCGAGACCATGCGGAAGC
mPai1	AGGTCAGGATCGAGGTAAACGAG	GGATCGGTCTATAACCATCTCCGT
mCol5a3	CCACCACTGTCACGATTGGA	GAGTCGTCTGCTCGGTTTCAG
mWisp1	GTCCTGAGGGTGGGCAACAT	GGGCGTGTAGTCGTTTCCTCT

Generation and treatment of 3D-LTCs

C57BL6/N mice of 8-12 weeks were instilled with 2 U/kg bleomycin and sacrificed at day 14 after instillation. 3D-LTCs were generated as previously described [4]. Briefly, lungs were flushed through the heart with sterile sodium chloride solution and filled with low gelling temperature agarose (2%, A9414; Sigma) in DMEM/Ham's F12 supplemented with 100 U·mL−1 penicillin, 100 µg·mL−1 streptomycin and 2.5 µg·mL−1 amphotericin B (Sigma Aldrich). Next, lobes were cut with a vibratome (Hyrax V55; Zeiss, Jena, Germany) to a thickness of 300 µm (speed 10–12 µm·s−1, frequency 80 Hz, amplitude of 1 mm). 3D-LTCs were treated with senolytic agents Dasatinib (500 nM, Selleck Chemicals, Houston, TX, USA) and Quercetin (50 µM, Sigma Aldrich) or respective DMSO control for 48 h in sterile cultivation medium containing 0.1% FCS. RNA was isolated and fibrotic gene expression was measured by qRT-PCR. Supernatants were collected, centrifuged at 14000 g for 10 min and stored at -80°C before analysis.

Western blotting

Cells or pulverized lung tissue were lysed with Tissue Protein Extraction Reagent (T-Per, Thermo Fisher) containing phosphatase and protease inhibitors (Roche Diagnostics,

Mannheim, Germany). Protein concentration was determined by BCA assay (Pierce, Thermo Fisher Scientific). Equal amounts of protein were loaded with 4× Laemmli loading buffer (150 mM Tris HCl [pH 6.8], 275 mM SDS, 400 nM dithiothreitol, 3.5% (w/v) glycerol, 0.02% bromophenol blue) and subjected to electrophoresis in 17% polyacrylamide gels and transferred to PVDF membranes. Supernatants from treated 3D-LTCs were collected and stored at -80°C. Samples were normalized to supernatant volume. 200 µL of supernatant from each sample was concentrated using Nanosep 10K OMEGA columns (Pall Corporation; Ann Arbor, MI, USA) at 15000 g for 20 min. Formed concentrate was diluted in 60 µL lysis buffer to form a 3:10 ratio. Western blotting was performed with 6% TRIS-based gels. Membranes were blocked with 5% non-fat dried milk solution in TRIS-buffered saline containing 0.01% (v/v) Tween (TBS-T) (Applichem) for 1h and incubated with primary antibodies (anti p21, MAB88058, Merck Millipore (Billerica, MA, USA); anti β-actin, A3854, Sigma Aldrich, anti proSP-C, ab40879, Abcam (Cambridge, UK), anti E-Cadherin BD 610181, anti Collagen1, 600-401-103, Rockland (Limerick, PA, USA) at 4°C overnight. Next, blots were incubated for 1 h at RT with secondary, HRP-conjugated, antibodies (GE-Healthcare) prior to visualization of the bands using chemiluminescence reagents (Pierce ECL, Thermo Scientific, Ulm, Germany), recording with ChemiDocTMXRS+ system and analysis using Image Lab 5.0 software (Biorad, Munich, Germany).

For analysis of protein expression, peripheral lung tissue samples from the lower lobe, from the subpleural region of the lung was used. Lung homogenates were prepared of shock-frozen lung tissue samples (size 1 cm^3) from IPF patients (N=16; mean age ± SD: 50.67 ± 12.010 years; 2 females, 14 males) and non-diseased control subjects (organ donors, N=11; mean age ± SD: 49.67 ± 7.615 years; 5 females, 5 males, 1 unknown) according to the protocol previously described [5]. The protein concentration in lung homogenates was determined according to the Pierce® BCA protein assay from Thermo Scientific.

For one-dimensional SDS-PAGE, lung homogenates were then diluted (1:3) in 4×SDS-sample buffer [leading to a final concentration 2% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 12.5 mmol/L tris-HCl [pH 6.8], 0.1% (w/v) bromophenol blue in samples] and heated for denaturation at 99°C for 15 min. Denaturated proteins from each sample (50 µg/lane) were then separated by 15% Laemmli-SDS-PAGE. Thereafter, the separated proteins were transferred to a PVDF membrane (Millipore) in a semi-dry blotting chamber according to the manufacturer´s protocol (Bio-Rad, Munich, Germany). Obtained immunoblots were then blocked by incubating at room temperature for 1 h in blocking buffer [1 × tris-buffered saline (TBS; 50 mmol/L tris-HCl, pH 7.5, 50 mmol/L NaCl) containing 5% (w/v) nonfat dried milk and 0.1 % (w/v) tween 20], followed by immunostaining for p16 (ab108349, abcam, diluted 1:250) or p21 (ab109520, abcam, diluted 1:500). Blots were incubated with primary antibody (diluted in blocking buffer) overnight at 4°C with gentle shaking. The blots were then washed four times in 1 × TBS containing 0.1 % (w/v) tween 20, and incubated with horseradish peroxidase-conjugated secondary swine anti-rabbit IgG (DakoCytomation, Hamburg, Germany; diluted 1:2000 in blocking buffer) for 2 h at rt. After four washes, blot membranes were developed with the Immobilon Western Chemiluminescent HRP substrate (Millipore), and emitted signals were detected with a chemiluminescence imager (Intas ChemoStar, Intas, Göttingen, Germany). Thereafter, blots were stripped using ″stripping buffer″ [2% (w/v) SDS and 50 mmol/L dithiothreitol in tris-buffered saline (TBS)] under gentle shaking at 55°C for 30 min, followed by reprobing the blots using antibodies against the loading control protein β-actin (ab8226, abcam, diluted 1:3000).

For quantification, band intensities in acquired TIFF-images were analyzed by densitometric scanning and quantified using ImageJ software (Version 1.46r, NIH). The band densities were normalized to β-actin.

145 **ELISA**

146 Supernatants were obtained from assays of primary mouse ATII cells or mouse 3D-LTCs,
147 centrifuged at 14000g for 10 min and only cell-free supernatant was used for the assay.
148 Samples were then transferred to the respective ELISA plate and the assays were performed
149 according to the manufacturer's instructions (WISP1 – DY1627, R&D, Minneapolis,
150 Minnesota, USA; IL6 – DY406, R&D, Minneapolis, Minnesota, USA, SP-C - CSB-E12639m;
151 Cusabio, Washington D.C., Columbia, USA).

152 **Immunohistochemistry (IHC)**

153 ZytoChem-Plus AP Kit (Fast Red) (Zytomed Systems, Berlin, Germany) was used for
154 immunohistochemical localization of P16, P21, proSP-C, cytokeratin-5 (KRT5) and α-SMA in
155 formalin-fixed, paraffin-embedded lung tissue sections from patients with sporadic IPF
156 (N=14) and organ donors (N=5), according to the manufacturer´s instructions and previous
157 published work [6]. Human lungs were placed in 4% (w/v) paraformaldehyde after
158 explantation (fixation was done for 12–24h), and processed for paraffin embedding. Sections
159 (3 µm) were cut and mounted on positively charged glass slides (Super Frost Plus,
160 Langenbrinck (Emmendingen, Germany)). Paraffin-embedded tissue sections of normal
161 donor and IPF lungs were deparaffinized in xylene and rehydrated in graded alcohol.
162 Antigens were retrieved by cooking the sections for 5 min in 10 mmol/L citrate buffer (pH 6.0)
163 using microwave irradiation (800 W). Thereafter, sections cooled down for 20 min at RT,
164 followed by repeated cooking (800 W, 5 min) and cooling (20 min at RT). This procedure was
165 performed three times. Importantly, the citrate buffer was freshly prepared by mixing 18 mL
166 100mmol/L citric acid monohydrate and 82 mL 100mmol/L sodium citrate tribasic dihydrate
167 with 900 mL distilled water.

168 For immunostaining, the streptavidin-biotin-alkaline phosphatase (AP) method with use of the
169 ZytoChem-Plus AP Kit (Fast Red) [Zytomed Systems, Berlin, Germany], according to the

manufacturer´s protocol, was employed. In the following, the primary antibodies used for IHC are listed, including the sources and dilutions: rabbit polyclonal for human proSP-C (1:750, Millipore, AB3786), rabbit monoclonal for human cytokeratin-5 [KRT5] (1:200, Abcam, ab75869), rabbit monoclonal for human cytokeratin-7 [KRT7] (1:200, used from Epitomics, #2303-1 as well as from Abcam, ab68459), rabbit polyclonal for human smooth muscle actin [α-SMA] (1:100, Abcam, ab5694), rabbit monoclonal for human p16INK4a (1:75, Abcam, ab108349), rabbit monoclonal for human p21 (1:100, Abcam, ab109520) and rabbit monoclonal for gamma H2A.X (phospho-S139) (1:100, Abcam, ab81299).

In general, sections were incubated for 2h at RT with primary antibodies, which were diluted in PBS containing 2% (w/v) BSA. Control sections were treated with PBS-2%BSA alone to determine the specificity of the staining. Detection was performed with a polyvalent secondary biotinylated antibody (rabbit, mouse, rat, guinea pig, provided by the ZytoChem-Plus AP Kit, 20 min incubation) followed by incubation with AP-conjugated streptavidin (20 min). Sections were then developed with Fast Red substrate solution, and the reaction was terminated by washing in distilled water. The stained sections were counterstained with hemalaun (Mayers hemalaun solution, WALDECK Division CHROMA GmbH & CO KG, Münster, Germany) and mounted in Glycergel (DakoCytomation). Lung tissue sections were scanned with a scanning device (Nano-Zoomer, Hamamatsu), and examined histopathologically using the ´NDP.view2 software´ at 100×, 200×, 400× and 800× original magnification. IHC for mentioned antibodies was undertaken in 14 IPF- and 5 control-donor lung samples.

Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) for senescence was performed using the GSEA Desktop Application software package from the Broad Institute [7] on previously published microarrays for whole mouse lungs of PBS- or bleomycin-treated animals (GSE16846) [8], fibroblasts isolated from PBS- or bleomycin-treated animals (GSE42564) [9] or isolated ATII

196 cells [1]. GSEA allows for computationally testing whether a defined set of genes, such as a
197 list of genes associated with senescence, are significantly enriched in one of two biological
198 conditions. Pre-ranked gene lists were generated from normalized data for the ATII cells
199 based on log2 fold change whereas fold change was used for generating the fibroblast pre-
200 ranked list. For whole lung homogenates, genes were ranked using the built-in Signal2Noise
201 function. The senescence list was obtained from Fridman *et al.* [10].

202 **Correlation analysis**

203 Data for the analysis were extracted from Lung Genomics Research Consortium (GSE47460
204 GPL4680) and correlated to diffusion capacity of the lung for carbon monoxide (%DL_{CO}) and
205 the forced vital capacity (FVC) in human patients as a measure of disease severity. Only
206 normal control patients and patients with confirmed IPF were used from the dataset. For
207 expression analysis of *P16* or *P21*, the expression data was extracted from the published
208 datasets.

209

210 **Immunofluorescence staining**

211 For immunofluorescence staining experiments, ATII cells were seeded on poly-l-lysin treated
212 coverslips. Cells were stopped at day 2 or after 48 h of treatment with senolytic drugs and
213 fixed with ice-cold acetone-methanol (1:1) for 10 min and washed 3 times with 0.1% BSA in
214 PBS. Next, cells were permeabilized with 0.1% Triton X-100 solution in PBS for 20 min,
215 blocked with 5% BSA in PBS for 30 min at room temperature and incubated with primary
216 antibodies (proSP-C 1:100 (AB3786, Millipore, Darmstadt, Germany), E-Cadherin 1:200
217 (610181, BD, Franklin Lakes, NJ, USA), Cytokeratin 1:500 (Dako, Glostrup, Denmark),
218 Cleaved caspase 3 1:150 (9661, Cell Signaling, Danvers, Massachusetts, USA**)**, Histone
219 H3K9me3 1:500 (8898, Abcam, Cambridge, MA 02139, USA), ZO-1 1:200 (400-2200,
220 Invitrogen, Waltham, Massachusetts, USA), αSMA 1:500 (AB5228, Sigma Aldrich, St. Louis,

Missouri USA), followed by secondary antibodies, 1 h each. DAPI (Roche, Basel, Switzerland) staining for 10 min was used to visualize cell nuclei. Next, coverslips were fixed with 4% PFA for 10 min, mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and visualized with an Axio Imager microscope (Zeiss, Oberkochen, Germany) or confocal microscope (LSM 710; Zeiss, Oberkochen, Germany).

3D-LTCs were fixed with the mixture of acetone-methanol (1:1) for 20 min and punched to a 4 mm diameter. Tissue was blocked for 1h with 5% BSA in PBS and incubated overnight with primary antibodies in 4°C, followed by incubation with appropriate secondary antibodies for 1h and with DAPI for 5 min. Then tissue was fixed for 30 min with 4% PFA. Staining was evaluated via confocal microscopy (LSM 710; Zeiss, Oberkochen, Germany).

Secretome analysis

Eight- to ten-week-old, pathogen-free female C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were used for the isolation of pmATII cells for secretome analysis. Lung fibrosis was induced in the animals by intratracheal instillation of a single dose of bleomycin (5 U/kg body weight), dissolved in 80 µl sterile phosphate-buffered saline (PBS). Control mice were treated with 80 µl PBS. Mice were sacrificed at day 14 after instillation and 3 mice per treatment were pooled for the isolation of pmATII cells. ATII cells were seeded in 12-well plates in DMEM media without phenol red and conditioned media (4, 6, and 8 h, respectively) were harvested either on the first day of culture or after 2 days of culture. (day 2 4 h; day 2 6 h; day 2 8 h; Bleo ATII: n=3; PBS ATII: n=2). Samples were snap frozen in liquid nitrogen and subjected to mass spectrometry analysis as previously described [11]. Briefly, proteins in conditioned media were digested in solution with trypsin and LysC into peptides, which were analyzed on a Q-Exactive mass spectrometer (Thermo Fischer). Mass spec raw data was processed using the MaxQuant software [12] and proteins were quantified using the embedded label free quantification algorithm MaxLFQ [13]. Statistical data analysis was performed using the Perseus software suite [14].

Flow cytometry (FCM)-based apoptosis assay in pmATII cells

FCM-based apoptosis assay was performed according to the manufacturer's instructions (Annexin V apoptosis kit, eBioscience, San Diego, CA). Briefly, pmATII cells from PBS- and bleomycin-treated animals (day 2) were incubated with vehicle (DMSO) or senolytic drugs Dasatinib (500 nM, Selleck Chemicals, Houston, TX, USA) and Quercetin (50 μM, Sigma Aldrich, St Louis, MA, USA) for 48 hours and were subsequently incubated with Bafilomycin A1 (100 nM, Enzo Life Sciences, Farmingdale, NY; USA) and C_{12}FDG (20 nM, Life technologies, Carlsbad, CA; USA) for 1 and 2 h, respectively. The cells were harvested with trypsin/EDTA (Life technologies), washed once with calcium chloride-free and magnesium chloride-free PBS (Life technologies), and once with the Annexin V-binding buffer (eBioscience). Cells were incubated with APC-conjugated Annexin V (eBioscience) for 15 min, washed, and resuspended in the binding buffer. Cells without C_{12}FDG treatment were further stained with propidium iodide solution (PI, eBioscience). The groups of the cells stained with Annexin V+PI or Annexin V+C_{12}FDG were analyzed with a FACS LSRII cell analyzer (BD Bioscience).

Supplemental Figures

Figure S1: Expression of senescence markers is upregulated in IPF patients.

Gene expression of *P16* and *P21* in IPF versus donor specimens. N=91 donor, N=122 IPF. Data extracted from the LGRC GSE47460 GPL4680. Data is presented as mean ± s.e.m.. Means were compared using Mann-Whitney U test. **(B)** Correlation between *P16* expression in the lung and the lung function parameter %predicted DL_{CO} (N=194) showed a significantly negative linear correlation (dashed line = 95% CI; data extracted from LGRC GSE47460 GPL4680).

Figure S1

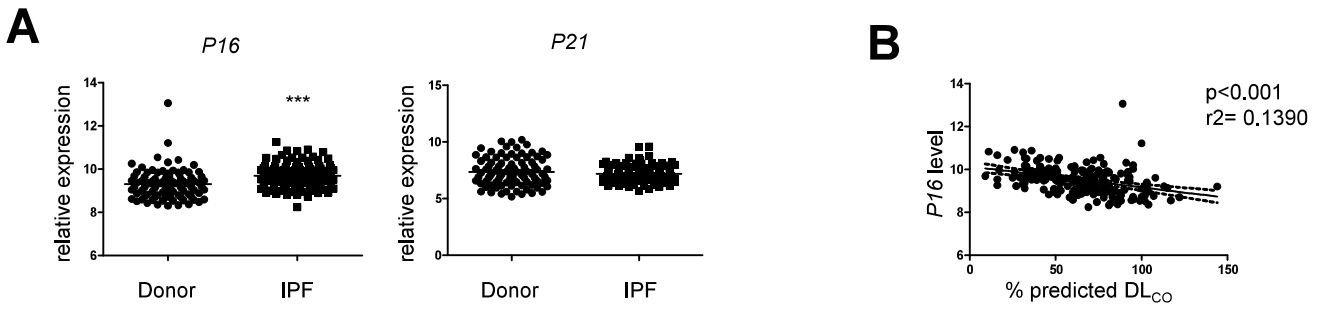

A
P16
P21
relative expression
***
Donor
IPF
B
p<0.001
r2= 0.1390
P16 level
% predicted DLCO

Figure S2: Expression and localization of P16 and P21 in IPF lungs.

(A, B) Immunohistochemical staining of serial sections of IPF lung tissue for α-SMA (marker for myofibroblasts/mesenchymal cells), proSP-C (marker for alveolar epithelial type II [ATII] cells), cytokeratin 5 (KRT5, marker for bronchiolar basal cells), cytokeratin 7 (KRT7, marker for simple epithelia) and P16 protein. $KRT5^+$ $KRT7^+$ basal cells expressing P16 are indicated by asterisks; α-SMA expressing mesenchymal cells which revealed no pronounced expression for P16 are indicated by hashmarks; proSP-C^+ $KRT7^+$ positive ATII cells expressing P16 **(B)** are indicated by arrows; proSP-C^- $KRT5^-$ $KRT7^+$ epithelial cells expressing P16 are indicated by arrowheads; proSP-C^+ $KRT5^+$ $KRT7^+$ positive epithelial cells are indicated by dashed arrows. The lung region shown in **(A)** did not exhibit ATII cells. The lung region shown in the left panel of **(B)** exhibited aberrant epithelial structures indicating KRT5 expressing basal cells (with abnormal superficial localization) in close proximity to proSP-C expressing ATII cells, as well as abnormal epithelial cells with co-expression of KRT5 and proSP-C, which all expressed P16. In contrast, the alveolar airspace shown in the middle panel of **(B)** indicated only a sparse amount of P16 expressing ATII. **(C, D)** Immunohistochemical staining of serial sections of IPF lung tissue for α-SMA (marker for myofibroblasts/mesenchymal cells), proSP-C (marker for alveolar epithelial type II [ATII] cells), cytokeratin 5 (KRT5, marker for bronchiolar basal cells), cytokeratin 7 (KRT7, marker for simple epithelia) and P21 protein. $KRT5^+$ $KRT7^+$ positive basal cells expressing P21 (**C**) are indicated by asterisks; α-SMA expressing mesenchymal cells which revealed no pronounced expression for P21 are indicated by hashmarks; proSP-C^+ $KRT7^+$ positive ATII cells expressing P21 are indicated by arrows; proSP-C^- $KRT5^-$ $KRT7^+$ epithelial cells expressing P21 **(C)** are indicated by arrowheads. The lung region shown in **(C)** exhibited aberrant epithelial structures indicating KRT5 expressing bronchiolar basal cells in close proximity to proSP-C expressing ATII cells, as well as epithelial cells with co-expression of KRT5 and proSP-C.

Figure S2

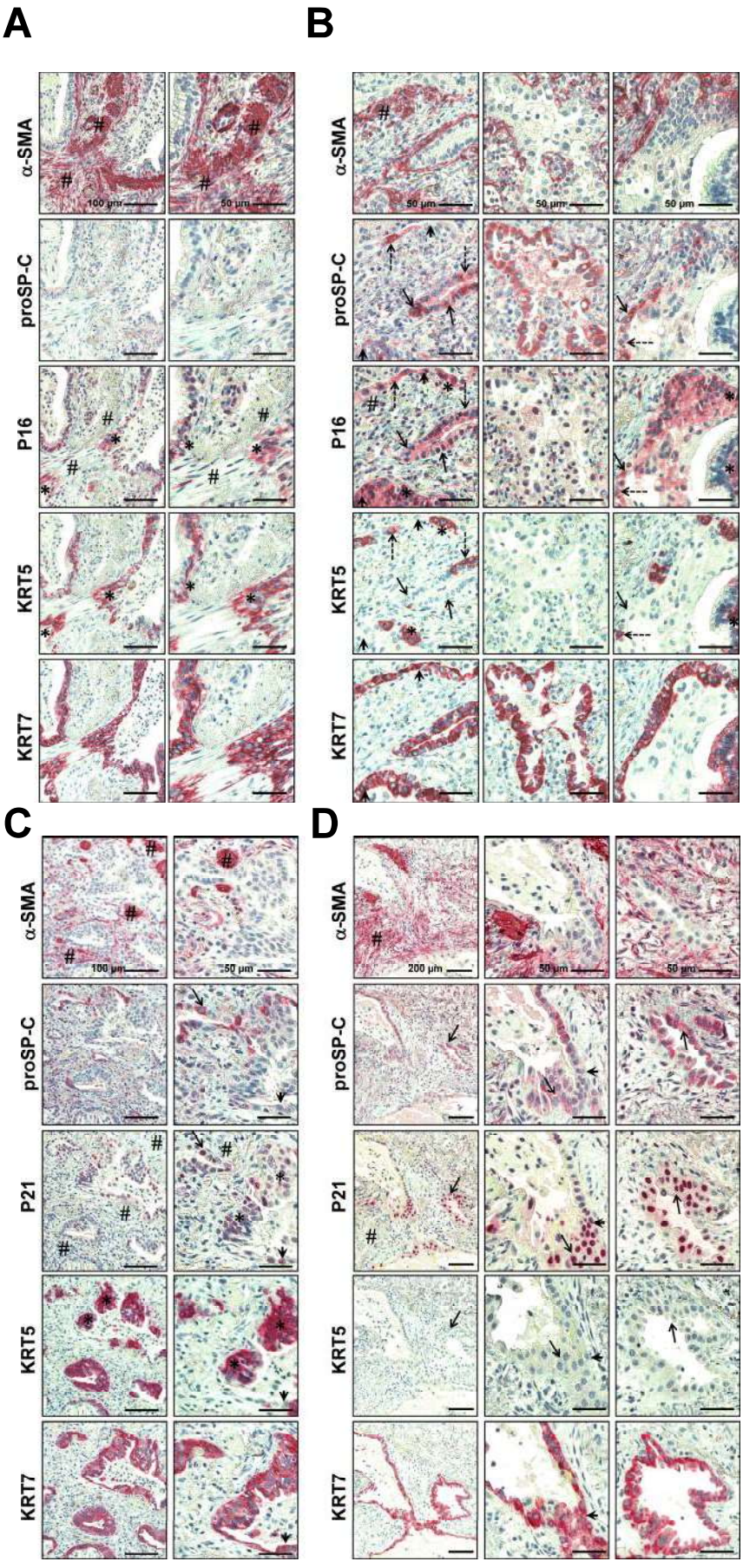


Figure S3: Induction and upregulation of γH2A.X (phospho-S139) in epithelial cells of IPF lungs.

(**A**-**E**) Immunohistochemical staining of serial sections of IPF (**A**, **B**, **D**, **E**) or donor lung tissue (**C**) for proSP-C (marker for alveolar epithelial type II [ATII] cells), cytokeratin 5 (KRT5, marker for bronchiolar basal cells), cytokeratin 7 (KRT7, marker for simple epithelia) and senescence markers γH2A.X (p-S139) and P16. In IPF (**A**, **B**, **E**), proSP-C^+ $KRT7^+$ ATII cells indicated robust nuclear staining for γH2A.X (p-S139) which co-localized with P16 overexpression (indicated by arrows). The same observations were made in proSP-C^- $KRT5^-$ $KRT7^+$ epithelial cells of alveolar spaces (indicated by arrowheads), whereas no substantial immunostaining could be observed in the fibrotic interstitium in IPF lungs **(C).**

The IPF-lung regions shown in **B**, **D** and **E** exhibited aberrant epithelial structures indicating KRT5 expressing basal cells (with abnormal superficial localization) in close proximity to proSP-C expressing ATII cells, which also revealed in part γH2A.X (p-S139) expression in co-localization with P16 (indicated by asterisks). In addition, proSP-C^+ $KRT5^+$ $KRT7^+$ positive epithelial cells in areas of aberrant re-epithelialization (**E**) indicated also concomitant expression of γH2A.X and P16 (indicated by dashed arrows). In general, induction of nuclear γH2A.X (p-S139) or P16 expression was frequently observed in bronchiolar basal cells in areas of bronchiolization in IPF **(B, D, E)**, but also in normal bronchioles of IPF lungs (not shown). In some instances, P16 overexpressing bronchiolar basal cells in IPF lungs did not indicate pronounced co-expression of γH2A.X (p-S139) (right panel of **B**, left panel of **D**). This phenomenon was also in part observed in proSP-C^+ $KRT7^+$ ATII cells as well as in other described epithelial cells (not shown). In age-matched normal donor lungs (**C**), minimal staining for P16 was observed; and no pronounced immunostaining for γH2A.X (p-S139) could be detected in any cells of donor lungs.

Figure S3

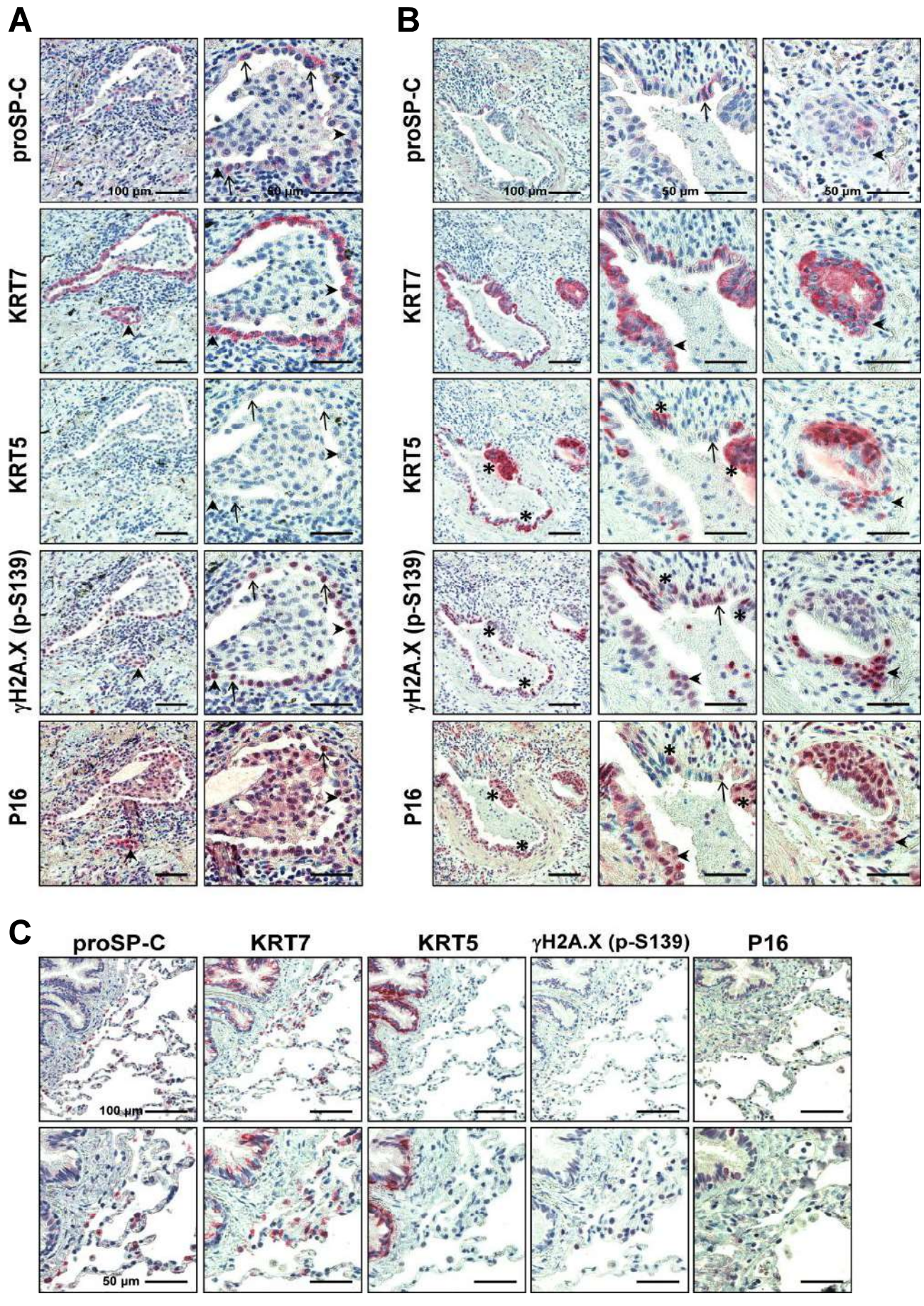


Figure S3 (continued)

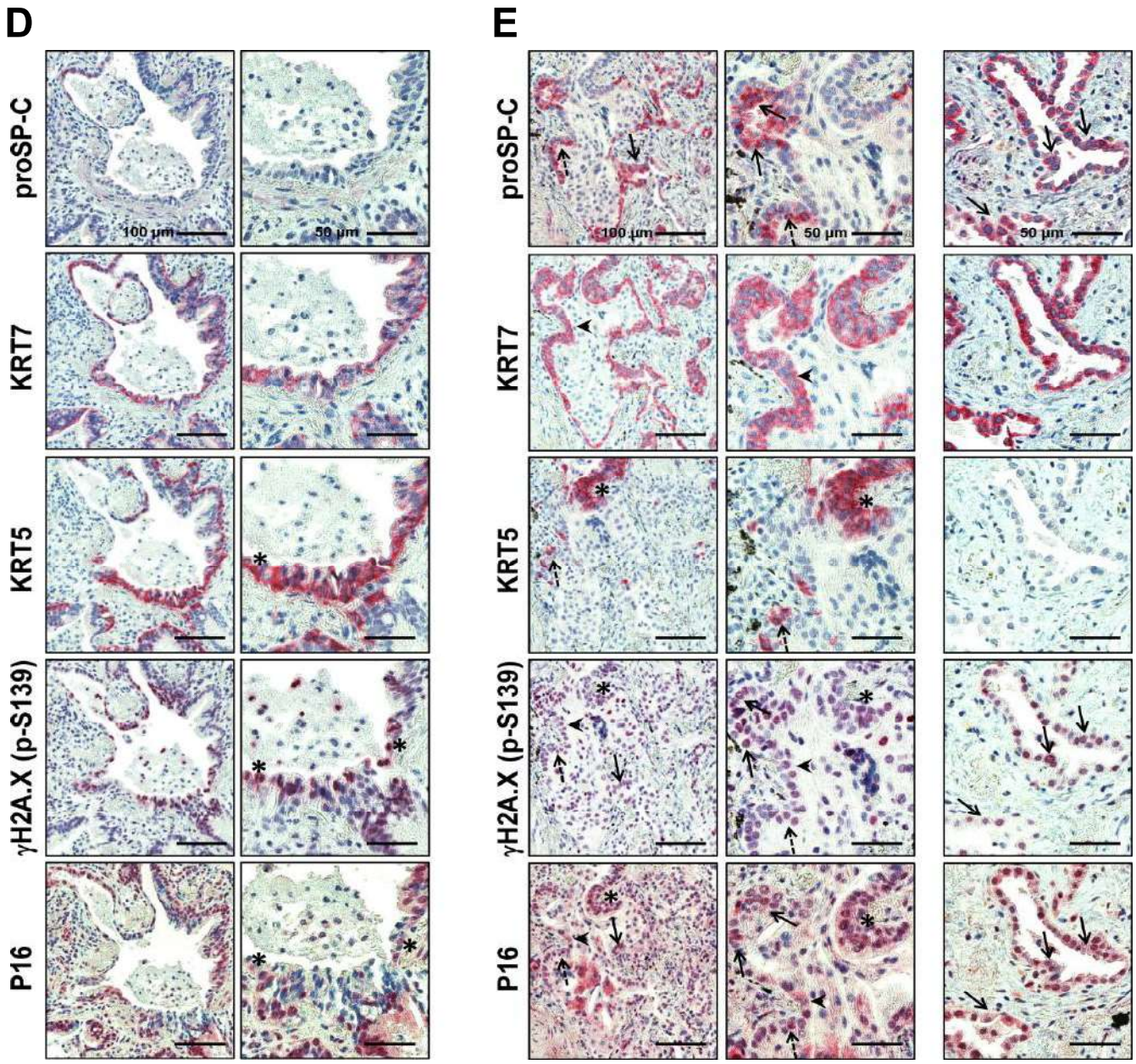
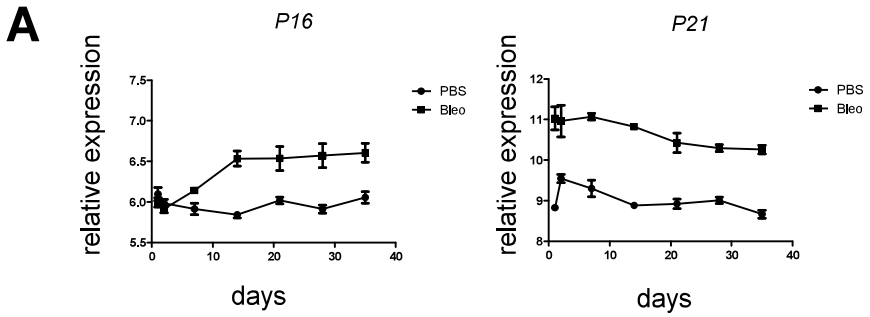
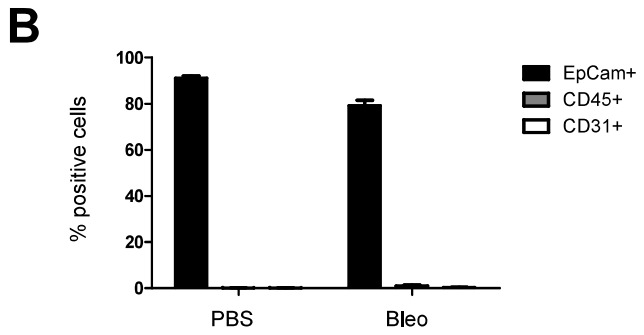


Figure S4: Senescent phenotype of ATII cells after bleomycin challenge. (A) Data for *P16* and *P21* was extracted from GSE40151 [15] for PBS/bleomycin timecourse. **(B)** Mice were instilled with PBS or Bleomycin. At day 14 after instillation mice were sacrificed and pmATII cells were isolated. Cells were stained for EpCAM, CD45 and CD31 and analyzed by FACS. Shown are mean ± s.e.m., n=3-6. **(C)** Representative images of immunofluorescence staining for senescence associated heterochromatic foci marker H3K9me3 and epithelial cell marker E-Cadherin in fibrotic and non-fibrotic pmATII cells at day 2 of culture. Fluorescent images represent a 630× magnification. Scale bar represents 20 µm. Representative of n=2.

Figure S4


A
P16
P21
relative expression
days
PBS
Bleo


B
% positive cells
EpCam+
CD45+
CD31+
PBS
Bleo

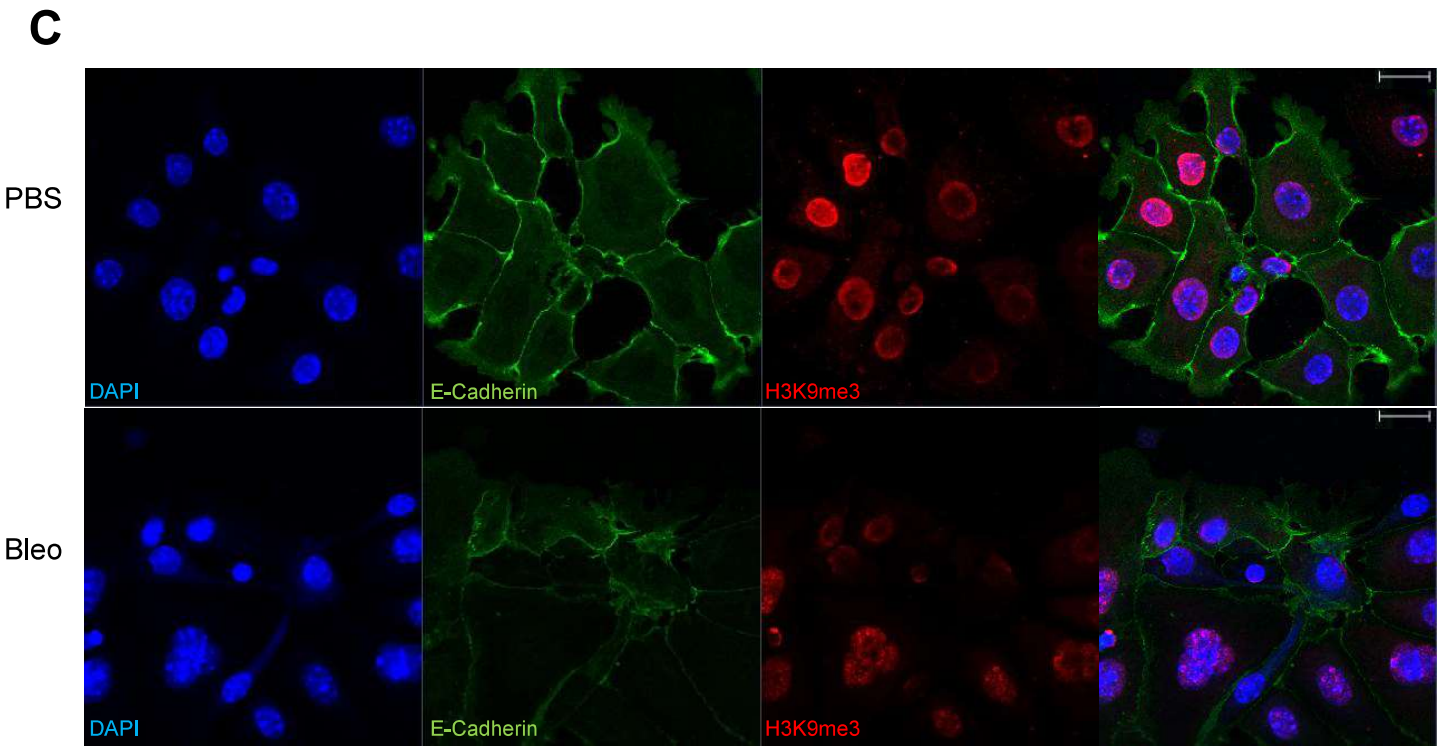

C
PBS
Bleo
DAPI
E-Cadherin
H3K9me3

Figure S5: Depletion of senescent cells in fibrotic pmATII cells

Mice were instilled with either PBS or bleomycin (Bleo). At day 14 after instillation mice were sacrificed and pmATII cells were isolated. **(A)** Gene expression analysis of fibrotic markers after 24/48 h of mock treatment was performed by qPCR. Data were normalized to *Hprt*. ΔCt is presented as mean ± s.e.m.. Significance was assessed with one-way Anova followed by Newman-Keuls's multiple comparison test, n=4. **(B-D)** Fibrotic pmATII cells were cultured in the presence of senolytic drugs Dasatinib (D; 200 nM) and Quercetin (Q; 50 µM). After 24 hours, expression of **(B)** *P16* **(C)** fibrotic markers and **(D)** epithelial cell markers was analyzed by qPCR. Data were normalized to *Hprt* levels. ΔCt is presented as mean ± s.e.m.. Significance was assessed with paired Student's t-test, n=4. Significance: *p<0.05, **p<0.01; ***p<0.001.

Figure S5

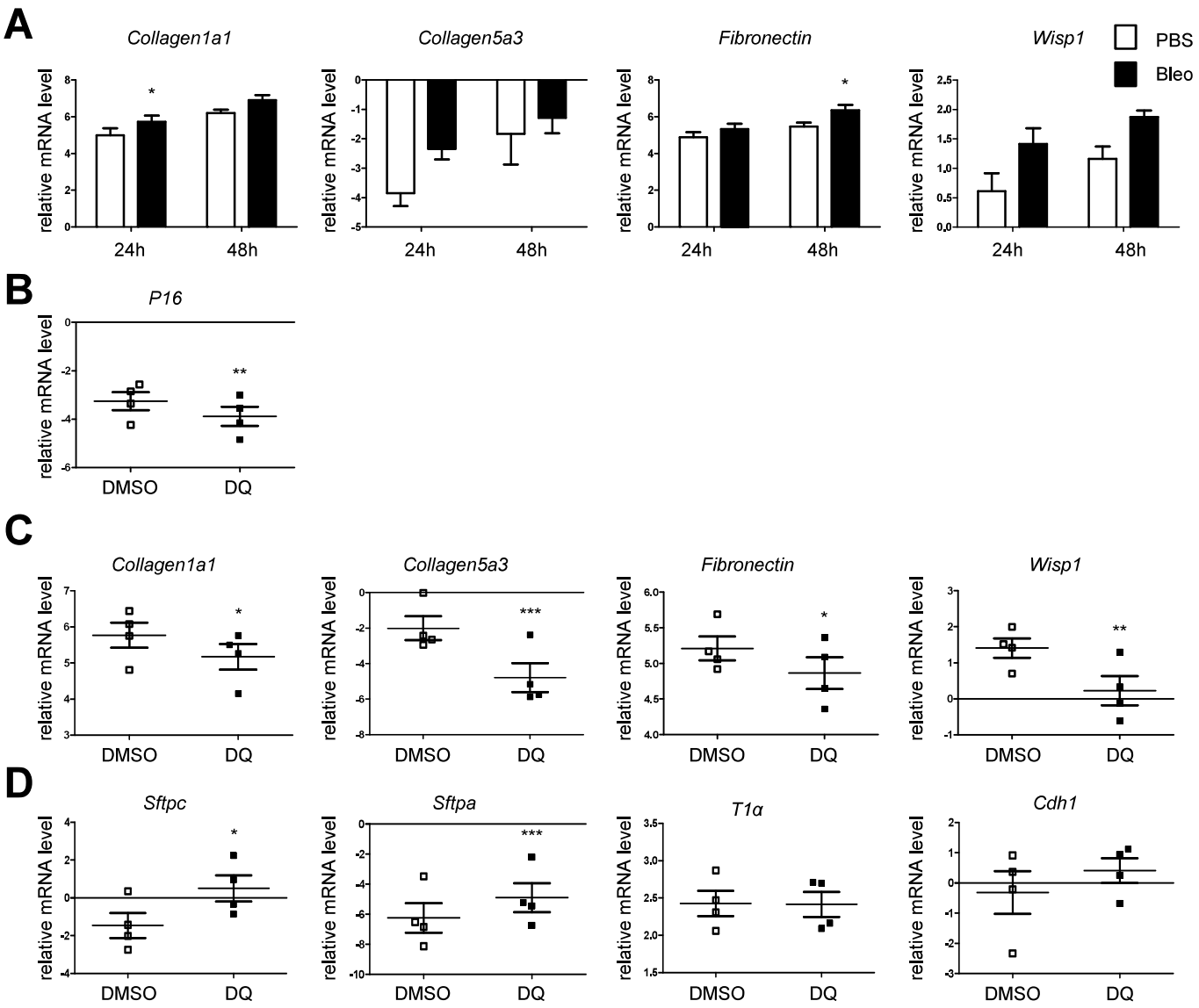

A
Collagen1a1
Collagen5a3
Fibronectin
Wisp1
PBS
Bleo
relative mRNA level
24h
48h
B
P16
DMSO
DQ
C
Collagen1a1
Collagen5a3
Fibronectin
Wisp1
D
Sftpc
Sftpa
T1α
Cdh1

Figure S6: Depletion of senescent cells in non-fibrotic pmATII cells

Mice were instilled with PBS. At day 14 after instillation mice were sacrificed and pmATII cells were isolated. Non-fibrotic pmATII cells were cultured for 48 h in the presence of senolytic drugs Dasatinib (D; 200 nM) and Quercetin (Q; 50 µM) and assessed for **(A)** cell numbers. Data are presented as normalized to DMSO control and as mean ± s.e.m. Significance was assessed with paired Student's t-test, n=3. **(B)** *P16* expression. Data were normalized to *Hprt* levels. ΔCt is presented as mean ± s.e.m, Significance was assessed with paired Student's t-test, n=6. **(C)** Representative images of immunofluorescence staining for the apoptotic marker cleaved caspase 3 and E-Cadherin. Fluorescent images represent a 630× magnification. The scale bar represents 20 µm. **(D-F)** qPCR analysis of non-fibrotic pmATII cells treated with senolytic drugs for the expression of **(D)** SASP markers, **(E)** fibrotic markers, **(F)** epithelial cell markers. Data were normalized to *Hprt* levels. ΔCt is presented as mean ± s.e.m. Significance was assessed with paired Student's t-test, n=6. *p<0.05, ***p<0.001.

Figure S6

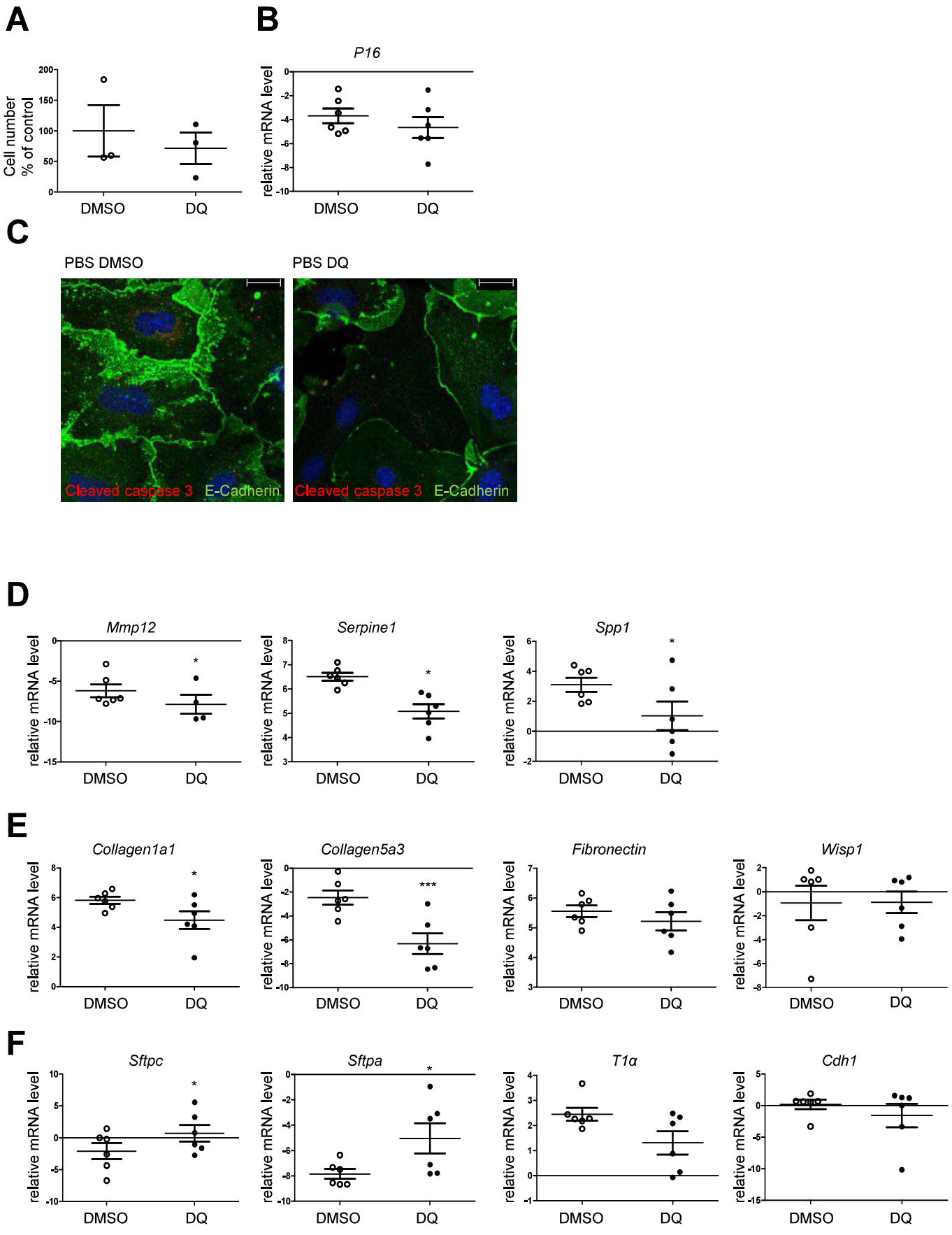

A
Cell number
% of control
DMSO
DQ
B
P16
relative mRNA level
DMSO
DQ
C
PBS DMSO
PBS DQ
Cleaved caspase 3
E-Cadherin
D
Mmp12
Serpine1
Spp1
relative mRNA level
DMSO
DQ
E
Collagen1a1
Collagen5a3
Fibronectin
Wisp1
F
Sftpc
Sftpa
T1α
Cdh1

Figure S7: Characterization of fibrotic markers in PBS/Bleo 3D-LTCs.

Mice were instilled with either PBS or bleomycin. At day 14 after instillation mice were sacrificed and 3D-LTCs were generated. **(A)** Gene expression of fibrotic markers of 3D-LTCs after 48 h of culture was analyzed by qPCR. Data were normalized to *Hprt*. ΔCt is presented as mean ± s.e.m.. Significance was assessed with Student's t-test, n=8.

Figure S7

A

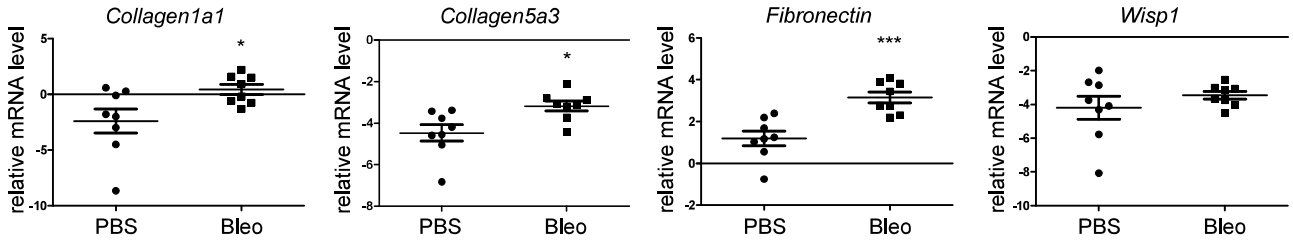
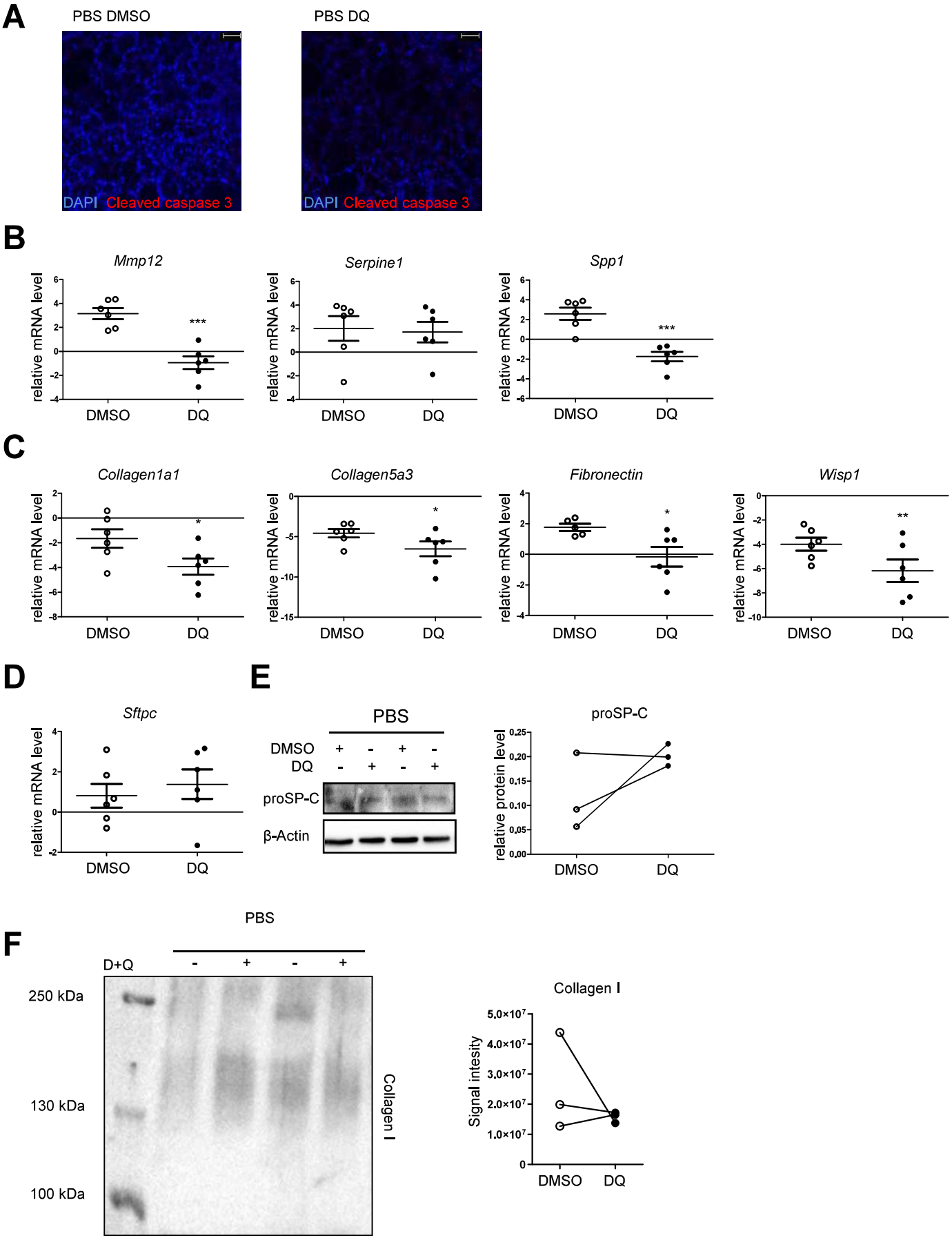

Collagen1a1
relative mRNA level
5
0
-5
-10
*
PBS
Bleo
Collagen5a3
relative mRNA level
0
-2
-4
-6
-8
*
PBS
Bleo
Fibronectin
relative mRNA level
6
4
2
0
-2
***
PBS
Bleo
Wisp1
relative mRNA level
0
-2
-4
-6
-8
-10
PBS
Bleo

Figure S8: Depletion of senescent cells in non-fibrotic 3D-LTCs.

Mice were instilled with PBS. At day 14 after instillation mice were sacrificed and 3D-LTCs were generated. 3D-LTCs were cultured for 48 h in the presence of senolytic drugs Dasatinib (D; 200 nM) and Quercetin (Q; 50 µM). **(A)** Representative images of immunofluorescence staining for apoptotic marker cleaved caspase 3. Fluorescent images represent a 200× magnification. The scale bar represents 50 µm. **(B-D)** 3D-LTCs from PBS animals treated with senolytic drugs were analyzed by qPCR for **(B)** components of the SASP **(C)** fibrotic markers **(D)** or *Sftpc***.** Data were normalized to *Hprt*. ΔCt is presented as mean ± s.e.m.. Significance was assessed with paired Student's t-test, n=6. **(E)** ProSP-C expression was assessed by Immunoblot. β-Actin was used as a loading control. Quantification of proSP-C protein relative to β-Actin. n=3. **(F)** Secreted Collagen I was assessed by Immunoblot. Quantification of secreted Collagen I normalized to supernatant volume. Significance was assessed with paired Student's t-test.n=3. Significance: *p<0.05, **p<0.01; ***p<0.001.

Figure S8


A
PBS DMSO
PBS DQ
DAPI Cleaved caspase 3
DAPI Cleaved caspase 3
B
Mmp12
Serpine1
Spp1
relative mRNA level
***
***
DMSO
DQ
C
Collagen1a1
Collagen5a3
Fibronectin
Wisp1
relative mRNA level
*
*
*
**
DMSO
DQ
D
Sftpc
relative mRNA level
DMSO
DQ
E
PBS
DMSO + - + -
DQ - + - +
proSP-C
β-Actin
proSP-C
relative protein level
0,25
0,20
0,15
0,10
0,05
0,00
DMSO
DQ
F
PBS
D+Q - + - +
250 kDa
130 kDa
100 kDa
Collagen I
Collagen I
Signal intesity
5.0×10⁷
4.0×10⁷
3.0×10⁷
2.0×10⁷
1.0×10⁷
0
DMSO
DQ

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9. Discussion

IPF is a progressive and devastating lung disease. Several hallmark features characterizing the disease have been identified, including impaired epithelial-mesenchymal crosstalk, increased myofibroblast activation and ECM production, genetic predispositions as well as, recently identified, senescence of different cell types in the lung (King, Pardo et al. 2011, Chilosi, Carloni et al. 2013, Lehmann M, Korfei M et al. 2017, Schafer, White et al. 2017). In the present work, we have identified a secreted matricellular protein WISP1/CCN4, which is upregulated in ATII cells as well as in lung fibroblasts, by pro-fibrotic stimuli to increase proliferation of lung fibroblasts, in part via an IL6-dependent mechanism (Klee, Lehmann et al. 2016). Moreover, we could show that senescence of ATII cells is highly increased in patients with IPF as well as in the murine model of bleomycin-induced lung fibrosis. Using murine 3D LTCs and isolated primary murine ATII cells, we could show that depleting senescent cells reduces the expression of pro-fibrotic factors including WISP1/CCN4 and upregulates epithelial markers, indicating that reducing senescence is a potential therapeutic approach in the treatment of lung fibrosis (Lehmann M, Korfei M et al. 2017).

9.1. Impaired epithelial-mesenchymal crosstalk

A number of potential mechanisms underlying IPF development have been postulated over the past decades. One of the features of IPF is an impaired epithelial-mesenchymal crosstalk that, by inducing an uncontrolled repair process of injured lung epithelium, contributes to IPF development and progression (King *et al.* 2001, Selman and Pardo 2002, Marchand-Adam *et al.* 2003, Noble and Homer 2005, Yang, Velikoff et al. 2014). Upon injury, an interaction between epithelial and mesenchymal cells is needed for wound closure, a process that is tightly controlled by the secretion of numerous effector proteins by both cell types in a paracrine manner (Crosby and Waters 2010, Chapman 2011, Akram, Samad et al. 2013, Chambers and Mercer 2015, Zheng *et al.* 2016). After wound repair, cellular processes are normally reverted to physiological conditions (Crosby and Waters 2010, Peng *et al.* 2015). However, the response to injury and the subsequent repair is less efficient and less tightly controlled with increasing age due to stem cell exhaustion, senescence and impaired epithelial-mesenchymal crosstalk (Selman and Pardo 2014). We have shown that inducing lung fibrosis using bleomycin increases the number of senescent ATII cells and augments their SASP production (Lehmann M, Korfei M et al. 2017). Moreover, it was shown that the

secretome of fibrotic/senescent ATII cells induces pro-fibrotic changes in mesenchymal cells leading to an increase in proliferation and ECM production (Selman and Pardo 2004, Shivshankar *et al.* 2012, Chilosi, Carloni et al. 2013, Schafer, White et al. 2017). By eliminating senescent cells using an anti-senescent cocktail of dasatinib and quercetin (DQ) in *in vitro* experiments of isolated primary ATII cells or in an *ex vivo* approach using murine 3D LTCs, the secretion of SASP components was reduced, which attenuated mesenchymal marker and increased epithelial marker expression (Lehmann M, Korfei M et al. 2017). Additionally, we have found that the secretion of WISP1/CCN4, a factor that so far has not been linked to SASP, is increased by fibrotic/senescent ATII cells. We have shown that WISP1/CCN4 is required for the proliferation of phLFs (Klee, Lehmann et al. 2016). Therefore, the increased secretion of WISP1/CCN4 by senescent ATII cells potentially contributes to the increased proliferation of fibroblasts as found in IPF. Moreover, the presence of WISP1/CCN4 potentiates signaling induced by TGFβ1 and TNFα in phLFs in regard to NF-κB target genes (Klee, Lehmann et al. 2016). Additionally, we show that both TGFβ1 and TNFα increase the production and secretion of WISP1/CCN4 in phLFs. This can further affect the phLFs in an autocrine manner, e.g. by increasing the production of ECM components, but also could potentiate the proliferation of ATII cells in a paracrine manner (Konigshoff, Kramer et al. 2009). Collectively, fibrotic stimuli change the secretory phenotype of ATII cells and fibroblasts, which influences both ATII cells and lung fibroblasts and thereby drives the progression of lung fibrosis.

9.2. Epithelial cell damage and its contribution to IPF

Epithelial cells are the first line of physical barrier in the lung and are continuously facing environmental challenges, including particles, viruses or bacteria. They are able to maintain a physiological balance by orchestrating multiple functions like the activation of the innate and adaptive immune system and induction of wound repair in response to continuous microinjuries (Chuquimia *et al.* 2013, Ganesan *et al.* 2013, Whitsett and Alenghat 2015). These injuries are continuously repaired by an interplay involving the ATII cells as well as the underlying mesenchymal and endothelial cells (Martin *et al.* 2005). Moreover, the repeated environmental insults lead to ATII cell apoptosis and ATII cells need to be replenished by progenitor cells (Martin, Hagimoto et al. 2005). However, with aging, an exhaustion of progenitor cells and increased senescence are observed. They represent known risk factors for the development of IPF and contribute to reduced ATII cell replenishment (Plataki,

Koutsopoulos et al. 2005, Chilosi, Carloni et al. 2013). We have shown in *in vitro* experiments with ATII cells as well as in *ex vivo* 3D LTCS experiments using anti-senescence drugs that the expression of mesenchymal markers was reduced while the expression of epithelial markers was restored when targeting senescent cells (Lehmann M, Korfei M et al. 2017). This indicates that by depleting senescent alveolar epithelial cells, a sufficient repair process could be induced leading to appropriate repair of the epithelial barrier without inducing lung fibrosis. In line with our findings, it was shown in bleomycin-induced lung fibrosis models that the inhibition of alveolar apoptosis or deletion of senescent cells reduced the fibrotic phenotype (Kuwano, Hagimoto et al. 1999, Schafer, White et al. 2017). It was shown that both stem cell exhaustion as well as increased levels of senescence can lead to changes in ATII cell behavior towards expression of mesenchymal markers (Chilosi *et al.* 2010, Perl *et al.* 2011). Although not the main source of ECM, ATII cells upregulate mesenchymal marker expression in the context of lung fibrosis (Kim *et al.* 2006, Yang, Velikoff et al. 2014), a process by which ATII cells potentially contribute to the increase in ECM formation. Additionally, changes in the underlying ECM are in part responsible for an increase of mesenchymal marker expression in ATII cells. It was shown in *in vitro* experiments using ATII cells isolated from healthy and IPF patients, that when cells are grown on Matrigel/collagen, ATII cells maintain their epithelial phenotype. However, when cells are grown solely on fibronectin, they lose expression of pro-SPC (ATII cells marker) but increase expression of mesenchymal markers (e.g. vimentin, αSMA and N-cadherin) (Marmai *et al.* 2011). This highlights the importance of the underlying ECM on ATII cell function.

9.3. Proliferation and activation of fibroblasts in IPF

Fibroblasts are the main source of uncontrolled ECM production in patients with IPF. These cells undergo continuous proliferative cycles, which is mainly observed in the so-called fibroblast foci. Moreover, these foci are also a place of fibroblast activation, generating (myo)fibroblasts, which are in turn producing large amounts of ECM components, including different types of collagens and fibronectin (King, Pardo et al. 2011). We and others have shown that WISP1/CCN4 is involved in the upregulation of ECM production by lung (myo)fibroblasts and ATII cells, but also in other cell types, underlining its pro-fibrotic character (Colston *et al.* 2007, Konigshoff, Kramer et al. 2009, Venkatachalam, Venkatesan et al. 2009, Yang *et al.* 2013). Additionally, we have demonstrated that WISP1/CCN4 is also

involved in the proliferation of phLFs. WISP1/CCN4 increases phLF proliferation in part via a mechanism that involves IL6 (Klee, Lehmann et al. 2016). This is in line with previous findings that show the pro-proliferative effects of WISP1/CCN4 in different cell types (Liu *et al.* 2013, Jian, Wang et al. 2014, Brzoska *et al.* 2015, Chiang *et al.* 2015). Therefore, WISP1/CCN4 contributes to both an increased pool of fibroblasts as well as an increased production of ECM components by phLFs. Another important function of WISP1/CCN4 is its potential to reduce apoptotic signals in different cells, including fibroblasts (Su *et al.* 2002, Venkatachalam, Venkatesan et al. 2009, Shang *et al.* 2012, Wang *et al.* 2012, Schlegelmilch *et al.* 2014). It was shown that the reduction of fibroblast apoptosis contributes to the disease pathogenesis of IPF (Horowitz *et al.* 2004, Maher *et al.* 2010, Sisson *et al.* 2012). However, how WISP1/CCN4 induces its effects in phLFs has not yet been elucidated. Due to the variety of functions of WISP1/CCN4 on phLF, it is very likely that the microenvironment for WISP1/CCN4-mediated signaling is of high importance. It was shown in different cell types that WISP1/CCN4 signals through different integrins (Ono *et al.* 2011, Chen, Ding et al. 2016, Jin *et al.* 2016). Interestingly, the expression of a number of integrins is upregulated in IPF. These integrins are involved in processes that contribute to the progression of lung fibrosis e.g. by activating TGFβ1 or mediating signals of pro-fibrotic cytokines like PDGF (Luzina *et al.* 2009, Todd, Luzina et al. 2012, Henderson and Sheppard 2013, Lu *et al.* 2017). These integrins could also be responsible for the pro-fibrotic and pro-proliferative signal induction mediated by WISP1/CCN4 in phLFs. As for WISP1/CCN4 in lung fibrosis, the responsible integrins required for the above-mentioned pro-proliferative and pro-fibrotic functions in lung fibroblasts have not yet been elucidated. Further studies will unravel these and thereby contribute to an increased understanding of the disease and the contribution of CCN proteins such as WISP1/CCN4 to disease pathogenesis.

9.4. ECM (-associated) proteins and their contribution to (lung) fibrosis

The extracellular matrix is not only composed of structure-giving proteins like collagens, proteoglycans and elastin (White 2015), but also contains numerous proteins that are continuously embedded and released in the ECM and function as signaling molecules. These proteins include numerous growth factors like TGFβ1, TNFα and PDGFs, but also MMPs or tissue inhibitor of metalloproteinases (TIMPs) (Bonnans *et al.* 2014, Hinz 2015, Watson *et al.* 2016). Dysregulation of these factors is implicated in a vast array of diseases (McAlpine and

Tansey 2008, Dooley and ten Dijke 2012, Heldin 2013). Importantly, deranged activation of molecules like TGFβ1 and TNFα, as well as a misbalance of MMPs and their respective inhibitors TIMPs has been shown in IPF (Mukhopadhyay *et al.* 2006, Fernandez and Eickelberg 2012, Pardo *et al.* 2016). However, direct targeting of pro-fibrotic factors like TGFβ1 or TNFα so far have not shown any efficacy in patients with IPF, which could be in part due to the fact that besides contributing to disease pathogenesis, they are involved in many physiological processes. Therefore, a deeper understanding of downstream processes and common targets of these and other pro-fibrotic factors like WNTs could help to improve therapy development. We and others have shown that WISP1/CCN4 is induced by canonical WNT signaling in ATII cells (Konigshoff, Kramer et al. 2009) and cell types of other origins (Pennica, Swanson et al. 1998, Xu *et al.* 2000, Blom *et al.* 2009). Additionally, we have shown here and in a recent publication from our laboratory that TGFβ1 and TNFα induce WISP1/CCN4 in phLFs mainly via NF-κB (Berschneider, Ellwanger et al. 2014, Klee, Lehmann et al. 2016). Moreover, an increase of WISP1/CCN4 was shown to be mediated by both cytokines in various other cell types (Venkatachalam, Venkatesan et al. 2009, Lukowski *et al.* 2010). Interestingly, it was also shown that other CCNs can be induced via activation of the NF-κB pathway (Sampath *et al.* 2001, Cras *et al.* 2012), indicating a conserved pathway for the induction of the CCN proteins. In line with this, it was shown in a number of studies that WISP1/CCN4 is involved in immune responses and contributes to an upregulation of pro-inflammatory cytokines (Hou *et al.* 2013, Murahovschi, Pivovarova et al. 2015, Chen, Ding et al. 2016, Tong *et al.* 2016, Barchetta *et al.* 2017). It is important to note that not only WISP1/CCN4, but also CyR61/CCN1 and CTGF/CCN2 are known to participate in inflammatory responses (Sanchez-Lopez *et al.* 2009, Bai *et al.* 2010, Lai *et al.* 2013, Elliott *et al.* 2015), as CCNs have a conserved function in increasing inflammation. In this context, IPF is characterized by increased activation of inflammatory pathways, which led to a number of clinical investigations on how to potentially target inflammation in lung fibrosis. A recent clinical trial investigated the effect of Etanercept, a recombinant soluble TNFα receptor, on the pathogenesis of IPF (Mohler *et al.* 1993, Kohno *et al.* 2007, Raghu, Brown et al. 2008). A beneficial effect of Etanercept was shown in different diseases such as rheumatoid arthritis and plaque psoriasis (Haraoui and Bykerk 2007, Nguyen and Koo 2009), however, it failed to show efficacy in IPF patients (Mease *et al.* 2000, Brandt *et al.* 2003, Leonardi *et al.* 2003, Raghu, Brown et al. 2008). This might be due to various reasons, including an underpowered study, the drug dose or blockage of potential beneficial

activities of TNFα. Therefore, it is important to understand the disease in more detail, especially to elucidate the contribution of inflammatory processes that are upregulated in IPF (Bringardner, Baran et al. 2008, Balestro, Calabrese et al. 2016), to develop more targeted approaches to treat the disease. WISP1/CCN4 is a potential candidate for this approach, as it acts downstream of a number of pro-fibrotic pathways. However, contrary to its pro-fibrotic role in several fibrotic diseases, WISP1/CCN4 also exerts protective functions in other diseases. It was shown that WISP1/CCN4 is required for sufficient epithelial cell barrier repair following injury-induced microdamages *in vivo* (Maeda *et al.* 2015). Moreover, although WISP1/CCN4 is upregulated in liver fibrosis and blockage using a neutralizing antibody reduced liver fibrosis development (Jian, Wang et al. 2014, Li, Chen et al. 2015), it was shown that WISP1/CCN4 knockout mice suffer from increased fibrotic burden upon tetrachloride-induced liver fibrosis (Pütter *et al.* 2015). Therefore, it will be of importance to understand which levels of WISP1/CCN4, which receptors for WISP1/CCN4 and additionally which microenvironment are required for a sufficient wound repair but also which domains of WISP1/CCN4 are responsible for its physiological and pathophysiological functions.

Interestingly, we have shown that the presence of WISP1/CCN4 is required for TGFβ1 and TNFα to induce several NF-κB-dependent cytokines, including IL6 (Klee, Lehmann et al. 2016). Similarly, effects on NF-κB-mediated signaling by WISP1/CCN4 were shown in different cell types, suggesting that there is a conserved mechanism of action of WISP1/CCN4 in different physiological and pathophysiological processes (Venkatachalam, Venkatesan et al. 2009, Hou, Tang et al. 2013, Wu *et al.* 2013), making WISP1/CCN4 an interesting target in inflammatory-related diseases. In this context, it was also shown that CyrR6/CCN1 and CTGF/CCN2 induce NF-κB-driven target expression (Tan *et al.* 2009, Liu *et al.* 2012, Chen *et al.* 2014, Kim *et al.* 2015), whereas Nov/CCN3 and WISP2/CCN5 were shown to downregulate NF-κB-mediated gene expression (Lin *et al.* 2010, Jeong *et al.* 2016). CyR61/CCN1 and CTGF/CCN2 are known to be pro-fibrotic (Ponticos *et al.* 2009, Grazioli *et al.* 2015), whereas Nov/CCN3 and WISP2/CCN5 have anti-fibrotic potential (van Roeyen *et al.* 2012, Jeong, Lee et al. 2016). Therefore, induction of NF-κB downstream of WISP1/CCN4, CyR61/CCN1 and CTGF/CCN2 could be one mechanism by which they contribute to fibrotic diseases, whereas Nov/CCN3 and WISP2/CCN5 are downregulating NF-κB-driven transcription and thereby, at least in part, potentially block fibrosis. Additionally, NOV/CCN3 was shown recently to downregulate the

production of different pro-fibrotic markers in kidney fibrosis models, to counteract TGFβ1 signaling and to reduce levels of CTGF/CCN2 and WISP1/CCN4 (Riser *et al.* 2010, van Roeyen, Boor et al. 2012, Abd El Kader *et al.* 2013). Moreover, a recent study revealed that WISP2/CCN5 exerts similar effects in the bleomycin model of lung fibrosis in mice (Zhang *et al.* 2014). Zhang and colleagues showed that the overexpression of WISP2/CCN5 in lung fibroblasts derived from IPF patients resulted in downregulation of αSMA and type I collagen. Moreover, the adenoviral-induced overexpression of WISP2/CCN5 *in vivo* in the bleomycin model resulted in a decreased total collagen and TGFβ1 levels as compared to the control mice. These results show that both NOV/CCN3 and WISP2/CCN5 could potentially be used to counteract the pro-fibrotic functions of WISP1/CCN4 in lung fibrosis and, furthermore, underlines the importance of targeting of the specific CCNs in the disease.

While NOV/CCN3 and WISP2/CCN5 block or downregulate TGFβ1, it was shown that the presence of CTGF/CCN2 is required for full activation of TGFβ1 target genes in different *in vitro* and *in vivo* models (Lipson *et al.* 2012). CTGF/CCN2 was shown to upregulate proliferation and ECM production in lung fibroblasts, similar to the effect observed when these cells were treated with recombinant WISP1/CCN4 *in vitro* (Konigshoff, Kramer et al. 2009). Additionally, epithelial cell function is affected by both proteins. Similar to WISP1/CCN4 (Konigshoff, Kramer et al. 2009), it was shown that targeting of CTGF/CCN2, either by a neutralizing antibody or a small interfering RNA, led to a reduction in the fibrotic burden *in vivo*, accompanied by a reduction in ECM component expression (Ponticos, Holmes et al. 2009). Moreover, clinical studies testing the effect of neutralizing CTGF/CCN2 in IPF patients are ongoing. This underlines the importance of both WISP1/CCN4 and CTGF/CCN2 in the development and progression of lung fibrosis and, more importantly, indicates that neutralization could be an effective IPF therapy. Importantly, approaches to neutralize WISP1/CCN4 in the setting of IPF are ongoing.

WISP1/CCN4 was shown to have positive functions on epithelial cell barrier repair, however, it also exhibits pro-fibrotic functions. As mentioned above, CCNs, including WISP1/CCN4, contain a total of four different functional domains. Understanding which of the domains of WISP1/CCN4 is responsible for its physiological functions and which is involved in the detrimental processes that lead to lung fibrosis will be of importance. One of those domains, the von-Willebrand domain, was shown to be required to interact with TGFβ1 (Abreu *et al.*

2002). However, the interaction of a different CCNs and TGFβ1 results in opposing effects, e.g. CTGF/CCN2 increases TGFβ1-mediated effects (Gressner *et al.* 2009, Parada *et al.* 2013), whereas NOV/CCN3 decreases these effects (Riser, Najmabadi et al. 2010). This suggests that a regulation of expression of the different CCNs is required to regulate each others´ functions and thereby to control a physiologic state to avoid disease progression. Therefore, specific targeting of one or more dysregulated CCN members, including WISP1/CCN4, could potentially restore the physiological balance and reverse pathological changes. Moreover, it was shown that NOV/CCN3 could downregulate the expression and function of WISP1/CCN4 thereby reducing WISP1/CCN4's pro-fibrotic functions. Therefore, treatment with NOV/CCN3 might also be an option to limit the detrimental properties of WISP1/CCN4 in IPF. Another feature shared among the CCNs is a hinge region between the domains 2 and 3, which is a potential target of post-translational modification (Perbal *et al.* 1999) and therefore can lead to changes in the functions of a CCN protein. It was shown that e.g. for NOV/CCN3, a truncated variant has anti-proliferative functions, whereas the full-length variant has pro-proliferative functions (Joliot *et al.* 1992). Similarly, different variants of WISP1/CCN4 have been found. One of these variants was shown to increase metastatic potential of gastric carcinoma cells and thereby enhanced the progression of scirrhous gastric cancer, whereas full-length version of WISP1/CCN4 did not exhibit this potential (Tanaka, Sugimachi et al. 2001). It is thus of importance to decipher the functions of the different variants of WISP1/CCN4 in the context of IPF, which further will help to distinguish if a variant found in a patient has pro- or anti-fibrotic functions and to more specifically target WISP1/CCN4 in total or to neutralize specific regions/domains of WISP1/CCN4 that are involved in IPF without affecting its potential beneficial functions in the lung. Additionally, WISP1/CCN4 was shown to contain multiple glycosylation sites (Pennica, Swanson et al. 1998) (own unpublished data). It is well known nowadays that changes in the glycosylation pattern of proteins can alter the cell functions, including cell-cell communication and cell adhesion (Scanlin and Glick 2001, Ohtsubo and Marth 2006, Marth and Grewal 2008, Dewald *et al.* 2016) and changes in glycosylation are linked to IPF (Lu *et al.* 2014, Westergren-Thorsson *et al.* 2017). Taken together, changes in the expression of different variants, the glycosylation pattern of WISP1/CCN4 as well as its time- and spatial-dependent expression potentially determines its pro- or anti-fibrotic properties. Future studies aiming at deciphering these details will lead to important insights into how to specifically target pro- or anti-fibrotic functions of CCNs therapeutically.

9.5. CCN family and senescence

IPF is a disease of the elderly, as the mean age at diagnosis is 68 years and only few patients are below 50 years of age (Raghu, Weycker et al. 2006, Garcia 2011). Recently, hallmarks of aging were described, which contribute to the development and progression of IPF (Lopez-Otin, Blasco et al. 2013, Meiners *et al.* 2015, Selman *et al.* 2016). Cellular senescence represents one of these features, contributing to aging and playing a significant role in IPF pathogenesis. Cellular senescence is characterized by the stable growth arrest along with the secretion of the SASP (Freund *et al.* 2010). Senescence is important as an anti-tumor mechanism, however, the chronic presence of senescent cells is the potential reason of age-related diseases (Campisi 2013). We and others could show that senescence-associated markers are upregulated in alveolar epithelial cells and lung fibroblasts of IPF patients (Minagawa, Araya et al. 2011, Hecker *et al.* 2014, Lehmann M, Korfei M et al. 2017). Moreover, *in vitro* experiments revealed that factors secreted by senescent lung epithelial cells can induce myofibroblast differentiation (Minagawa, Araya et al. 2011). We further found these markers to be upregulated in whole lung tissue and specifically in ATII cells derived from bleomycin-instilled mice (Lehmann M, Korfei M et al. 2017). Treatment of either murine 3D LTCs or isolated ATII cells with drugs depleting senescent cells (dasatinib and quercetin) resulted in reduction of fibrosis-associated markers like collagens and fibronectin, but also increased epithelial markers. This suggests that the depletion of senescent (epithelial) cells could be beneficial in the treatment of lung fibrosis.

The secretion of WISP1/CCN4, though not yet directly associated with the SASP, is highly upregulated by fibrotic ATII cells (Lehmann M, Korfei M et al. 2017). It was recently shown that WISP1/CCN4 is involved in the upregulation of ROS (Premat C *et al.* 2015), one of the inducers of cellular senescence (Campisi 2013). Therefore, WISP1/CCN4 might be involved in the induction of senescence, however, this requires further investigation. As described above, WISP1/CCN4 belongs to the CCN family of total 6 members, with the most well characterized members being CyR61/CCN1 and CTGF/CCN2. Both CyR61/CCN1 and CTGF/CCN2 have been described to induce senescence in different cell types in physiological and pathophysiological processes (Jun and Lau 2010, Capparelli *et al.* 2012, Kim *et al.* 2013, Jang *et al.* 2017). Interestingly, it was shown that CTGF/CCN2 can contribute to the senescence of lung epithelial cells. Overexpression of CTGF/CCN2 in HBECs led to a growth arrest accompanied

by increased expression of senescence marker (senescence-associated β-galactosidase, p16) (Jang, Chand et al. 2017). Moreover, CTGF/CCN2 was also shown to induce cellular senescence in skin fibroblasts, by which it reduces the ECM production (Jun and Lau 2017). However, it was shown that the SASP expressed by lung fibroblasts reduces the proliferation of lung epithelial cells (Fogarty *et al.* 2017). Therefore, the potential induction of senescence of lung fibroblasts by CTGF/CCN2 could be another explanation of how CTGF/CCN2 contributes to disease progression.

Additionally, the SASP secreted by lung fibroblasts was shown to further increase fibrotic marker expression including collagens, αSMA and FN1, suggesting an autocrine mechanism induced by the SASP of lung fibroblasts (Schafer, White et al. 2017). One of the components of the SASP is TGFβ1, a cytokine that, as shown here, can induce WISP1/CCN4 expression in lung fibroblasts, but also in ATII cells (data not shown). Moreover, another well-established component of the SASP is IL6, which expression is dependent on the presence of WISP1/CCN4 in lung fibroblasts. Thereby, it is possible that an increased expression of WISP1/CCN4 contributes to a senescent phenotype of both the ATII cells as well as the lung fibroblasts and thus further impairs crosstalk of epithelial and mesenchymal cells and contributes to a progression of lung fibrosis. Importantly, we could show that senolytic drugs reduce the expression of WISP1/CCN4, further indicating its role in the SASP as well as in senescence-associated signaling.

9.6. New potential therapeutics in IPF treatment

Recently, two drugs were approved for mild-to-moderate IPF, namely Pirfenidone (Esbrit®; (King, Bradford et al. 2014)) and Nintedanib (OFEV®; (Richeldi, du Bois et al. 2014)). These drugs were shown to significantly reduce the lung function decline occurring during disease. Moreover, recently published retrospective studies of the phase 3 clinical trials performed for approval of both drugs suggest a beneficial effect on prolonged survival of IPF patients (Richeldi *et al.* 2016, Fisher, Nathan et al. 2017). However, both drugs show a long profile of side effects, which lead to either changing of the treatment (from Pirfenidone to Nintedanib or vice versa) or in about 15-25% of the cases to the discontinuation of the treatments (Costabel *et al.* 2014, Milger *et al.* 2015, Hughes *et al.* 2016, Galli *et al.* 2017). Although a number of factors potentially leading to the development of IPF and their underlying

molecular mechanisms have been revealed (King, Pardo et al. 2011, Selman and Pardo 2014), the existing therapies lack efficacy to finally halt or reverse disease progression.

It was shown that fibrotic ATII cells downregulate epithelial markers while increasing the expression of mesenchymal proteins (Marmai, Sutherland et al. 2011). Here, we show that targeting senescence *in vitro* or *ex vivo* leads to the reduction of pro-fibrotic markers along with a rescue of epithelial markers (Lehmann M, Korfei M et al. 2017). In a recent report, it was shown that the depletion of senescent cells prolongs the life span of mice by 20% without increasing risks for tumorigenesis (Baker *et al.* 2016). Moreover, it was shown that using a senolytic cocktail consisting of dasatinib and quercetin in the bleomycin model of lung fibrosis in mice resulted in an improvement of lung function and an overall physical health (Schafer, White et al. 2017). Importantly, the safety and efficacy of both dasatinib and quercetin was shown in different diseases, including lung cancer (Paller *et al.* 2015, Hahn *et al.* 2016, Lu *et al.* 2016, Kelley *et al.* 2017, Schuetze *et al.* 2017). Thus, both drugs represent a potential therapeutic option for IPF patients. Moreover, as we and others show that epithelial cells undergo senescent changes which contribute to lung fibrosis, a more targeted approach to eliminate this particular cell population would be desirable for the treatment of IPF. There are a number of potential therapeutic options available to target specific cell types. These include the use of viral vector systems, nanoparticles in combination with integrin-binding molecules and the recently developed CRISPR-Cas system (Harrop and Carroll 2006, Chen and Chen 2011, Marelli *et al.* 2013, Donohoue *et al.* 2017). These methods have been mainly used in the development of cancer therapies. However, these techniques still require further investigations as the efficacy of targeting cells still needs improvement as well as the safety for patients cannot be fully guaranteed yet (Nayerossadat *et al.* 2012, Shi *et al.* 2017). Another approach for direct targeting of senescent epithelial cells could be cell-penetrating peptides (CPPs), which show high selectivity towards their target cells and thereby limit potential off-target effects (de Keizer 2017). Recent phase 1 clinical studies have shown the tolerability of the CPPs (Deloche *et al.* 2014, Beydoun *et al.* 2015) and might be an appropriate tool to target senescent cells and thereby improve pathogenic conditions in IPF. Future studies will show if the efficacy and specificity of these methods can be improved so they will be viable tools in the treatment of patients with IPF.

We additionally show that WISP1/CCN4 is involved in the proliferation of lung fibroblasts and is a common downstream target of multiple pro-fibrotic pathways, including TGFβ1, TNFα and WNT signaling (Klee, Lehmann et al. 2016). Therapies directly targeting TGFβ1 or TNFα have been shown to have too many side effects or being ineffective (Raghu, Brown et al. 2008). Potential reasons for the inefficacy might be that both TGFβ1 and TNFα also are important in various physiological processes, which are also blocked by the treatment. Therefore, targeting downstream effectors of these pathways appears to be more efficient in treating lung fibrosis. Recently, the results of an open-label phase 2 clinical trial have been published that investigated the effects of a neutralizing antibody targeting CTGF/CCN2 (Pamrevlumab) (Raghu *et al.* 2016). It was shown that the antibody is safe for use in IPF patients and showed promising results in regard to changes in FVC and radiographic patterns. Importantly, follow-up study with 103 IPF patients (phase 2b; PRAISE study, randomized, double-blinded) further confirmed the anti-fibrotic effects of this treatment, as patients showed a significantly lower reduction of lung function compared to placebo-treated patients (Gorina *et al.* 2017). Furthermore, the effect of Pamrevlumab are comparable to the effects seen for Pirfenidone and Nintedanib, but Pamrevlumab showed less side effects in these two phase 2 studies compared to the approved drugs for IPF treatment. These results are encouraging regarding targeting of WISP1/CCN4, as it shows that ECM-bound molecules can be targeted using an antibody approach. As we have shown here (Klee, Lehmann et al. 2016) and in previous publications (Konigshoff, Kramer et al. 2009, Berschneider, Ellwanger et al. 2014), WISP1/CCN4 contributes to different pro-fibrotic mechanisms connected to lung fibrosis and targeting WISP1/CCN4 *in vivo* in a fibrosis mouse model led to an overall improvement of multiple measures, incl. survival, lung function and lung morphology. Therefore, developing a human antibody targeting WISP1/CCN4 is a promising strategy in IPF therapy (NIH Grant N° 1R43HL122078-01A1).

The data presented here further deepen our understanding of the pathological mechanisms of IPF. This knowledge will further contribute to the development of personalized medicine in the treatment of IPF, which will finally result in improved therapeutic strategies that ultimately could halt or even reverse the progression of IPF. Further studies will be needed to identify the challenges and opportunities in targeting senescent cells or WISP1/CCN4 in IPF and to further develop the presented findings into therapeutical approaches.

9.7. Limitations and future directions

In the present work, I could show that WISP1/CCN4 is a downstream target of several pro-fibrotic pathways involved in lung fibrosis, including TGFβ1 and TNFα, in primary human lung fibroblasts. However, if this is a general mechanism still requires further analysis.

I also showed that the expression of IL6 is dependent on the presence of WISP1/CCN4 in phLFs. Additionally, the removal of senescent cells in *in vitro* assays of primary mouse ATII cells reduced the levels of both WISP1/CCN4 and IL6. However, if there is a direct link between these proteins in regard to senescence was not subject of investigation in the presented work. Here, the results suggest that WISP1/CCN4 has an effect on NF-κB-mediated signaling. It will be of interest to elucidate how WISP1/CCN4 potentially influences NF-κB in regard to its expression, translocation and activity. Thereby, WISP1/CCN4 could potentially affect the expression of IL6 and other NF-κB target genes. Moreover, I could not reveal a receptor for WISP1/CCN4, which will be important for further understanding the mechanisms of WISP1/CCN4-mediated signaling, especially in regard to therapeutic options. Blocking the receptor, which WISP1/CCN4 requires for its pro-fibrotic signaling, would offer new treatment strategies for IPF patients.

Moreover, the results presented in this thesis originate mainly from *in vitro* and *ex vivo* studies. A knockout model of WISP1/CCN4, either general or cell type-specific, could bring further insides on how WISP1/CCN4 affects signaling *in vivo*, especially in regard to the development and progression of lung fibrosis. Future studies of this model will reveal how WISP1/CCN4 affects different signaling cascades *in vivo*. Moreover, WISP1/CCN4 is mainly produced by ATII cells. A cell-type specific knock out model like the SPC-Cre mice offers the possibility to specifically delete WISP1/CCN4 in ATII cells (by flanking the WISP1/CCN4 gene with loxP sites). This could help to understand the role of WISP1/CCN4 in these cells on different aspects, including the effect of WISP1/CCN4 on senescence in ATII cells and the effect of ATII cell-derived WISP1/CCN4 on the development of lung fibrosis.

10. References

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Affidavit

I hereby declare, that the submitted thesis entitled:

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Biberach, 14.03.2018	Stephan Klee
Place, date	Signature of doctoral candidate

Confirmation of congruency between printed and electronic version of the doctoral thesis

I hereby declare, that the electronic version of the submitted thesis, entitled:

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is congruent with the printed version both in content and format.

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