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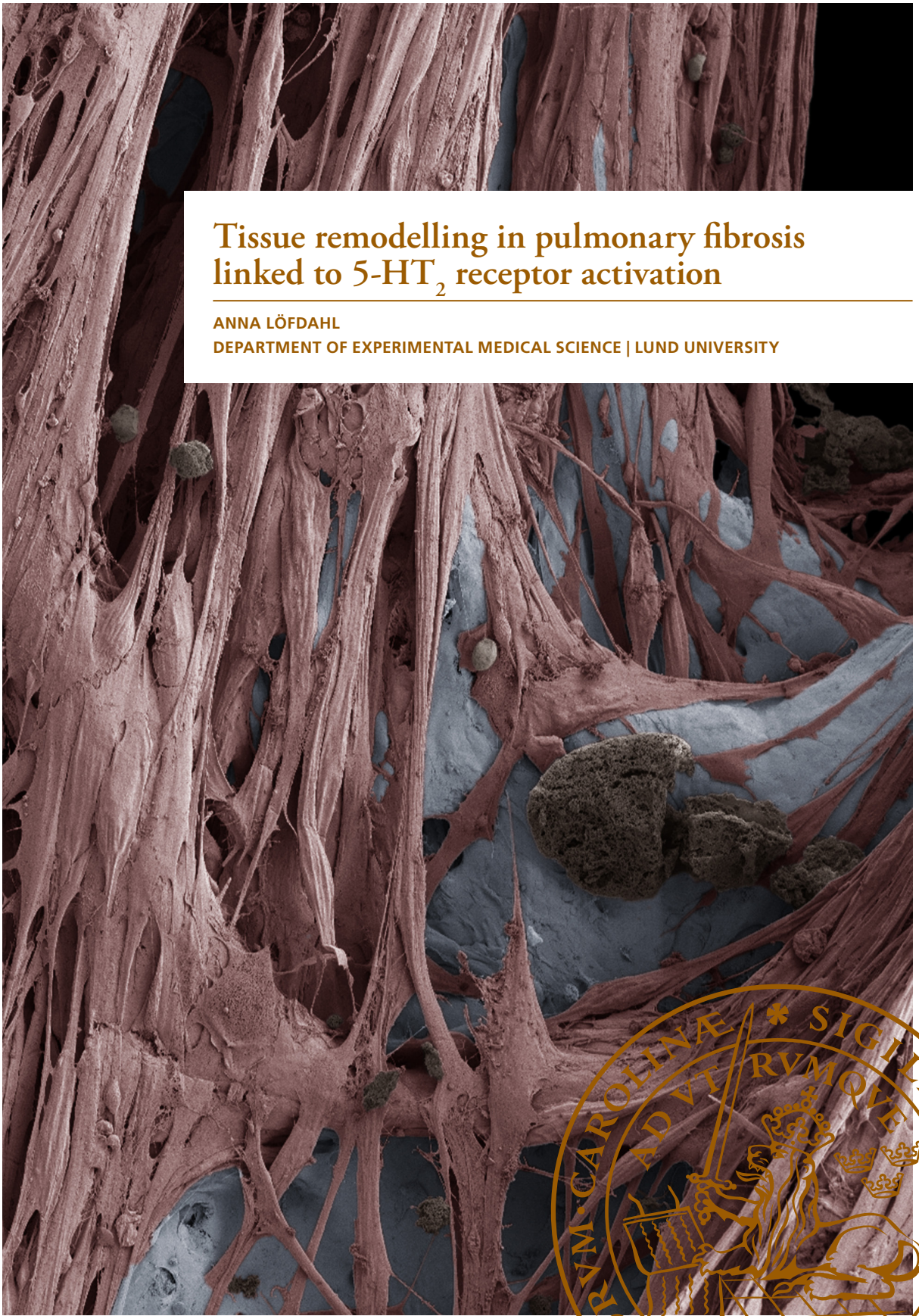

Tissue remodelling in pulmonary fibrosis linked to 5-HT_2 receptor activation

ANNA LÖFDAHL
DEPARTMENT OF EXPERIMENTAL MEDICAL SCIENCE | LUND UNIVERSITY

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Anna Löfdahl

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

After an injury, an immediate reparative response is triggered to replace the damaged tissue, however, in fibrosis, cells remain active despite wound resolution causing a steady build-up of fibrotic tissue. In pulmonary fibrosis, there is a constant regeneration and remodelling of lung tissue where the thin architecture of alveoli become thickened, hampering efficient diffusion of oxygen, as seen in diseases like idiopathic pulmonary fibrosis (IPF). IPF is a detrimental respiratory disease marked with enhanced extracellular matrix (ECM) deposition generating structural alterations in the lung. To provide new insight into the underlying pathological mechanisms of pulmonary fibrosis, this thesis has examined how pro-fibrotic cellular responses may be driven by a specific receptor and how surrounding ECM may affect the disease progression of IPF. Serotonin (5-HT) and its 5-HT_{2B} receptor have been implicated in several fibrotic conditions, acting as important mediators during fibrosis. In our studies, examination of 5-HT_{2B} receptor antagonists showed several anti-fibrotic effects, reducing both myofibroblast differentiation and fibroblast proliferation as seen in human cell cultures, as well as attenuated production and deposition of ECM as observed in mouse models of pulmonary fibrosis. Beneficial add-on effects with the 5-HT_{2B} receptor antagonists on bronchodilation and immune modulation were shown *ex vivo* and *in vivo*, respectively. The continuous induction of fibroblast activity in IPF renders an altered ECM with a unique protein composition that influenced cellular behaviour, as shown in this thesis. To elucidate the interplay between cells and ECM we have examined the fibroblast response to IPF-derived ECM, using decellularized lung scaffolds repopulated with healthy lung fibroblasts. The IPF-ECM with enhanced tissue density and stiffness, programmed the fibroblasts to rebuild a IPF-like matrix, lacking normal production of basement membrane proteins as well as enhanced early induction of disease associated proteins such as periostin and tenascin-C, and the proteoglycans decorin, versican and biglycan. Collectively these results show that several profibrotic responses can be attenuated by 5-HT_{2B} receptor antagonism, identifying the 5-HT_{2B} receptor as a promising target for pulmonary fibrosis. Fibroblast activity is evidently influenced by surrounding ECM, triggering pathological remodelling of the lung resulting in cellular activation. Taken together, the ECM niche plays an important role in the disease mechanisms leading to progression of IPF.

Key words Serotonin, serotonin class 2 receptor antagonist, fibroblast, pulmonary fibrosis, extracellular matrix

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**contributed equally as authors*

Selected Abbreviations

5-HT	5-hydroxytryptamine
α-SMA	Alpha-smooth muscle actin
BLM	Bleomycin
BM	Basement membrane
ECM	Extracellular matrix
GPCR	G-protein coupled receptor
IL-1β	Interleukin-1beta
ILD	Interstitial lung disease
IPF	Idiopathic pulmonary fibrosis
PCLS	Precision cut lung slices
SMC	Smooth muscle cell
TNF-α	Tumour necrosis factor-alpha
TGF-β1	Transforming growth factor-beta 1

Background

Pulmonary fibrosis

Pulmonary fibrosis is a condition characterized by the build-up of connective tissue in the lung. Imbalanced levels of pro-fibrotic and anti-fibrotic mediators cause a shift in the cellular production and deposition of extracellular matrix (ECM) proteins, resulting in gradual replacement of healthy tissue with fibrotic tissue. Resident cells such as lung fibroblasts assist in the accumulation of fibrotic tissue in parenchymal areas, creating a barrier that hinders efficient oxygen exchange. Newly synthesized ECM proteins become incorporated into the tissue and affect lung elasticity and structural organisation. As a result of the constant regeneration of tissue and the lack of proper cellular regulation, the remodelling process is able to remain active with formation of fibrotic tissue. Collectively, these processes result in severe breathing difficulties and lung function decline. Pulmonary fibrosis may develop as a pathological feature in autoimmune diseases such as rheumatoid arthritis or systemic sclerosis, and is the main pathological feature in interstitial lung diseases (ILDs) such as idiopathic pulmonary fibrosis (IPF) [1-3].

Idiopathic pulmonary fibrosis

A serious type of ILD is IPF, a progressive and fatal disease with a mean survival of 2-3 years post diagnosis [3]. The aetiology of IPF, as the name implies, is unknown, however, risk factors have been associated with the disease such as age, gender, environmental exposures, genetic factors and smoking [4-6]. It was estimated from five large database analyses, collected from 1991 to 2012 that the incidence lies between 3-9 cases of IPF per 100 000 inhabitants per year in Europe, with a predisposition to men above 55 years of age [6, 7]. Diagnosis of IPF is based on combinatory radiological characterisation, lung function testing and clinical symptoms, sometimes supported with histopathological investigations. On high resolution chromatography (HRCT), patients with IPF demonstrate a usual interstitial pattern (UIP) of the lung, showing honeycombing (cystic air cavities separated by thick fibrotic walls), traction bronchiectasis and reticular changes, with basal and subpleural distributions [8]. The fibrotic areas are often heterogeneously distributed, with lung tissue areas appearing healthy, localized adjacently to dense fibrotic tissue (Fig. 1). When HRCT, lung

function testing and clinical symptoms are unclear for diagnosis, lung biopsies may be needed for histological analysis. IPF is characterized by the UIP pattern, featuring areas of densely packed avascular fibrotic tissue lined with proliferative fibroblasts known as fibrotic foci [9].

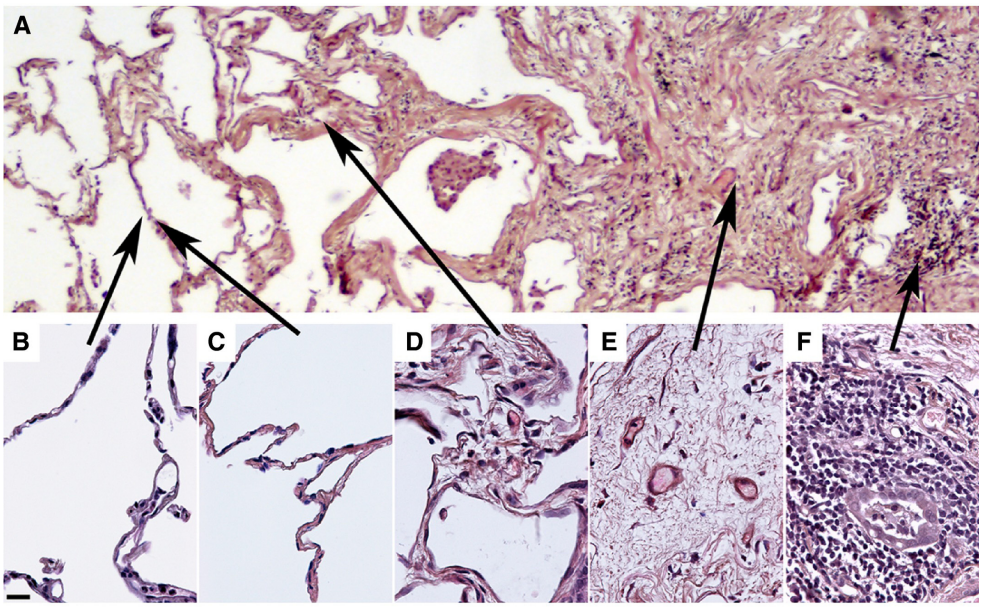


Figure 1. Border zones of healthy and fibrotic lung tissue.
Images of parenchymal IPF tissue (A) and healthy control (B). Enlargements of specific structures in IPF tissue with parenchyma illustrating border zones of normal-looking areas (C) situated adjacent to densely fibrotic areas (D). Densely fibrotic zones (E) were situated next to areas with inflammatory infiltrates (F). Scale bar = 20 µm. Reproduced image from Physiological Reports 2016 Mar; 4(5): e12727; DOI 10.14814/phy2.12727.

Today, IPF is an irreversible disease with lung transplantation as the only available curative treatment, as current pharmacological treatments are limited to attenuate disease symptoms with moderate effects on disease progression and life expectancy. Pharmacological treatment of IPF is currently concentrated on nintedanib - a tyrosine kinase inhibitor that inhibits vascular endothelial growth factor (VEGF) receptor, platelet derived growth factor (PDGF) receptor and fibroblast growth factor (FGF) receptor [10]; and pirfenidone - a small anti-fibrotic agent with multiple and unidentified molecular targets. Pirfenidone and nintedanib have been shown to enhance pulmonary function in patients with IPF and improving forced vital capacity (FVC) [11-13]. Several side effects have been reported with these treatment options such as nausea, skin rashes, dyspepsia, photosensitivity and diarrhea, which could result in dose reductions in affected patients. However, a recent study by Crestani *et al.* supports the long-term usage (median 44.7 months) and tolerability of nintedanib in patients with IPF [14].

Recent discoveries have also identified galectin-3 inhibitors as a new potential strategy in treating IPF, with initial results showing the drug as safe and well tolerated [15]. This small molecule, showing promising results in mouse models of pulmonary fibrosis [16], has now entered a phase IIb study to evaluate efficacy and safety in IPF patients.

Collectively, the current therapeutics are targeted on reducing cell-mediated fibrotic responses, which ultimately halters the accumulation of fibrotic tissue. However, no treatment shows the ability to reverse already established fibrosis. To be successful in designing effective treatments, it is essential to understand the underlying disease mechanisms that propagate the manifestation of fibrotic tissue and the continuation of pro-fibrotic cellular activity. Studies have shown that fibroblasts are highly influenced by which substrate they are cultured on, regardless of cellular origin [17], emphasising an important interplay between cells and their microenvironment. It is becoming more evident that the ECM acts as a powerful regulator of cell behaviour and that the ECM structure and composition affect repair responses and disease progression [17, 18]. Therefore, the development of new anti-fibrotic treatments must take into account the influential effects of the fibrotic ECM as an important disease mechanism driving the dysregulated cellular response in pulmonary fibrotic tissue.

The pulmonary tissue

The lung is composed of a branched structure of airways descending from the trachea to the two major central bronchi (approx. 14 mm lumen diameter) [19], to the bronchioles, which organize into smaller terminal and respiratory bronchioles. These structures connect to small alveolar compartments, the alveoli (200 µm diameter) - structures that are surrounded by capillary vessels to facilitate oxygen exchange in the respiratory zone [20]. The alveoli are lined with alveolar epithelial cells (AEC) type I and type II, which are surrounded by the interstitium, a compartment between alveoli and capillaries composed of pulmonary cells and ECM. The ECM is an intricate network of a wide range of proteins that in its unique composition constitutes a tissue-specific microenvironment for resident cells. Besides providing structural stability, the ECM has an active role in directing cellular functions, providing both mechanical and biochemical signals [21-23]. The interactions between cells and matrix are fundamental in the regulation of cellular behaviour such as differentiation, migration and proliferation. The collective protein ensemble of the ECM, the matrisome, consists of main structural core proteins such as collagens, proteoglycans and glycoproteins, together with ECM regulators, ECM affiliated proteins and secreted factors, which constitute the matrisome-associated proteins [24]. The pulmonary ECM is mainly organized into two compartments: the basement membranes (BM), lining basal sides of epithelial and endothelial cells; and the interstitium, forming the space between alveoli and capillaries. The interstitium together with the alveoli and respiratory

bronchioles, form the parenchymal tissue, which carries out the pulmonary gas exchange. A summary of key pulmonary structures and components are described below, which relates to the studies conducted in this thesis.

ECM and cellular structures of airways and vessels

Basement membrane

The BM is a thin sheet structure of ECM lining the airways, alveoli and blood vessels, with epithelial or endothelial cells anchored to it. The alveolar BM, mainly composed of collagen type IV intertwined with proteoglycans and nidogen/laminin complexes, acts as a structural barrier that physically separates the epithelial cell layer from underlying mesenchyme [25]. The BM is an important structure shaping the pulmonary tissue through connections to adjacent ECM components and facilitating cellular attachments [26]. Integrins are receptors expressed on cellular surfaces, and bind to laminins in the BM to form focal adhesions and complexes which through connections to intracellular structures affect cellular behaviour [27, 28]. In the lung, epithelial and endothelial cells anchor to the underlying BM that acts as a signalling platform for growth factors, modulating cellular responses locally [29].

Epithelial cells

Epithelial cells are attached to the BM and form the first line of defence against inhaled harmful particles. This physical barrier of airway epithelium consists of multiple cell phenotypes showing a distinct spatial distribution on airway surfaces (Fig. 2). The conducting airways (descending to terminal bronchioles) are lined with ciliated epithelial cells, mediating mucociliary clearance of pathogens along with goblet cells that produce and secrete mucins into the airway lumen to form a protective mucus layer [30]. In the airway basal lamina, underlying the epithelial layer, basal cells form a separate layer able to differentiate and regenerate the epithelial layer above [31].
The lower airways are coated with AEC I and AEC II, which forms the alveoli. AEC I is the most abundant cell type that creates the area for gas diffusion. AEC II are considered progenitor cells able to transdifferentiate into AEC I and replenish an injured epithelium. Another important function of the AEC II is the production of large amounts of surfactants, a mixture of substances that reduce surface tension in the alveoli. The alveolar epithelial cells are implicated as important regulators in matrix remodelling, providing sources of fibroblast-like cells through epithelial-mesenchymal transition (EMT), as seen in a mouse model of pulmonary fibrosis as well as in fibroblastic foci in patients with IPF [32, 33].

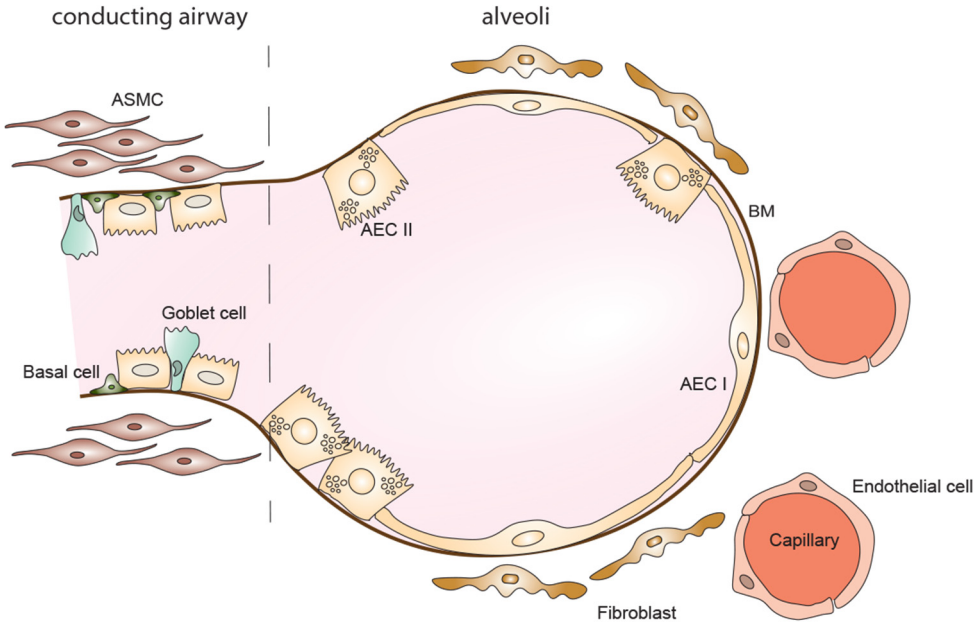


Figure 2. Schematic figure of conducting respiratory airway.
Conducting airways descend from bronchi to narrower formations of bronchioles. Several subtypes of epithelial cells, anchored to the underlying basement membrane (BM), line the conducting airways. Intertwined in the epithelial layer are the mucin producing goblet cells and situated underneath are the basal cells, which are progenitors able to renew the airway epithelium. Airway smooth muscle cells (ASMC) surround conducting airways, regulating bronchoconstriction and bronchodilation. Descending segments of terminal bronchioles connect to respiratory bronchioles to distal alveolar sacs, which form the respiratory zone. In the parenchymal space, resident fibroblasts regulate the production and degradation of ECM. The endothelial cells lining the capillaries are in close proximity to the alveoli, composed of alveolar epithelial cells type I (AEC I) and type II (AEC II), in order to facilitate efficient oxygen exchange.

Endothelial cells

Surrounding the alveoli are the pulmonary capillaries facilitating pulmonary transportation of deoxygenated and oxygenated blood. Endothelial cells compose the inner lining of blood vessels and are described as a multifunctional cell type regulating vessel tone, inflammation and remodelling processes with several pathological conditions associated with endothelial damage or dysfunction [34, 35]. In animal models mimicking pulmonary fibrosis, the properties of endothelial cells are shifted, showing increased expression of fibrotic mediators and heightened response to transforming growth factor (TGF)-β1, resulting in elevated levels of collagens and alpha-smooth muscle actin (α-SMA) [36]. The anatomically close distance between epithelial and endothelial cells suggests an important cellular interplay between these cell types in fibrotic tissue development. Microvascular injuries have been confirmed in lung biopsies derived from IPF patients with an increased amount of circulating antibodies against endothelial cells [34]. Following vessel injury, exposed BM components such as collagen type IV, provide attachment and aggregation of

circulating platelets. Activated endothelial cells secrete platelet activating factor (PAF), fibrinogen and von Willebrand factor, providing further activation and adhesion of platelets resulting in blood clot formation and release of mediators such as serotonin (5-HT) [37]. To minimize bleeding during injury, there is an immediate local contraction of vessels, a function executed by the surrounding smooth muscle cells (SMC).

Smooth muscle cells

Vascular and airway motor tones are regulated by sheets of SMC, a cell type able to shift between a synthetic phenotype, secreting mediators affecting cellular proliferation and growth; and to a contractile phenotype, controlling dilatation and contraction [38]. With ongoing inflammation and cellular damage, airways, as well as pulmonary vessels, undergo chronic remodelling with increased SMC layer causing airway narrowing with restricted airflow as a result [39, 40]. The pathobiology of airway SMC has been extensively studied in asthma, a disease marked with bronchial SMC hyperplasia [41]. The bronchial SMC are, besides their evident effect on airway size, responders to and producers of inflammatory stimuli, portraying the SMC as an inflammatory cell able to induce mast cell degranulation [42]. Release of mast cell mediators may influence ECM deposition, as the mast cell mediator tryptase has been shown to increase collagen synthesis in human lung fibroblasts [43] .

The lung interstitium

The lung interstitium constitutes the region between the alveolar epithelial cells in airways and the endothelial cells constituting the capillaries. This matrix space is composed of an intricate network of ECM proteins such as collagens, proteoglycans and glycoproteins, and resident cells such as matrix-producing fibroblast.

Collagens

The most abundant group of proteins expressed in the ECM contains the collagens, a class of proteins composed of three polypeptide chains forming a triple helix [44]. In the form of procollagens, the precursors, triple helix structures are secreted into the extracellular space where intermolecular crosslinking and larger macromolecular organization occur. Depending on structure, collagens are divided into different groups; fibril-forming collagens, fibril-associated collagens, network-forming collagens, anchoring fibrils, transmembrane collagens as well as other structural groups, all with specialized tissue functions and organisations [45]. Fibril-forming collagens are the most biologically represented, that generate thick collagen fibres from staggered covalently cross-linked fibrils. Running parallel to alveolar walls, collagen fibres maintain alveolar stability and elasticity through mechanical interactions with elastins and proteoglycans [46].

Proteoglycans

Proteoglycans constitute critical components of the lung ECM, controlling cellular biological functions and tissue mechanics to maintain tissue homeostasis. Proteoglycans are negatively charged macromolecules, composed of a core protein with attached glycosaminoglycans (GAGs). Proteoglycans are distributed throughout the lung and are integrated in the ECM, in cellular plasma membranes or intracellularly [47]. The ECM proteoglycans are divided into large chondroitin sulphate proteoglycans, such as versican; small leucine-rich chondroitin sulphate proteoglycans, such as decorin and biglycan; and heparan sulphate proteoglycans such as perlecan. Versican is predominantly expressed in the parenchymal ECM with regulating functions on myofibroblast differentiation [48]. Interestingly, versican was found within the fibroblastic foci in IPF lungs, further indicating its functional role in tissue remodelling [49]. Decorin and biglycan are bound to collagens and influence remodelling processes through effects on fibroblast migration and modulation of TGF-β1 activity [50, 51]. In border zones in IPF lung tissue, where more and less remodelled parenchymal areas align, the expression of highly sulphated heparan sulphates were found co-localized with the core protein of perlecan. Perlecan is an important BM protein in pulmonary vessels [52] and influenced by VEGF [53]. Interestingly, the total amount of GAGs were increased in IPF lungs in comparison to healthy lungs, seen in both dense and less dense fibrotic tissue areas [54].

Glycoproteins

Glycoproteins are composed of a core protein with glycosylated attached carbohydrates, a protein group that constitutes 197 out of the 274 core matrisome proteins identified in human *in silico* based proteome [55]. The glycoproteins fibrillins are important in the assembly of elastic fibres and maintenance of elastic recoil, a vital pulmonary function in organizing tissue at mechanical stretch during physical breathing. In IPF an imbalanced presence of elastic fibres has been described and is believed to affect tissue stiffness and disease progression [56]. The fibrillin microfibers act as a signalling platform affecting the bioavailability of ligands such as TGF-β1 [57], a pro-fibrotic and multifaceted cytokine able to induce the production of several ECM proteins such as tenascin-C [58, 59]. Tenascin-C is a glycoprotein expressed upon tissue injury with effects on fibroblast migration, differentiation and ECM production as seen in human skin fibroblasts [60]. The incorporation of tenascin-C into the ECM is assisted by periostin, a multifunctional secreted glycoprotein that is able to promote allergic inflammation and fibrosis [61, 62]. In patients with IPF, periostin in lung tissue was increased along with elevated plasma levels, which correlated with clinical progression [61]. The progressive worsening in disease may be governed by the ability of periostin to induce myofibroblast differentiation, as collagen I production and TGF-β activation was upregulated in human lung fibroblasts treated with periostin [63].

Fibroblasts

Fibroblasts are the main producers of ECM proteins and crucial in fibrotic diseases where they contribute to deposition and remodelling of ECM. In the distal lung, fibroblasts are situated in the interstitium, in close proximity to alveoli, as well as around airways and pulmonary vessels (Fig. 2). In pulmonary fibrosis an increased number of fibroblasts are found and the suggested sources of fibroblasts are many such as originating from bone marrow-derived fibrocytes, pericytes, and EMT or endothelial-mesenchymal transition [8, 64, 65] The elongated fibroblasts are distributed throughout the connective tissue of the lung with evident heterogeneity and plasticity, as distally and centrally derived primary human lung fibroblasts differ in their ability to proliferate and synthesize ECM proteins in health and during disease such as asthma [66] and chronic obstructive pulmonary disease (COPD) [52]. Murine lung fibroblasts display two subpopulations, based on the expression of the surface receptor Thy1, showing cell types with different morphological traits and antigen-presenting functions [67]. Further identification of fibroblast subtypes was recently shown in murine breast cancer tumours showing three subpopulations of cancer-associated fibroblasts, identified with distinct spatial localisations [68]. Fibroblasts from IPF patients demonstrate an altered *in vitro* secretion of hepatocyte growth factor, as well as prostaglandin E_2, which may delay alveolar epithelial repair procedures thus influencing disease progression [69, 70]. A contributing factor to the altered cell behaviour in IPF is believed to be tissue stiffness [18]. Lung fibroblasts show enhanced content of α-SMA and increased migratory capacity when cultured on stiff matrix, along with an altered cell morphology [71], events that enhance recruitment of fibroblasts into fibrotic areas.

Upon tissue injury, resident fibroblasts differentiate into myofibroblasts, a spindle shaped cell with SMC characteristics. Myofibroblasts are contractile cells containing intracellular stress fibres of α-SMA, which through integrin and matrix connections assist in wound closure [72]. Fibroblasts derived from IPF patients demonstrate an increased resistance to oxidative stress with senescent-like features [73]. Senescent fibroblasts are larger in their cellular shape and do not replicate, but remain metabolically active. With age, the ability for repair resolution following injury is reduced as seen in young and old bleomycin (BLM)-treated mice with the accumulation of senescent myofibroblasts [74]. A hallmark in IPF is the presence of fibroblastic foci identified as heterogeneous and independent structures at sites of lung injury and repair, showing large complexity and plasticity of both composition and shape [75]. These foci are able to alter the gas diffusion area in the lung by pushing capillaries away from the AEC, an event which may restrict blood flow to alveolar septa [76]. A first step in developing fibrosis is believed to be the differentiation of fibroblasts to myofibroblasts, an event that can be triggered by several potent mediators such as TGF-β1.

Mediators affecting ECM

There are several factors affecting ECM and the following section highlights some of the important mediators in pulmonary fibrosis, which have been studied in this thesis.

Transforming growth factor-beta 1

One of the most widely recognized pro-fibrotic mediators in fibrosis is TGF-β1. This protein is secreted in a biologically inactivated form bound to latency-associated protein (LAP). TGF-β1 becomes incorporated into the tissue through latent TGF-β1 binding protein (LTBP) and retained by integrins or proteoglycans such as decorin, creating a growth factor reservoir in the tissue [77]. TGF-β1 becomes biologically active when LAP is removed, exposing the receptor binding site of the protein. Cell-mechanical tensions and matrix elasticity influence the release and activation of TGF-β1 through interactions with integrins [78, 79]. Following the release of the protein by physical liberation via conformational alteration of the LTBP/LAP complex, TGF-β1 activates the heterodimeric receptor formation of TGF-βRI and TGF-βRII, initiating Smad-dependent signalling pathways as well as Smad-independent pathways, such as MAPK kinase signalling [80]. The third type of TGF-βR, a co-receptor named betaglycan, is known to influence ligand binding to TGF-βRI/II and to both induce and inhibit TGF-β signalling [81, 82]. TGF-β1 induces several biological processes such as cell proliferation, differentiation and metabolic remodelling [83]. In the lung, TGF-β1 is expressed in mast cells, alveolar macrophages, endothelial and bronchial epithelial cells as identified with mRNA transcripts, along with its presence in platelets [84-86]. In fibrosis, TGF-β1 stimulates myofibroblast differentiation, ECM production and inhibition of proteases, thus controlling the turnover of the ECM [87, 88].

Tumour necrosis factor –alpha and interleukin -1beta

The pro-inflammatory cytokines tumour necrosis factor–alpha (TNF-α) and interleukin-1 beta (IL-1β) have been implicated in many pulmonary diseases with described pleiotropic effects. In patients with IPF, mRNA levels of pulmonary TNF-α and IL-1β were increased, specifically in macrophages and epithelial cells of the alveoli [89, 90]. Several pulmonary cells produce IL-1β and TNF-α which are primarily released upon inflammatory stimuli. When systemically overexpressed, TNF-α induces remodelling processes in the lung with increased collagen deposition and loss of small airspaces along with lung infiltrates of inflammatory cells [91, 92]. Overexpression of IL-1β, through adenoviral gene transfer, triggers an acute inflammatory reaction with a successive fibrotic tissue response with elevated levels of TGF-β1 and collagen deposition [93]. Fibroblasts respond to TNF-α with enhanced cell proliferation and increased secretion of IL-6 and lumican, with the latter being able to promote fibrocyte differentiation [94, 95]. However, the functions of TNF-α are contradictive with studies identifying anti-fibrotic properties in its capacity to accelerate resolution of pulmonary fibrosis in mice [96]. TNF-α also interacts with proteoglycans such as

decorin and biglycan, thus influencing matrix turnover [97, 98]. The systemic production/release of TNF-α has in addition been shown to be influenced by endogenous exposure to serotonin (5-HT) [99].

Serotonin

The pathogenesis of pulmonary fibrosis has not been fully elucidated, however, the monoamine serotonin (5-HT) has been implicated in the development of fibrosis, recognized as an important ECM mediator. 5-HT (5-hydroxytryptamine), a multifunctional signalling molecule synthesized from the amino acid L-tryptophan, which is either incorporated into newly synthesized proteins or undergoes metabolism via two pathways of rate limiting enzymes; tryptophan hydroxylase (TPH) or indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) [100]. Following an initial hydroxylation by TPH and decarboxylation, L- tryptophan is converted to 5-HT. There are two isoforms of TPH; TPH1, expressed in neural cells and enterochromaffin cells in the gastrointestinal (GI) tract, and TPH2, expressed predominantly in the central nervous system (CNS). The major source of 5-HT is found outside the nervous system, synthesized by the enterochromaffin cells. As such, reported effects on GI motility have proven 5-HT as a potent target in the treatment of patients with irritable bowel syndrome [101, 102]. Upon secretion and release from the GI tract, 5-HT is rapidly taken up by circulating platelets via the serotonin re-uptake transporter SERT, and stored in dense granules (Fig. 3) [103]. At sites of injury, where platelets aggregate and degranulate, 5-HT stimulates initial phases of wound healing by restricting blood flow to the injured site as well as the formation of new blood vessels [104, 105], proving a regulatory role of 5-HT during wound repair.

In bronchoalveolar lavage fluid (BALF) from murine lungs, levels of 5-HT are increased during fibrosis [106], probably linked with an enhanced platelet degranulation at injured sites [107]. Additionally, both mast cells and pulmonary neuroendocrine cells have intracellular storages of 5-HT, providing another pulmonary source of 5-HT [108, 109]. Several serotonergic effects are coupled with pulmonary functions, as 5-HT induces vasoconstriction in the pulmonary circulation [110, 111]. This effect was shown to be mediated by activation of 5-HT class 2 receptors, thus highlighting their implication in the pathobiology of arterial hypertension (PAH) and tissue remodelling [107, 112, 113]. In addition, 5-HT increases pro-inflammatory and nociceptive activity as reported *in vivo* with intraplantar injections of 5-HT in rats [114], showing the vast and pleiotropic effects of 5-HT signalling.

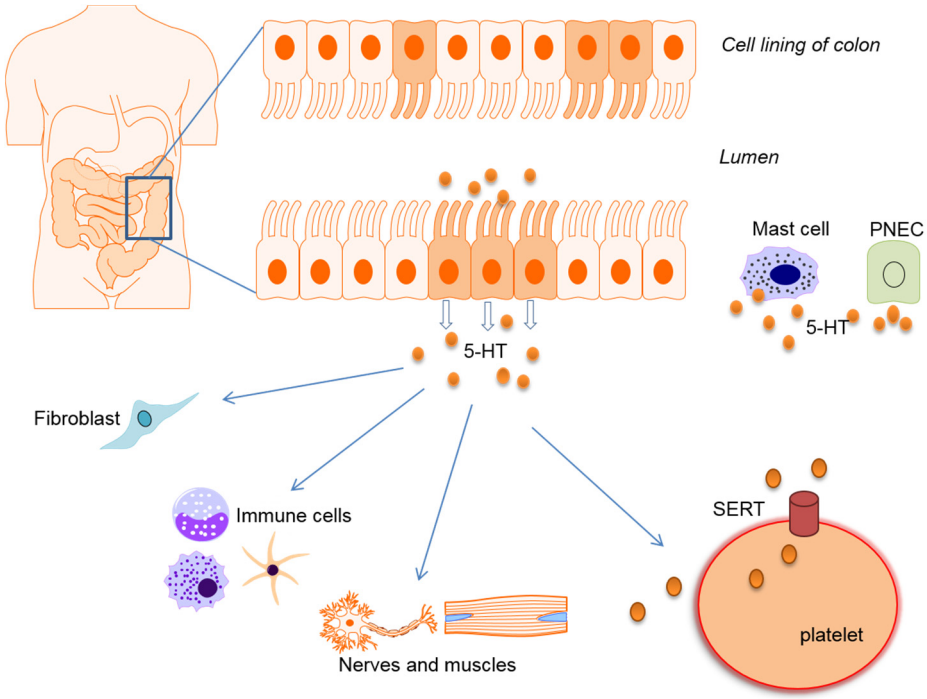


Figure 3. The release and uptake of 5-HT.
Following pathogen exposure, mechanical or chemical stimuli, enterochromaffin cells in the GI tract secrete 5-HT. 5-HT is rapidly taken up by circulating platelets via the serotonin reuptake transporter (SERT). Stored in secretory dense granules, 5-HT is released upon activation of platelets, mast cells and pulmonary neuroendocrine cells (PNEC). 5-HT activates fibroblasts and immune cells such as macrophages, dendritic cells and mast cells, along with modulating effects on pain and gut motility.

5-HT class 2 receptors

5-HT binds to at least 14 human classes of 5-HT-receptors, all with specific tissue distribution [115]. The receptors are divided into subgroups, where the class 2 receptors are further subdivided into 2A, 2B and 2C, all of which are G-protein coupled receptors (GPCRs) (Fig. 4). By binding to its receptor, 5-HT initiates a conformational change in the intracellular trimeric structure of the GPCR, triggering a signalling cascade. The activated 5-HT_2 receptor propagates the signal by interacting with effector proteins such as phospholipase (PLC) and inositol 1, 4, 5-trisphosphate (IP_3), which ultimately triggers Ca^{2+} release. Elevated levels of Ca^{2+} in the cytoplasm regulate gene expression and influences cellular responses [116].

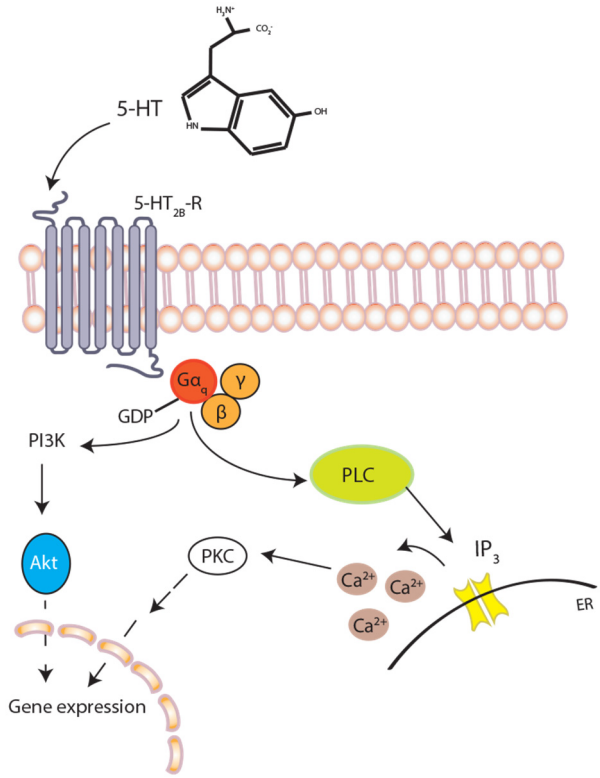


Figure 4. GPCR signalling cascade mediated through 5-HT$_{2B}$ receptor activation.
The binding of 5-HT to the 5-HT$_{2B}$ receptor initiates an intracellular signalling cascade with subsequent activation of Akt and enhanced cytoplasmic levels of Ca^{2+}, all of which influence gene expression and cellular responses.

The distribution and function of 5-HT$_2$ receptors are diverse. In the vasculature system, 5-HT$_{2A}$ receptor activation on SMCs results in vasoconstriction, while the activation of 5-HT$_{2B}$ receptors on arterial endothelial cells results in vasodilation [117]. In the respiratory system, 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors are found on bronchial and vascular SMCs, and on endothelial cells, respectively [118]. However, in lungs from patients with IPF, the 5-HT$_{2A}$ receptors have also been described to be localized on interstitial fibroblasts. Expression of 5-HT$_{2B}$ receptors was mainly localized to fibroblasts in fibroblastic foci, as compared to 5-HT$_{2A}$ receptors, and in areas of fibrotic tissue in lungs of patients with IPF [106]. Alveolar and interstitial macrophages also express the 5-HT$_{2C}$ receptor, as seen in models of pulmonary fibrosis in mice [119]. The distribution and function of 5-HT$_2$ receptors have been evaluated across species identifying variations in receptor expression and physiological responses between humans and other species [120]. This biological discrepancy needs to be recognized when studying serotonergic signalling in animal models in order to provide translationally relevant models and defendable pharmacology studies.

5-HT in fibrosis

In recent years, several studies have demonstrated potent fibrotic effects elicited through 5-HT signalling, stimulating several cellular processes that are associated with the development of tissue fibrosis. In a recent study in a mouse model of pulmonary fibrosis, 5-HT aggravated disease progression as observed in a THP-1 knock-out model [121]. These mice deficient in peripheral 5-HT were protected against experimental-induced pulmonary fibrosis, showing a marked reduction in fibrotic lesions, pro-inflammatory cell infiltration and cytokines in BALF such as interleukin 6 (IL-6) and TNF-α. Markers for oxidative stress were also downregulated in mice deprived of systemic 5-HT. Interestingly, the systemic levels of 5-HT have marked effects on dermal fibrosis, where reduced levels of 5-HT resulted in protective effects against fibrotic manifestation in skin [107], results which further identify 5-HT-associated signalling as a promising target in regulating several pro-fibrotic cellular responses in multiple organs. In normal human pulmonary conditions, levels of 5-HT are usually low due to high pulmonary expression of SERT [122]. In pulmonary fibrosis, gene expression of the rate limiting enzyme *Tph1* is increased along with downregulation of *Sert*, thus influencing 5-HT homeostasis with locally elevated levels of 5-HT, as studied in BLM-induced pulmonary fibrosis in rats [123]. Enhanced circulation of plasma 5-HT has also been shown to be coupled with cardiac changes with enhanced ECM production and thickened heart valves, mechanisms believed to be mediated through 5-HT_{2B} receptor activation [124, 125].

Inhibition of 5-HT signalling

With receptor antagonists, the binding of 5-HT to specific receptors can be blocked, thereby inhibiting serotonergic signalling. Selective as well as non-selective, 5-HT_2 receptor antagonists have been used to closely study the role of 5-HT. In BLM-treated mice, lung fibrosis was reduced with decreased lung collagen and protein levels of TNF-α, TGF-β1 and VEGF following treatment with the 5-HT_2 receptor antagonist terguride [119]. The beneficial effect of 5-HT_2 receptor antagonists as an anti-fibrotic treatment is recognized in other biological systems, as seen in heart, skin and liver. In hepatic stellate cells in the liver, a 5-HT_{2A} receptor antagonist attenuated liver fibrosis in mice through a reduction of pro-collagens and TGF-β1, events that were shown to be associated to p-Smad3 and pERK pathways [126]. The same effects were also shown with 5-HT_{2B} receptor antagonism, where inhibition of 5-HT_{2B} receptor activation with SB-204741 caused a reduction in TGF-β1 and improved liver functions in a mouse model of liver fibrosis [127]. Similar responses were also observed in scleroderma, where TGF-β1-mediated pathways were reduced following treatment with terguride in human dermal fibroblasts [128]. Still, usage of 5-HT_2 receptor antagonists as pharmacological agents are mostly focused on systemic sclerosis, schizophrenia, migraine and CNS-related disorders [129-131] where the compounds are considered safe and tolerable in patients [132].

Aims of the study

This study aimed to investigate the role of 5-HT and 5-HT_2 receptors in pulmonary fibrosis, as well as how fibrotic cellular processes may be influenced by receptor antagonism or by the ECM niche of the lung.

The specific aims of the studies were:

- To evaluate possible anti-fibrotic effects of 5-HT_{2B} receptor antagonist treatment in experimental pulmonary fibrosis *in vitro* and *in vivo*
- To further specify the underlying mechanism of the anti-fibrotic effects mediated by 5-HT_{2B} receptor antagonism in modelled pulmonary fibrosis *in vivo*
- To identify alleviating effects of 5-HT_{2B} receptor antagonism on pulmonary remodelling and airway function *ex vivo*
- To characterize tissue properties of the IPF lung and the effect of IPF ECM on lung fibroblast responses

Methods

Several experimental techniques and biological tissues have been implemented in this thesis to study isolated and precise cellular responses and pharmacological interventions to inhibit pulmonary fibrosis. In paper I-III human cell culture systems portraying key pulmonary fibrotic events were studied in connection with animal models to manifest in depth studies of the anti-fibrotic mechanism generated by 5-HT_{2B} receptor antagonism. In Paper IV, *ex vivo* culturing of decellularized human lung tissue generated a unique approach in deciphering ECM-driven disease mechanisms in IPF by using a novel human 3D-model. This model system shows promising translational features as the model mimics pulmonary physiology with patient characteristics. This chapter describes a methodological overview of important methods used in this thesis. Detailed descriptions of selected methods are found in individual papers. Human materials and animal studies were approved by local ethical committee and conducted in accordance with the ethical permission

Pulmonary cell cultures

Commercially available human fetal lung fibroblasts (HFL-1) (CCL-153, ATCC, U.S.) and primary distal lung fibroblasts, isolated from healthy human lung biopsies, were used in Paper I-II and Paper IV, respectively. The primary fibroblasts were extracted by culturing small pieces of distal parenchymal lung tissue in DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10 % fetal clone serum. The outgrowing adherent cell layer, protruding from the tissue, was further expanded and classified as primary fibroblasts. For experiments, HFL-1 cells and primary fibroblasts were used between passage 15-22 and 5-7, respectively. In Paper III, *in vitro* studies were performed with commercially available human primary bronchial smooth muscle cells (HBSMC) (CC-2576; Lonza, Switzerland).

Experimentally induced pulmonary fibrosis *in vivo*

BLM is an anti-tumour agent with pulmonary fibrosis as a serious side-effect, a feature that is believed to be the result or the induction of reactive oxygen species (ROS), causing DNA stress [133]. This cytostatic is widely used to induce experimental pulmonary fibrosis *in vivo*, a model commonly used for pharmacological evaluations

[106, 134]. The administration route and dosage of BLM are crucial for the formation and development of pulmonary fibrosis in murine models. Intratracheal (i.t.) or intranasal (i.n.) administration of BLM generates an acute model of pulmonary fibrosis that produces an initial massive inflammatory response in the lung, followed by the formation of tissue fibrosis [135]. However, repetitive subcutaneous (s.c.) injections of BLM result in a mild to moderate pulmonary fibrosis, where inflammation and fibrosis develop in parallel [135], perhaps more closely mimicking a chronic development of the condition in humans. In Paper I, C57/Bl6 mice (female, 12.5 weeks) were injected s.c. with BLM (50 IE/ animal) three times a week for a duration of two weeks. Mice received daily peroral (p.o.) administrations of 5-HT_2 receptor antagonists for the evaluation of potential anti-fibrotic effects using a prophylactic treatment approach. Lungs were removed and extracted for RNA and analysed with histology. Serum was analysed for cytokines.

Ex vivo models

Precision-cut lung slices

Precision cut lung slices (PCLS) generated from lung tissue are commonly used to examine SMC activity in peripheral airways [136, 137]. With these thin (>250 µm) tissue slices, bronchial and pulmonary vessel responses can be simultaneously studied *ex vivo.* In Paper III, low melting agarose solution filled lungs *in situ* were extracted from C57/Bl6 mice (female, >10 weeks), chilled on ice and sliced with a Krumdieck tissue slicer (Alabama Research and Development, Alabama, USA). The agarose from the solidified lung slices was removed by melting the agarose with continuous medium replacement at 37°C. Experiments were performed the following day to ensure an intact receptor expression profile of the PCLS. Since the contractile ability of an airway is affected by the size of the airway lumen [138], PCLS containing airways of similar sizes were selected for experiments and positioned in a fixed position with platinum weights. PCLS were pre-treated with 5-HT_2 receptor antagonists (20 min) followed by cumulative addition of increasing concentrations of the bronchoconstriction agent 5-HT (0.01 µM- 100 µM) with 5 minute intervals. Live images of airways were taken continuously to monitor changes in area of airway lumen (Fig. 5).

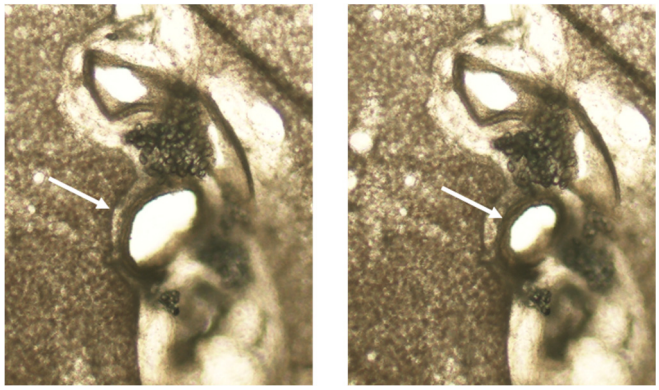


Figure 5. Airway contraction of murine bronchial airways.
PCLS derived from murine lungs were treated with cumulative doses of 5-HT to induce bronchoconstriction. Live imaging of the airways was monitored comparing untreated PCLS (to the left) to PCLS treated with 100 µM 5-HT (to the right).

Decellularized lung tissue (scaffolds)

In order to perform translatable pre-clinical research, tissue characterisations and patient variations have to be considered when examining cellular responses, aspects that are not reflected in general *in vitro* settings using artificial culturing substrates. To study isolated cell-matrix interactions we used an *ex vivo* model that studies a selected cell population, the lung fibroblast, cultured in a maintained framework of acellular human tissue, known as scaffolds. For efficient removal of cells and residual nucleic acids, cryosectioned frozen lung slices (350 µm) were treated with a detergent solution (CHAPS 8 mM, NaCl 1 M, EDTA 25 mM in D-PBS) for 4 h and benzonase nuclease for 30 min [139, 140]. It is important to consider how a detergent treatment of lung scaffolds affects the ECM with studies showing loss of several critical components following decellularization. This decellularization procedure creates acellular scaffolds with a well preserved structure and composition of the ECM [139, 140]. In Paper IV, scaffolds from parenchymal lung tissue derived from patients with IPF and healthy individuals were mounted on holders (8 mm in diameter) to maintain scaffolds in a stretched formation during fibroblast repopulation, since fibroblasts in culture contract surrounding matrix. Primary lung fibroblasts originating from a healthy donor were cultured up to nine days on each type of scaffold. This 3D-*ex vivo* model maintains a stretched formation of the lung tissue during culture along with the complex characteristic of the ECM, thus enabling more translatable studies of cell-matrix interactions seen in humans.

Compounds for evaluation

5-HT stimuli

5-HT is a strong agonist with an EC_{50} of 5 nM and the *in vitro* and *ex vivo* settings examined in Paper I-III utilized the application of 1 µM or 10 µM 5-HT. The added concentrations of 5-HT were used to study distinct agonistic effects in complex model systems, as expression patterns and turnover rates of 5-HT_2 receptors have not been clarified. Since the uptake of 5-HT is rapid and little is known about its half time, the stable derivate of 5-HT, alpha-methyl-5-HT was also examined in Paper III. Studies have shown that 5-HT may solicit its effect in synergy with other mediators, acting as a helper agonist. Pulmonary cells and PCLS were treated with 5-HT alone or in combination with TGF-β1 (10 ng/ml) to evaluate pro-fibrotic responses.

5-HT_2 receptor antagonists

In Paper I-III, the 5-HT_{2B} receptor antagonists EXT5 and EXT9 (AnaMar AB, Lund, Sweden) were evaluated for anti-fibrotic effects *in vitro*, *ex vivo* and *in vivo.* Compounds were evaluated *in vitro* at 10 µM to provide sufficient receptor occupancy for observing and detecting possible effects in cell model systems, with the exclusion of compound-mediated cellular toxicity. The receptor binding and functionality of EXT5 and EXT9 were determined *in vitro*, utilizing cells overexpressing the different 5-HT_2 receptors. EXT5 and EXT9 demonstrated an evident ability to bind and antagonize 5-HT_{2B} receptors, however, moderate affinity to 5-HT_{2A} and 5-HT_{2C} receptors were also detected. However, the interpretation of receptor functionality remains challenging as IC_{50} (half maximal inhibitory concentration) and Ki (inhibition constant) values reported *in vitro* (described for EXT5 and EXT9 in Paper I) are used as reference when studying compounds in more complex model systems, such as *in vivo* and *ex vivo* settings, creating an insufficient translational comparison between functionality analysis *in vitro*.

EXT5 and EXT9 are orally available compounds, with slightly different functionality profiles toward the 5-HT_2 receptors. Commercially available 5-HT_{2B} receptor antagonists were used as reference compounds; RS127445 (Tocris, Bristol, UK) and PRX 08066 (eNovation Chemicals LLC, Bridgewater, U.S.) and the 5-HT_{2A} receptor antagonist ketanserin (Tocris).

Biochemical analysis

Proteoglycan extraction

For studying proteoglycan synthesis *in vitro,* HFL-1 cells were cultured with the radio isotope ^{35}S-sulfate in sulfate poor medium, allowing for the incorporation and labelling of ^{35}S into newly sulfated proteoglycans. Negatively charged ^{35}S-labeled proteoglycans were isolated and extracted using diethylaminoethyl (DEAE)-52 cellulose anion exchange resin column, as further described in Paper I [52]. Individual proteoglycans perlecan, versican, biglycan and decorin were separated with gel electrophoresis. The ionizing radiation was measured with a scintillation counter and the relative increase or decrease of individual proteoglycans between samples of cell culture medium were identified, given that the number of sulfate groups on each proteoglycan is constant.

Protein synthesis with mass spectrometry

In Paper IV, scaffolds repopulated with fibroblasts were cultured in SILAC medium containing "heavy" 13C6 labelled L-Arginine-HCl and "heavy" 13C6 15N2 -labelled L-lysine-2HCl. At protein synthesis, the heavy labelled amino acids become incorporated into newly synthesized proteins, creating a shift in the molecular mass. With mass spectrometry (LC-MS/MS analysis), separating proteins based on ion mass to charge ratio, the composition of the ECM was analysed separating newly synthesized proteins (heavy) from pre-existing proteins (light) in the scaffolds [139]. Only the spanned scaffold area was isolated and examined for matrisome composition according to Paper IV. In this study, the protein levels were adjusted for tissue density to eliminate the discrepancy in tissue morphology between healthy and IPF derived scaffolds. Scaffold area, height and weight were used to calculate tissue density of scaffolds. This novel adjustment for tissue density facilitates an equal comparison of examined tissue area and cellular content in repopulated healthy and IPF scaffolds.

Proliferation assays

HBSMC and HFL-1 cells were examined for cell proliferation with three different methods. With a colorimetric BrdU ELISA assay; cells were cultured with BrdU, a synthetic nucleoside that becomes incorporated into the DNA of newly dividing cells. With a sandwich ELISA, using antibodies binding to BrdU in permeabilized cells, the amount of BrdU was measured, correlating to the relative amount of active dividing cells. Another method for proliferation measurement utilizing DNA quantification is the usage of crystal violet, a dye that binds to double stranded DNA. This colorimetric assay determines the total relative amount of cells per sample, including dividing and non-dividing cells.

Cell cycle progression can be more closely studied using flow cytometry, a method distinguishing cell cycle phases. In Paper II, HFL-1 cells were incubated for 10 min with the nuclear fluorescent stain 7AAD and examined with flow cytometry, analysing

the emitted fluorescence light by each cell in suspension. During cell cycling, dividing cells (entering S, G2/M phases) contain more DNA (7AAD labelling) than non-dividing cells (G0/G1 phases). Distinguished with flow cytometry, the fluorescent light of 7AAD separates the dividing cell from the non-dividing cells into two cell cycle peaks. Cellular counts of the two peaks separate the cell cycle distribution of S, G2/M phases from GO/G1 phases.

Viability assays

To evaluate cellular toxicity in cells treated with 5-HT_2 receptor antagonists, measurements of cell necrosis and cell viability were performed *in vitro*. Upon cell necrosis, the plasma membrane breaks down resulting in cytoplasmic leakage of intracellular content such as enzyme lactate dehydrogenase (LDH). The enzymatic activity of LDH was measured in cell medium using a colorimetric assay, which correlates to the relative amount of LDH in cell medium. Cell viability was evaluated with tetrazolium salt, WST-1, which is cleaved to formazan, by the succinate-tetrazolium reductase system expressed in mitochondria in metabolically active cells. Formazan is measured by a colorimetric assay, relating to the relative amount of viable cells.

Protein quantification

With enzyme-linked immunosorbent assay (ELISA) medium from cultured PCLS exposed to 5-HT and 5-HT_2 receptor antagonists were analysed for TGF-β1, as described in Paper III. This assay measures the total content of TGF-β1, not taking into consideration active or non-activated forms of TGF-β1.

By using a Multiplex ELISA, multiple analytes can be studied simultaneously in a small sample volume. In a 96-well plate, wells are coated with 10 individual areas with antibodies directed to a specific analyte. With electrochemiluminescence technology, signal from secondary antibody labelling is amplified and analyte quantity is analysed. Serum extracted from BLM-treated mice in Paper I was analysed with a panel of 10 pro-inflammatory cytokines; interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, keratinocyte chemoattractant/growth-regulated oncogene (KC-GRO) or interferon gamma (INFγ) and tumour necrosis factor-alpha (TNF-α).

With western blot, the relative quantity of specific proteins in a sample was assessed through combination of gel electrophoresis and antibody labelling. In short, proteins from cell lysates were separated using standard gel electrophoresis methodology. For detailed information, see Method section in Paper I and Paper II. Bands of proteins were electrostatically transferred to a membrane and labelled with fluorescent conjugated antibodies targeting proteins of interest. Protein bands, made visible at specific excitation wavelength of light were quantified with densitometry, a method

allowing for multiple comparison of proteins simultaneously in different types of samples.

Gene expression

Gene expression of a selective number of genes can easily and swiftly be studied using real time -quantitative polymer chain reaction (rt-qPCR), where the genes are amplified and detected with sequence-specific primers, binding to genes of interest. In the cell, messenger RNA in the cytosol, the copied DNA sequence of a gene, is translated to proteins in the ribosome. With rt-qPCR, the mRNA, retrieved and isolated from cell lysates with RNeasy Mini Kit (Qiagen) was converted to complementary DNA (cDNA), a sequence of nucleotides reflecting the protein-coding gene. With probes constructed with a fluorescent reporter dye, binding to specific complementary nucleotide sequences, genes of interest were amplified and the relative gene expression was quantified based on the PCR cycle where threshold of fluorescent intensity was reached.

In Paper II, a whole genome microarray was performed at SCIBLU (Lund, Sweden), examining distal lung tissue samples extracted from mice used in the BLM-model (Paper I). With Illumina BeadChips, RNA samples were converted to florescent-labelled complementary RNA (cRNA) strands, and bound through direct hybridization to the BeadChips, consisting of over 45 000 probes with each bead coated with the complementary gene sequence of a protein. Fluorescent intensity was analysed at each bead location, corresponding to transcript quantity of the sample.

Histology

With standard immunohistochemistry and immunofluorescent methodology, proteins e.g. ECM associated proteins (proteoglycans and collagens) and 5-HT_2 receptors were detected in monolayer cell cultures and in lung tissue. Myofibroblasts were identified as cells double-positive for α-SMA and prolyl-4hydroxylase (P4H), the enzyme used for collagen synthesis. Staining with Masson's Trichrome was used to identify collagen fibres in lung tissue through the acidophilic properties of the stain. To maximize antigen-antibody binding in samples that were fixed and embedded in paraffin, a process which may mask the antigens, antigen retrieval was performed with either enzymatic degradation or with heat-induction. For detailed information, see Method section in Paper I, II and IV.

Biomechanical measurements

In Paper IV, biomechanical properties of lung tissue were evaluated for tissue elasticity and stiffness with an organ bath (EMKA technologies), a system that enables to study small changes in tissue tension in a controlled environment such as tissue contraction and dilatation, via isometric force-displacement transducers using the IOX acquisition

system (EMKA technologies, France). Native lung tissue slices and scaffolds derived from healthy donors and patients with IPF were mounted with hooks and stretched 0.1mm/s until physically breakage of the tissue or that the tissue reached maximum 10 mm. This measurement translates into the ultimate force (mN) of the tissue. Tissue stiffness was measured in scaffolds with applied consecutive increase in vertical displacement (5 % increase strain, at 0.1 mm/s rate), allowing tissue fibers to stabilize and align in between stretching. Data were analysed by the software program Data analyst (EMKA, France). Tissue stiffness (N/m) was calculated from the linear region of the force displacement curve ($k=F/\delta$).

Results

Paper I

Inhibition of the 5-HT$_{2B}$ receptor attenuates pulmonary fibrosis in human cell models and in BLM-treated mice

The lung fibroblast, which upon TGF-β1 exposure differentiates towards a myofibroblast, is regarded as a major player in fibrosis, by contributing and orchestrating enhanced tissue deposition. Commercially available human fetal lung fibroblasts were used to examine myofibroblast differentiation, by implementing a combinatory stimulation with TGF-β1 and 5-HT. The number of myofibroblasts, identified by increased intracellular levels of α-SMA, was increased following treatment with TGF-β1 (10 ng/ml) and 5-HT (1 μM) (Figure 6A, C), - an event shown to be markedly reduced upon treatment with 5-HT$_{2B}$ receptor antagonists EXT5 and EXT9 (Fig. 6B), along with a reduced production of total proteoglycans, in particular decorin.

By modulating the activation of 5-HT$_2$ receptors, beneficial effects have been observed in experimentally induced models of fibrosis, attenuating fibrosis both in skin and lung [106, 107]. BLM-induced pulmonary fibrosis is a common murine model to mimic fibrotic processes in the lung with a central pulmonary exposure to the static agent by using i.t. or i.n. administrations. However, to incorporate 5-HT-mediated responses *in vivo*, a systemic administration of BLM was implemented, as the major source of 5-HT is distributed by circulating platelets. It has been shown that after intravenous injections of BLM in mice, platelets are markedly localized to the lung with enhanced contact with the alveolar capillaries [141]. In patients with IPF, the remodelling is found in more distal regions of the lung, with inwards expansions towards central areas of the lung Hence, an *in vivo* model with the influential aspects of the pulmonary circulation to the initial fibrotic establishment may reflect early fibrotic processes. Additionally, to increase drug exposure to distal parenchymal areas, an oral delivery of anti-fibrotic compounds was used, which may enhance drug efficiency, as compared to inhaled administrations. In Paper I, we used repetitive s.c. administrations of low dosages of BLM in mice to promote a mild to moderate model of pulmonary fibrosis, where inflammation and fibrotic events overlap during disease manifestation. Mice were treated with or without daily p.o. administrations of EXT5 or EXT9 (30 mg/kg). After 14 days, EXT treatment resulted in reduced amount of pulmonary myofibroblasts with

consequential reduction in production of ECM proteins (Fig. 7). To further characterize the deposited ECM in BLM-treated mice, decorin was further evaluated showing less amounts of positive antibody-labelled tissue areas of decorin, results that correlated with our previous *in vitro* findings.

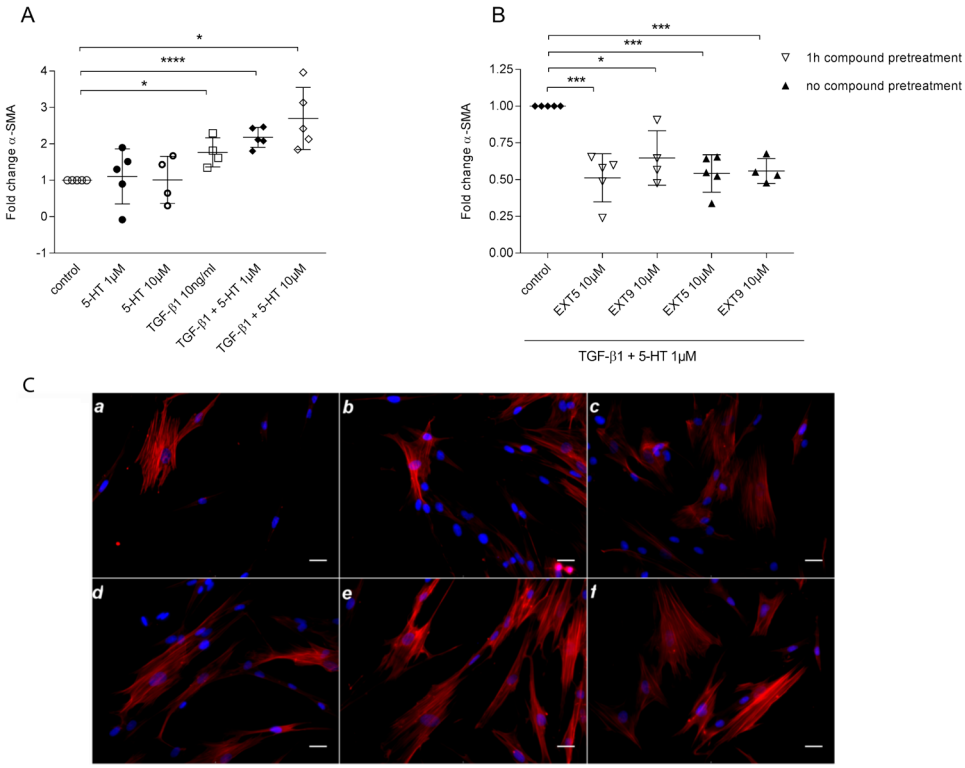


Figure 6. Myofibroblast differentiation of human lung fibroblasts.
Quantification of intercellular α-SMA in human lung fibroblasts cultured 24 h with 5-HT (1 μM ,10 μM) in combination with TGF-β1 (10 ng/ml) from western blot analysis of cell lysates (A). Fibroblasts treated with the combinatory stimuli (5-HT 1 μM + TGF-β1 10 ng/ml) were treated with receptor antagonists, EXT5 or EXT9 (10 μM), with or without 1 h pre-treatment (B). Visualization of intracellular α-SMA (red) in fibroblasts cultured in medium (a), 5-HT 1 μM (b), 5-HT 10 μM (c), TGF-β1 10 ng/ml (d), TGF-β1 + 5-HT 1 μM (e), TGF-β1 + 5-HT 1 μM (f). Scale bar = 30 μm. One sample t-test $p<0.05$*, $p<0.01$**, $p<0.001$***.

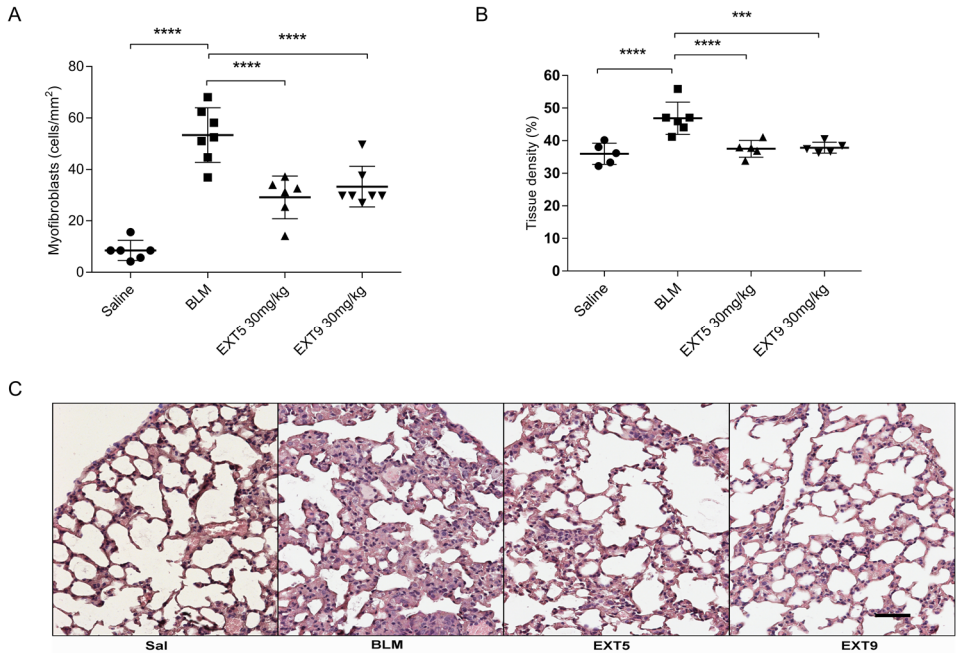


Figure 7. Pulmonary cell count and tissue deposition in BLM treated mice.
Myofibroblast count (A) in lungs of s.c. BLM-treated mice treated p.o. with EXT5 or EXT9 (30 mg/kg). Saline (Sal) was s.c. administered to animals, representing controls. Lung tissue density was quantified and visualized with hematoxylin/eosin staining (C). Scale bar =50 µm. Kruskal-Wallis with LSD post hoc test p<0.05*, p<0.01**, p<0.001***.

Furthermore, immune modulating effects were detected in serum extracted at the end of study after BLM administrations, showing reduced levels of the pro-inflammatory cytokines TNF-α and IL-1β in EXT-treated mice (Fig. 8).

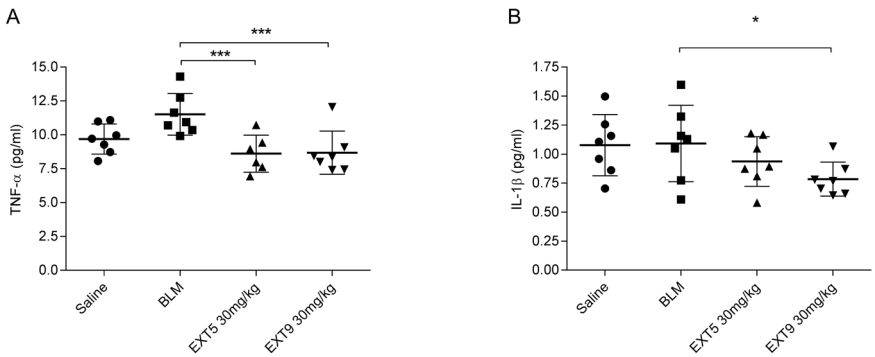


Figure 8. Serum levels of TNF-α and IL-1β in BLM-treated mice with or without daily p.o. treatment with EXT5 or EXT9.
Immune modulating effect of 5-HT_{2B} receptor antagonists EXT5 (30 mg/kg) and EXT9 (30 mg/kg) in BLM-treated mice, 14 days post study initiation, was analysed in serum with a multispot sandwich immunoassay. Protein levels of TNF-α (A) and IL-1β (B) are presented as pg/mL, n=6-7, with Kruskal-Wallis test with LSD post hoc test. *p≤ 0.05, ***p≤ 0.005.

Paper II

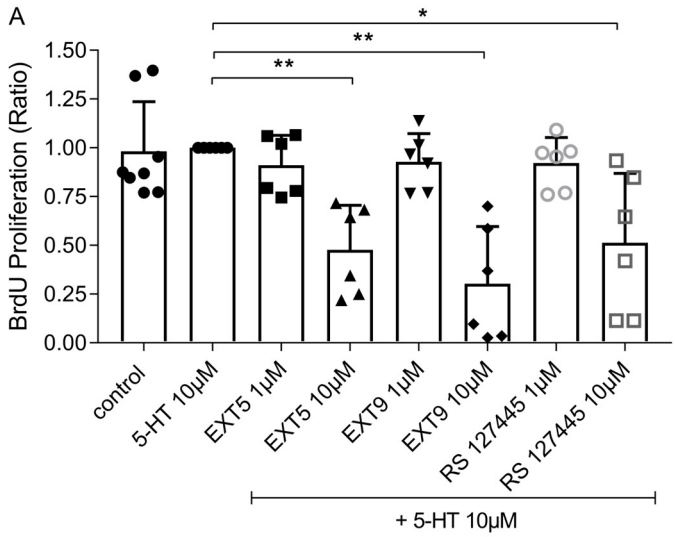
Anti-proliferative effects of 5-HT$_{2B}$ receptor antagonism on lung fibroblasts may explain anti-fibrotic mechanisms in vivo

Several cellular responses are directed through the binding of 5-HT to its cellular receptors, of which the 5-HT$_{2A}$ receptor activation has been coupled with mitogenic effects [142]. A hallmark in IPF is fibroblastic foci, a restricted area characterized with proliferating fibroblasts and myofibroblasts assisting in the deposition of ECM proteins. The subsequent thickened alveolar compartments in distal pulmonary regions of IPF results in low oxygen tension creating a negative feedback loop, with hypoxic conditions triggering fibroblast proliferation and myofibroblast differentiation [142-144]. Distally derived lung tissue from BLM-treated mice, displayed enhanced expression of *Cdkn1α*, a gene encoding the protein p21 (Table I). Treatment with EXT, especially EXT9, showed a modest reduction of *Cdkn1α*, however, not statistically significant.

Table I. Significant differentially expressed genes in BLM-, EXT5- and EXT9-treated mice, in comparison to saline controls.
Probe expression levels shown as fold change of gene symbol. Significance analysis of microarrays (SAM) was used to detect differentially expressed genes.

Fold change			
Gene symbol	**BLM**	**EXT5**	**EXT9**
Cdkn1a	2.73	2.25	2.10
Gdf15	1.55	1.52	1.51
Prc1	1.56	1.67	1.65
Cdkn1a	2.04	2.00	1.51
Cdkn1a	1.88	1.82	1.50
Phlda3	1.47	1.62	1.24
Igfbp2	1.85	1.64	1.71
LOC665235	-1.38	-1.30	-1.43

The protein p21 is upregulated in association with cellular damage and during pulmonary fibrosis, and has regulatory effects on the cell cycle. As 5-HT and 5-HT$_2$ receptors have been shown to be elevated in mice with pulmonary fibrosis, a potential link between cell cycle progression and serotonergic signalling was further examined using a human *in vitro* setting. Proliferation of human lung fibroblasts was reduced following treatment with 5-HT$_{2B}$ receptor antagonists EXT5 or EXT9 (Fig. 9A), an effect caused by the inhibition of cell dividing phases in fibroblasts (Fig. 9B).



B

Sample	Counts (%)
Control	6.08
5-HT	8.74
5-HT + EXT5	7.87
5-HT + EXT9	6.62
5-HT + RS 127445	6.6

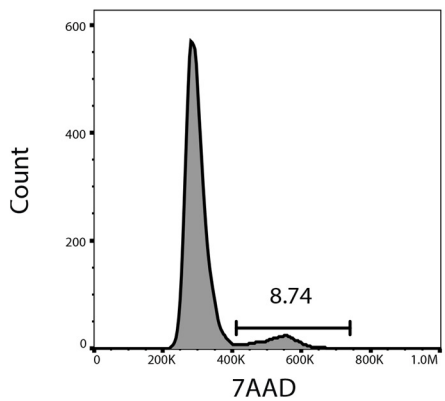


Figure 9. Cell proliferation of lung fibroblasts.
Fibroblasts were cultured 48 h with 5-HT (10 µM) with or without 5-HT2B receptor antagonists (EXT5, EXT9, RS 127445) (10 µM) (A). Quantification of total amount of cycling cells in S/G2/M phases, with distinct cell cycle peaks using flow cytometry (B). Image shows 8.74% cycling lung fibroblasts following treatment with 5-HT 10 µM. One sample t-test $p<0.05$*, $p<0.01$**, $p<0.001$***.

Cells exposed to 5-HT displayed increased intracellular p21, which was reduced with EXT-treatment. Similar tendencies were found in levels of pAkt, the upstream target of p21 (Fig. 10). These results indicate a connection between the pAkt/p21 signalling pathway and 5-HT_{2B} receptor antagonism.

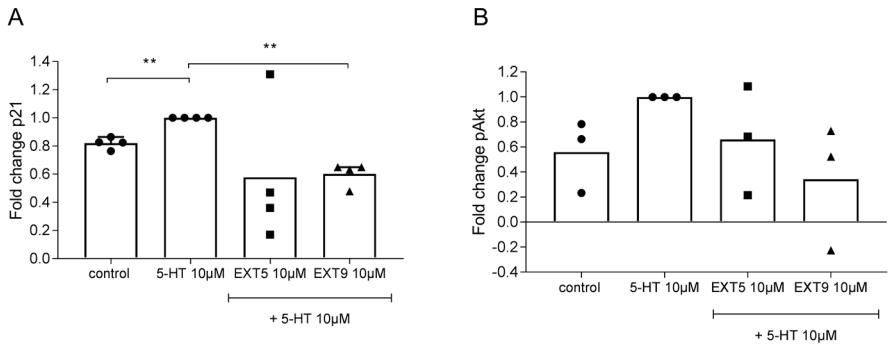


Figure 10. Protein levels of p21 and pAkt in lung fibroblasts treated with 5-HT and 5-HT$_{2B}$ receptor antagonists. Quantification with western blot of p21 and pAkt in cell lysates collected from lung fibroblasts treated 15 min with 5-HT and receptor antagonists. One sample t-test p<0.05*, p<0.01**, p<0.001***.

Paper III

5-HT$_{2B}$ receptor antagonists alleviate bronchoconstriction and pulmonary remodelling processes

Several pulmonary reconstructing processes are governed by the tissue remodelling mediator TGF-β1, a mediator which has been shown to be elevated trough 5-HT$_{2B}$ receptor activation [145]. Pathologically remodelled airways become narrowed with increased SMC layer affecting pulmonary responsiveness and functions [146], features that are commonly shown in patients with asthma. The enhanced SMC activity results in restricted air supply in pulmonary fibrotic conditions. Located adjacent to the airways and SMC are mast cells, providing local delivery of 5-HT upon cellular degranulation. In the *ex vivo* model, using murine PCLS, 5-HT induces effective bronchoconstriction (Fig. 11A), an event shown to be directed via the 5-HT$_2$ receptors and especially via the 5-HT$_{2A}$ receptor, which was blocked with ketanserin (Fig. 11B). However, the 5-HT$_{2B}$ receptor antagonists EXT5 and EXT9 also reduced airway narrowing (Fig 11C-D).

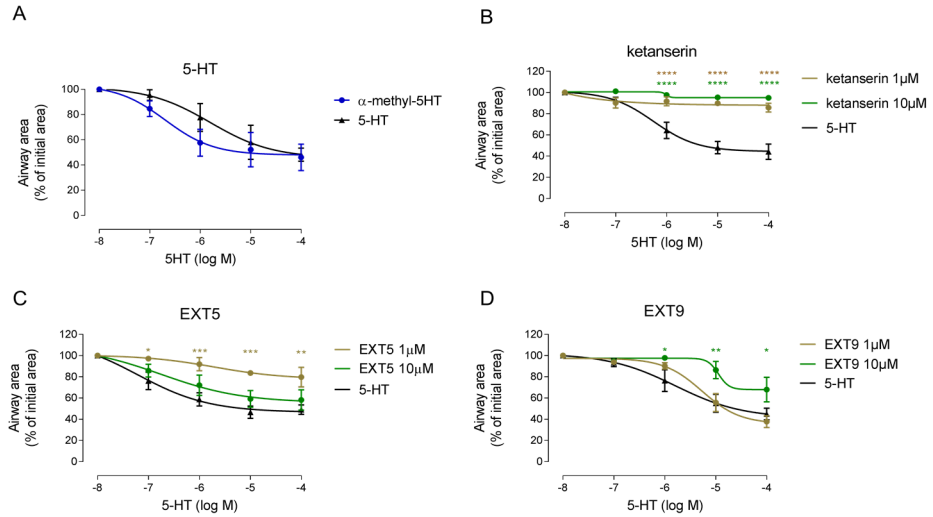


Figure 11. Effect of 5-HT$_2$ receptor antagonists on bronchoconstriction *ex vivo*.
Murine PCLS were treated with cumulative concentrations of 5-HT (A). Preventive treatment (20 min) of PCLS with 5-HT$_{2A}$ receptor antagonist (ketanserin) (B) and 5-HT$_{2B}$ receptor antagonists (EXT5, EXT9) (C-D) were performed prior addition of 5-HT. Alterations to initial airway area were monitored, with significantly differences presented in comparison to 5-HT treatment alone. Values represented as means ± SEM and analysed with two-way analysis of variance with Dunnett's multiple comparisons test. p<0.05, p<0.01**, p<0.001***.

Contractile forces are connected to pulmonary remodelling processes, as bronchoconstriction enhances the release of matrix-bound TGF-β1 [22]. 5-HT was shown to moderately increase the release of TGF-β1 in murine lung tissue (Fig. 12A) and to increase the proliferation of human bronchial SMCs, as shown in combination with TGF-β1, events that were inhibited by EXT5 or EXT9 treatment (Fig. 12B).

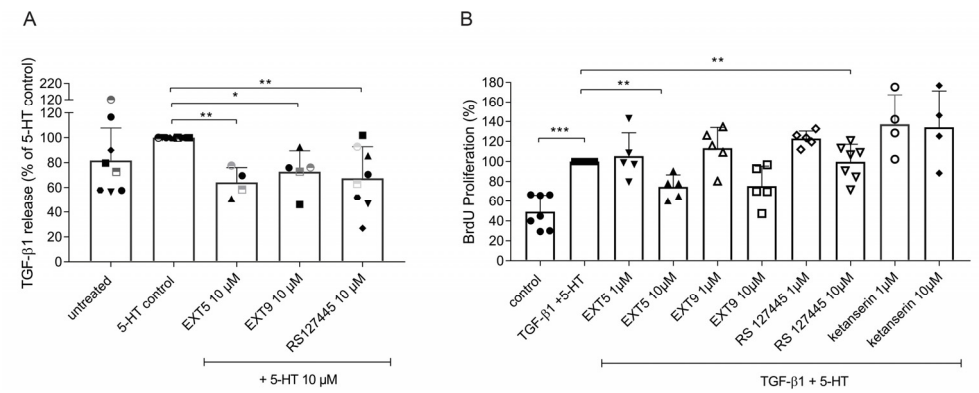


Figure 12. Pulmonary remodelling processes inhibited by 5-HT$_{2B}$ receptor antagonism.
Release of TGF-β1 from murine lung tissue following treatment with 5-HT 10 μM and 5-HT$_{2B}$ receptor antagonists (EXT5, EXT9 and RS127445) (A). Proliferation of human bronchial SMCs treated with TGF-β1 10 ng/ml + 5-HT 1 μM in combination with 5-HT$_{2A/2B}$ receptor antagonists. One sample t-test p<0.05*, p<0.01**, p<0.001***.

Paper IV

The lung matrisome in IPF directs fibroblast responses, promoting the maintenance of disease-specific matrix through targeted ECM production

Continuous deposition of ECM proteins forms a stiff tissue with altered structural and mechanical properties, changes shown to heavily influence cellular behaviour [23, 71, 147]. As biomechanical and compositional properties of the tissue may propagate disease progression, studying cell–matrix interactions become essential in deciphering pathological mechanisms in IPF. Following the cellular removal through decellularization, the matrisomal properties of the lung ECM can be more closely studied. Distally derived lung tissue from patients with IPF showed an enhanced stiffness and tissue density with a specific ECM composition in comparison to lung scaffolds from healthy individuals (Fig. 13).

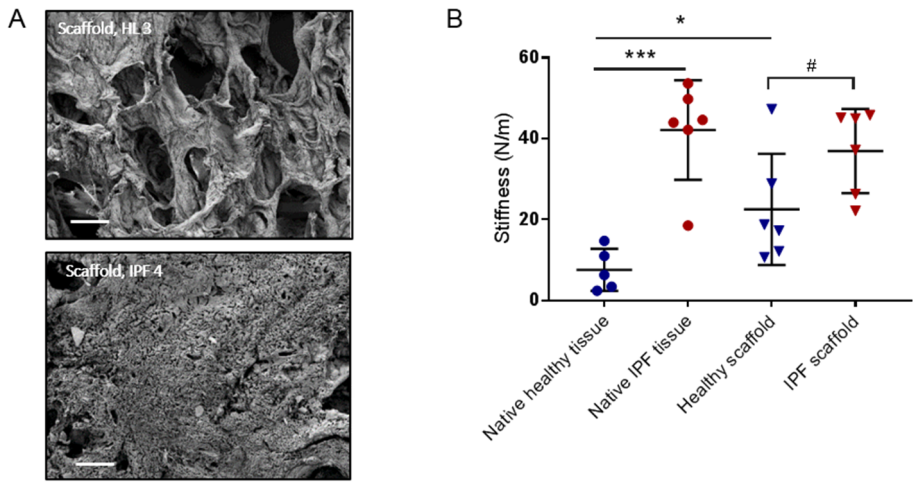
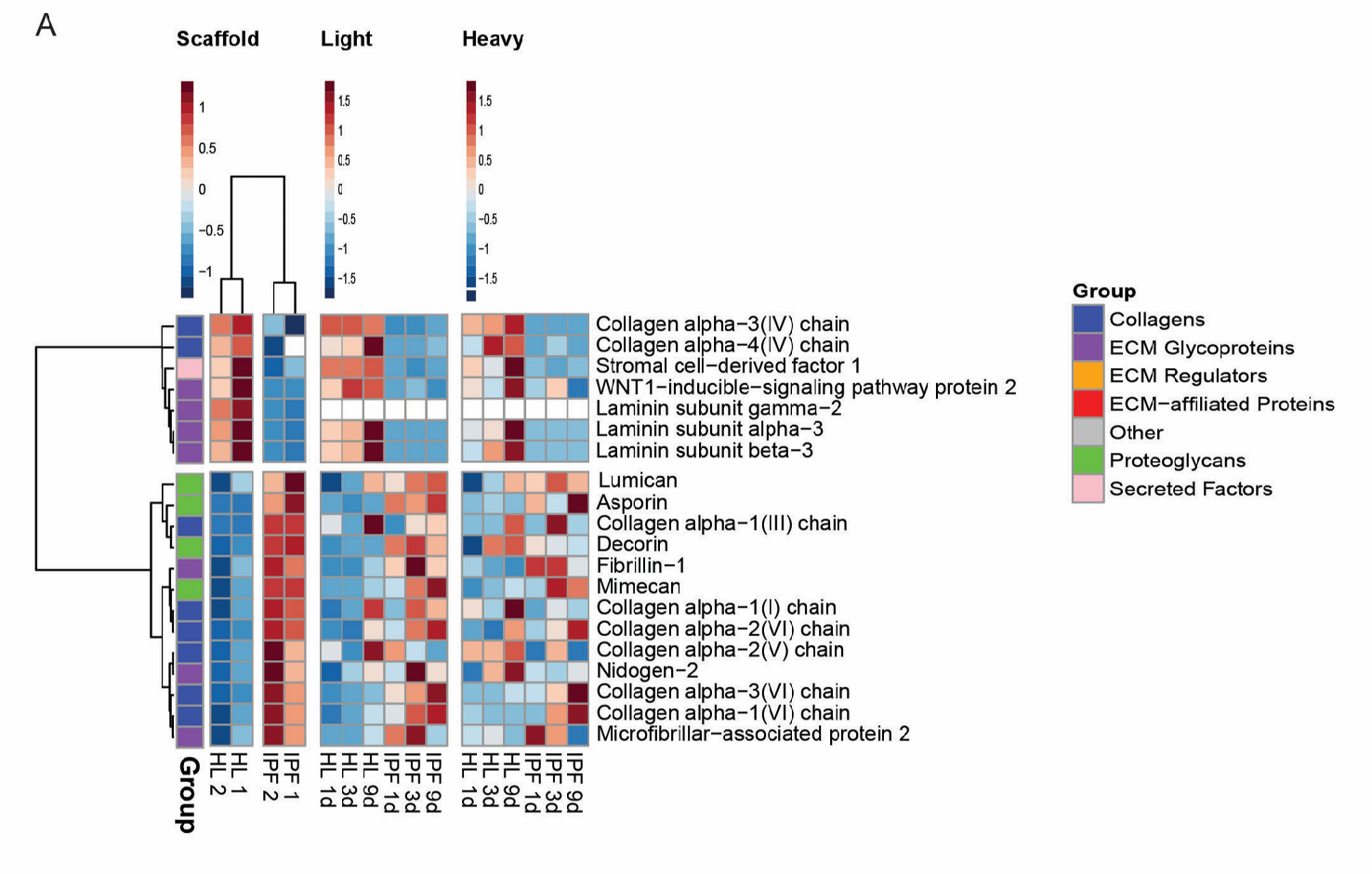


Figure 13. Tissue properties of IPF and healthy scaffolds.
Representative SEM images of healthy scaffold (HL 3) and IPF scaffold (IPF 4). Scale bar = 100 µm (A). Stiffness of native healthy and IPF lung tissue with corresponding scaffolds from four individuals per group (including 1-2 technical replicates per individual). Unpaired t-test for significance between groups $p<0.05$*, $p<0.01$**, $p<0.001$*** (B).

Healthy fibroblasts grown on IPF lung matrix continued to re-build an IPF-like tissue with distinct ECM features, as compared to healthy tissue (Fig. 14A). Changes in ECM production were seen as early as after one day in culture, with proteoglycans and BM proteins as the major affecters in IPF tissue supporting the evidential loss of normal BM architecture in IPF scaffolds verified with histology (Fig. 14B).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Stromal cell−derived factor 1
WNT1−inducible−signaling pathway protein 2
Laminin subunit gamma−2
Laminin subunit alpha−3
Laminin subunit beta−3
Lumican
Asporin
Collagen alpha−1(III) chain
Decorin
Fibrillin−1
Mimecan
Collagen alpha−1(I) chain
Collagen alpha−2(VI) chain
Collagen alpha−2(V) chain
Nidogen−2
Collagen alpha−3(VI) chain
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Microfibrillar−associated protein 2
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ECM Glycoproteins
ECM Regulators
ECM−affiliated Proteins
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Secreted Factors

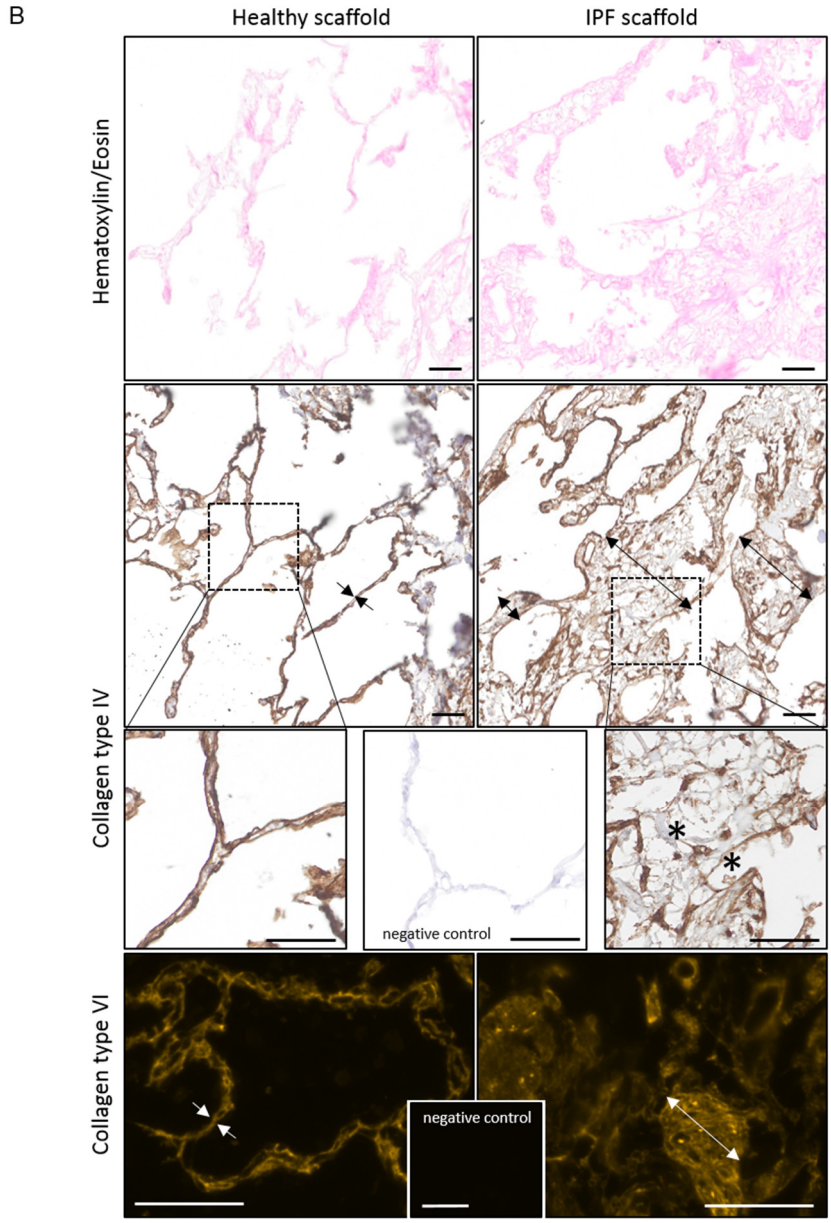

Figure 14. Proteomic characterization of matrisome proteins in lung scaffolds repopulated with SILAC labelled fibroblasts.

Heat map with unsupervised hierarchical clustering of Z-scored values of matrisome proteins over time, demonstrating scaffolds and repopulated scaffolds (light and heavy) at day 1, day 3 and day 9 of culture. Patient group mean intensities, based on the groups of biological replicates (n= 2-4), are presented (A). Histological verification and spatial distribution of BM proteins (collagen type IV (brown) and collagen type VI) along with corresponding hematoxylin/eosin staining of scaffolds, showing thin alveolar septa in healthy scaffolds (marked with arrows) compared to thickened fibrotic remodelled septa in IPF scaffolds. Areas lacking or showing reduced intensity of collagen IV staining were marked with *. Scale bar = 50 µm.

The proteoglycan decorin and disease associated proteins such as tenascin-C and periostin showed an enhanced synthesis at day one with selective spatial tissue distribution of decorin and periostin, forming prominent accumulations in IPF scaffolds, seen with antibody labelling (Fig 15).

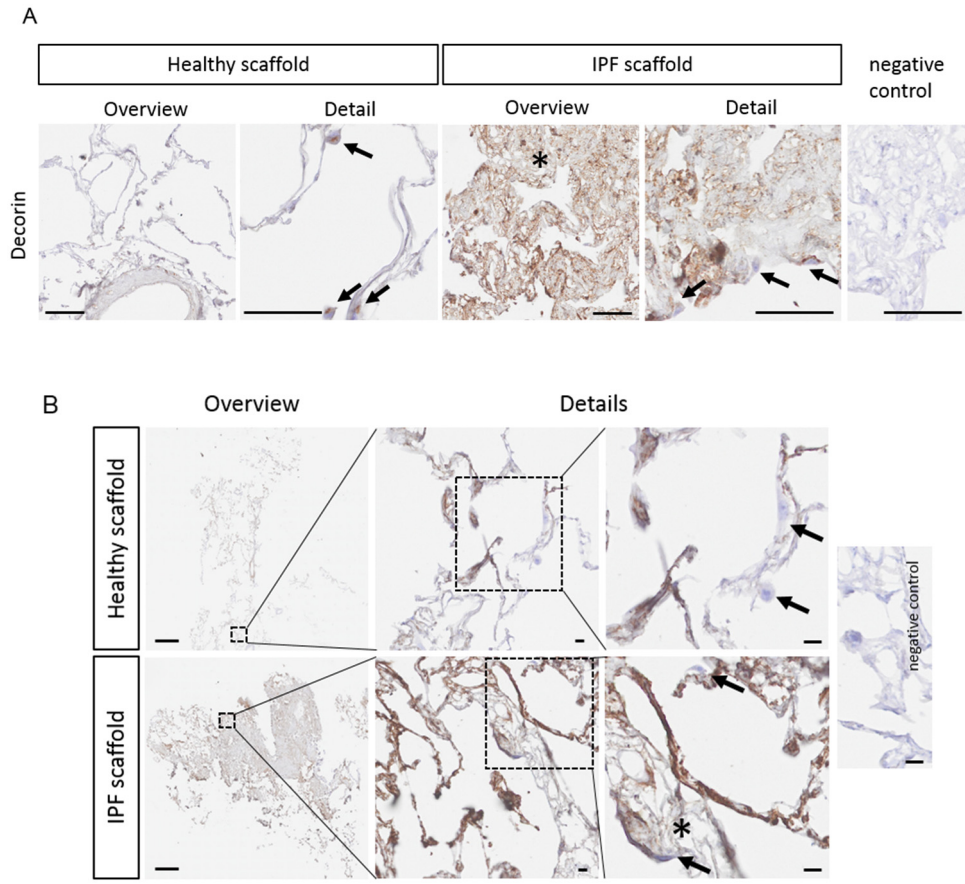

Figure 15. Decorin and periostin distribution in repopulated lung scaffolds.
Antibody staining illustrating decorin (A) and periostin (B) distribution in healthy vs. IPF scaffolds at day one, showing newly synthesized proteins (brown) including original scaffold composition. * = extracellular deposition, arrow= intracellular, newly produced protein in cells of repopulated scaffolds. Scale bar = 50 µm (A). Scale bar = 500 µm (overview) and 10 µm (details) (B).

Discussion

The focus of this thesis is to identify disease mechanisms important for the progression of pulmonary fibrosis, with emphasis on the role of 5-HT_2 signalling and the interplay of cells with underlying ECM. The first and major part of the thesis investigates how 5-HT/5-HT_{2B} signalling influences key tissue remodelling processes in lung by implementing several experimental systems using *in vitro*, *ex vivo* and *in vivo* models. As fibroblasts are the main producers of ECM, we examined how their proliferation and differentiation into myofibroblasts were affected by 5-HT_{2B} receptor antagonism, as well as by production and deposition of ECM proteins.

Platelet delivery of 5-HT

Several fibrotic conditions show increased levels of 5-HT, seen both locally and systemically, with circulating platelets acting as the major carriers of 5-HT. As alveoli and pulmonary capillaries are closely located to each other, the source and delivery of 5-HT may originate from the pulmonary circulation through aggregation and degranulation of platelets. Platelets aggregate upon vascular injury to form blood clots to minimize bleeding. This event has been shown to be closely linked to IPF, with increased levels of coagulation factors such as fibrin and thrombin, which are found locally in lungs of patients with pulmonary fibrosis [148, 149]. Additionally, pulmonary haemorrhage and thromboembolism have been shown to be associated with acute exacerbations in patients with IPF [150], suggesting a potential link between activation of the endothelial layer and progression of disease. Increased local pulmonary concentration of 5-HT would enhance the activation of 5-HT_2 receptors on nearby cells, causing a conceivable 5-HT-driven pro-fibrotic response in the parenchymal lung tissue. Expression of both 5-HT_{2A} and 5-HT_{2B} have been shown to be clearly increased during pulmonary fibrosis, localized on fibroblasts, endothelial cells and bronchial epithelial cells [106, 118]. As such, we examined the anti-fibrotic effects of 5-HT_{2B} receptor antagonists in a s.c. BLM-model of pulmonary fibrosis. This model is different from direct pulmonary exposure to BLM using i.t. or i.n. routes of delivery, showing earlier signs of fibrosis, which coincides with mild inflammation. By using a s.c. administration, the distal regions of the lung as well as the central parts become equally exposed to BLM with an initial interaction with the endothelial cell layer. Attenuating effects were identified on inflammation and fibrosis following p.o. treatment with 5-HT_{2B} receptor antagonist. These promising anti-fibrotic effects seen after two weeks

indicate the 5-HT_{2B} receptor as a promising target for pulmonary fibrosis. However, to establish whether the compounds have a therapeutic effect, treatment should be initiated in later stages of the model since few therapeutic treatments have effect on already established fibrosis. BLM-models are commonly used to evaluate novel drugs for IPF treatment. However, a recently published study shows that BLM-treated mice, with either s.c. or oropharyngeal administrations, have enhanced pulmonary levels of the ATP-binding cassette transporters in comparison to patients with IPF [151]. These membrane proteins enhance the efflux of endogenous substrates causing a reduced drug exposure, in this case nintedanib [152]. Results that demonstrate the limitations of the model for translatable pharmacological evaluations for therapeutics acting as substrates for ABC transporters, highlighting the need for more relevant *in vivo* models for IPF.

5-HT_{2B} signalling in fibrosis

Through its 5-HT receptors, 5-HT stimulates a vast repertoire of physiological responses, however, the exact mechanisms induced by serotonergic signalling during fibrosis remain unresolved. We found a close connection between TGF-β1 and 5-HT_{2B} receptor signalling in induction of pulmonary myofibroblast differentiation and deposition of ECM proteins. It has been shown that 5-HT may act as a mediator for TGF-β1 signalling by enhancing intracellular levels of pSmad3 and expression of TGF-β1 itself, as seen in dermal fibroblasts [107]. A possible signalling pathway was also described in cardiac fibroblasts where 5-HT_{2B} antagonism arrested the non-canonical pathway of TGF-β1 by sequestering pSrc, thereby reducing α-SMA and SM22α [145]. We demonstrate that reduced 5-HT_{2B} receptor activity inhibits proliferation of both bronchial SMC and lung fibroblasts, with the latter possibly mediated via the pAkt/p21 pathway. Still, the induced transduction pathways triggered by 5-HT_{2B} receptor activation are diverse with possible subjections to cell subtypes and local tissue interactions. Our study supports the 5-HT_{2B} receptor as a promising target in regulating fibrotic responses, as lung fibroblast proliferation and myofibroblast differentiation were inhibited by 5-HT_{2B} receptor antagonism.

5-HT_{2B} receptor antagonist as potential treatment for fibrosis

The clinical development of selective 5-HT_{2B} receptor antagonists are focusing on selective antagonists with peripheral effects and good pharmacokinetic profiles. Structural similarities of class 2 receptor subtypes create difficulties in developing receptor-specific antagonists as non-selective antagonists may produce unwanted effects. Receptor binding and functionality studies *in vitro* identified EXT5 and EXT9 as 5-HT_{2B} receptor antagonists with a minor binding to the 5-HT_{2A} and 5-HT_{2C} receptors. The slightly different profiles of the two compounds may therefore reveal specific characteristics favourable for regulating diverse anti-fibrotic events as seen in this study with altered attenuating effects on bronchoconstriction and cytokine release between the two compounds. Nevertheless, a major beneficial property in using a pure

receptor antagonist to modulate receptor functions is the requirement of an agonist, limiting the spatial function of the compound. As such, the effect of a receptor antagonist is directly connected with presence of its agonist, a feature that minimizes prolonged receptor inactivation and adverse effects. This pharmacological feature may be well implemented in the wound healing response, which has sequential and transient phases of repair that generates temporal changes in local levels of mediators such as 5-HT.

Immune modulating effects by 5-HT_{2B} receptor antagonists

In the initial phase of a wound healing response there is a rapid localisation of platelets to the injured site, which is followed by a release of 5-HT and an influx of inflammatory cells. IPF, however, is not regarded as an inflammatory disease as little effects have been shown with corticoid steroid treatment and with no requirement of inflammatory infiltrates at IPF diagnosis. Importantly, due to the insidious nature of the disease, diagnosis and pharmacological treatment are commonly initiated late when fibrosis has been developing for several years. It is possible that inflammation may occur in early phases in IPF, since the condition is thought to arise from repeated damage to epithelial cells with subsequent triggered inflammatory response. However, inflammation appears not to be a requirement for fibrotic establishment *in vivo*. An αvβ6 integrin knock-out model with mice lacking the ability to activate TGF-β1, demonstrates evident pulmonary inflammation with no signs of fibrosis following exposure to BLM [153]. Whether inflammation has a critical effect on the pathology in IPF is still debatable with evidence pointing towards fibroblast dysfunction and imbalanced repair process. Even so, IPF patients with a rapid deterioration display an increased infiltration of inflammatory cells [154], which has also been seen in models of pulmonary fibrosis with enhanced circulating and pulmonary levels of IL-1β [155]. A pilot study also demonstrated immune regulatory properties of pulmonary fibroblasts derived from patients with IPF, as IPF fibroblasts *in vitro* were shown to inhibit differentiation and functions of dendritic cells during co-culture, a cellular-interaction which may alter immune responses in IPF [156]. Interestingly, we demonstrated that 5-HT_{2B} receptor antagonism attenuated pulmonary fibrosis in BLM-treated mice together with a reduced systemic level of pro-inflammatory cytokines TNF-α and IL-1β.

The immune modulating properties of 5-HT_{2B} receptor antagonists may have a beneficial effect in anti-fibrotic treatment, since chronic inflammatory processes and enhanced release of pro-inflammatory mediators contribute to tissue destruction and remodelling, as seen in other diseases such as rheumatoid arthritis and systemic sclerosis [2, 157]. Conversely, 5-HT_{2B} receptor activation has been shown to modulate human macrophage polarisation, promoting M2 macrophages representing a pro-fibrotic phenotype with immunosuppressive and wound reparative characteristics. Macrophages that are recruited to sites of injury express increased levels of TNF-α while levels of TGF-β1 are increased at later stages of wound healing [158]. These temporal

alterations in inflammatory and fibrotic mediators, orchestrated by the wound healing response, further emphasize the complex mechanistic role of 5-HT_2 receptor activation during fibrosis development and optimal windows for effective therapeutic treatments.

Airway contraction inhibited by 5-HT_{2B} receptor antagonist

In another model system, using PCLS originating from mouse lungs [159], we identified an interesting effect solicited by the 5-HT_{2B} receptor antagonists EXT5 and EXT9. These antagonists demonstrated inhibitory effects on bronchoconstriction, which is a prominent feature in asthma. Although 5-HT is described to not induce bronchoconstriction in human PCLS [120], elevated plasma levels of 5-HT are seen in patients with asthma and asthmatic symptoms are improved following reduction of free 5-HT in plasma using the drug tianeptine [160, 161]. Interestingly, IL-1β potentiated 5-HT-induced bronchoconstriction in mice [162], and our results show that EXT9 reduced systemic levels of IL-1β. Additional serotonergic effects were shown on airway SMC proliferation with anti-proliferative effects demonstrated by EXTs *in vitro*. The enhanced bronchoconstriction together with SMC hyperplasia enhance the contractile response in the tissue, which has been shown to trigger the release of ECM-stored mediators such as TGF-β1 [78]. The tissue contraction caused by 5-HT was shown to enhance the release of TGF-β1, measured in medium from 5-HT-treated murine distal lung tissue. This effect may in part be the result of reduced contractile fibers in cells (as seen in lung fibroblasts) or/and via direct inhibitory effects on SMC activity. Interestingly, mechanical stretch has been shown to induce the release of 5-HT from pulmonary neuroendocrine cells via mechanosensitive channels [109], further emphasizing the importance of mechanical cues in lung diseases.

Specific ECM features related to IPF

The mechanical property of a tissue is fundamental in organizing structural support and directing cellular activity. The final part of the thesis focuses on how the matrisome properties of the tissue influence cellular responses in IPF. The interplay between cells and ECM was studied in scaffolds from healthy or IPF derived lung tissue. The enhanced tissue density and the altered ECM composition collectively contributed to a stiffer matrix. Our studies show that subpleural parenchymal lung regions of IPF patients have an altered ECM composition in comparison to healthy individuals along with increased tissue stiffness. With altered mechanical properties, mechanosignalling pathways become selectively activated, driving a matrix stiffening feedback-loop amplifying pro-fibrotic cellular responses such as larger cell spreading, increased collagen production, enhanced migration and activation of fibroblasts as seen in other studies [71, 147, 163-166]. In IPF, both the regional increase in collagen production and deposition as well as the altered post-translational modifications of collagen fibrils generate structural and mechanical changes in the lung [165]. Collagens fibers in IPF have been shown to be smaller in diameter and stiffer with differential expression of

crosslinking enzymes, thus highlighting the need to target dysregulated fibril formations to inhibit mechanotransduction pathways in pulmonary fibrosis [165, 167]. Similar characteristics were demonstrated in remodelled airways in patients with asthma, showing altered structural fibril collagen formations with fibroblasts producing increased circular fibers with enhanced directional dispersion [168]. Overtime we observed that a modified ECM architecture directs fibroblasts to produce a disease-specific ECM, with distinct temporal production of proteins as seen in our *ex vivo* model of IPF. These findings were supported by a previous study, showing that the ECM dictates cell behaviour, showing fibroblasts expressing enhanced levels of α-SMA when cultured on acellular IPF scaffolds [18].

ECM dictates fibroblast response in IPF

A study by Parker *et al.* demonstrated that fibroblasts derived from healthy or IPF lung tissue generate similar gene expression profiles when cultured on equivalent ECM matrices [17]. Seemingly, IPF is a disease controlled by its microenvironment with matrisome properties highly affecting cellular behaviour, regardless of cellular origin. The heterogeneous IPF tissue intertwined with areas of normal histological appearance with dense fibrotic areas [9] emphasize how local cues may influence cellular phenotypes and responses. The fibroblast foci, complex 3D-structures portraying areas of active fibrosis were shown to predict IPF patient survival [75, 169, 170]. The presence of versican and decorin in these foci may imply a pivotal role in early phases of the disease [171], a notion further recognised in our scaffold models, where fibroblasts grown on IPF scaffolds produced increased levels of versican and decorin as early as after one day in culture. The same pattern was also present *in vivo* where 5-HT_2 receptor antagonist treatment attenuated fibrosis in BLM-treated mice along with reduced amount of decorin in the lung. Furthermore, treatment with the 5-HT_2 receptor antagonists resulted in reduced synthesis of decorin in TGF-β1 activated fibroblasts *in vitro*, supporting a selective and temporal deposition of ECM components by fibroblasts in fibrosis.

Basement membrane defects in IPF

We demonstrated that pre-existing matrisome characteristics in IPF scaffolds overlap with newly synthesized ECM proteins produced by healthy fibroblasts repopulated on IPF scaffolds. The clear shift in the production of BM proteins would further provide a disorganized BM and the loss of barrier integrity, as seen in our present study with IPF scaffolds. In alveolar epithelial cells, the formation of BM is in part regulated by TGF-β1, which at higher concentration obstruct a normal architecture of the BM [172]. Without proper assembly, an abnormal BM may contribute to enhanced EMT and subsequent active production of matrix proteins. Alterations in collagen IV assembly, the most abundant collagen in the BM, stimulate increased cellular expression of TGF-β1, as observed in tubular epithelial cells [173], potentially

propagating EMT. As the underlying BM construct fails to support epithelial attachment and homeostasis, the protective physical barrier of the epithelial layer breaks, thus enabling the entry of pathogens and the activation of host defence immune responses. Pulmonary bacterial attachments perpetuate fibrotic tissue development [174] with matrix abnormalities as key elements in regulating pathogen survival [175], thus highlighting the importance cell-matrix interplay in chronic lung diseases. Collectively, our results further support a pivotal role of the ECM in IPF and specifically the BM as an important component in disease progression of IPF.

The altered production and turnover of important ECM elements provide a constantly fluctuating extracellular environment that controls local cellular response. The spatial complexity of the ECM enhances the cellular plasticity in pulmonary fibrosis, which in addition renders therapeutic treatment of IPF patients challenging. Modifications of the underlying pathologically structured matrix would strengthen the foundation of a signalling platform for healthy tissue generation, creating a balanced cellular behaviour in a fibrotic lung. In the pathological state, at enhanced detrimental levels of ligand, a restricted receptor activation mediated by the 5-HT_{2B} receptor antagonists would further inhibit remodelling of tissue and potentially terminating the constant fibrotic feedback loop between cells and matrix in pulmonary fibrosis.

Conclusions

In conclusion, these studies suggest an important role of 5-HT in pulmonary fibrosis with several pro-fibrotic events directed by 5-HT_{2B} receptor signalling (Fig. 16). Fibroblast proliferation, myofibroblast differentiation and ECM deposition are reduced by blocking 5-HT_{2B} receptors with selective receptor antagonists. Collectively, these findings identify the 5-HT_{2B} receptor as a potential molecular target in the treatment of pulmonary fibrosis.

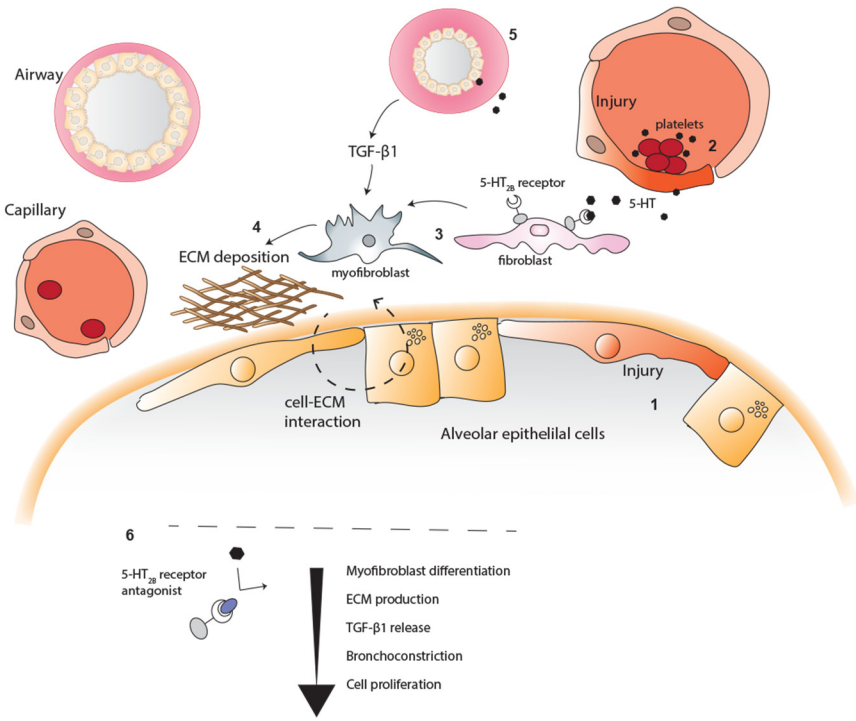

Figure 16. Matrix production by fibroblasts in pulmonary fibrosis.
Upon tissue damage to the epithelial or endothelial cell layers, resident fibroblasts become activated initiating a wound healing response (1). Insults to the adjacent pulmonary vascular system stimulates platelet aggregation and release of 5-HT (2). Fibroblasts differentiate towards contractile myofibroblasts (3), secreting massive amounts of ECM proteins and causing the release of TGF-β1 (4). Enhanced levels of 5-HT cause airway narrowing through bronchial SMC contraction and proliferation (5). Ongoing cell activation in pulmonary fibrosis results in the formation of fibrotic tissue with a pathological remodelled interstitium, acting collectively as a cell-matrix feedback loop progressing the development of fibrotic tissue. Blocking the binding of 5-HT to its receptor, with 5-HT_{2B} receptor antagonism, results in several anti-fibrotic events, thus highlighting the 5-HT_{2B} receptor as a promising target for pulmonary fibrosis (6).

Beneficial add on effects were shown with EXTs, where the receptor antagonists attenuated bronchoconstriction *ex vivo* and airway remodelling *in vitro*. The pathological remodelling of lung tissue directs fibroblast behaviour by stimulating specific synthesis of ECM proteins, and thereby progressively creating a diseased matrix. The evidential effect of compositional and structural alterations of the ECM on cellular responses creates a cell-matrix feedback loop that suggests surrounding ECM niche and 5-HT_{2B} receptor activation as fundamental driving forces in the development of IPF.

Future perspectives

These studies have provided novel insights into the cellular regulation of pulmonary fibrosis and highlighted new targets for therapeutic approaches. Nonetheless, to further understand underlying disease mechanisms leading to pulmonary fibrosis, the following issues need to be addressed.

Fibroblasts have been the primary focus of this thesis, and have been extensively studied as a monoculture, however, a co-culture approach would include important cell-cell communications. It would be interesting to study the combination of fibroblasts and pulmonary endothelial cells as well as fibroblasts and alveolar epithelial cells, as the latter being regarded as main and initial players in IPF. Fibroblasts, which express 5-HT_{2A} and 5-HT_{2B} receptors, might also be heavily influenced by residential mast cells, as these cells harbour large amounts of 5-HT and have been identified in diseased areas of IPF lung [176]. It is evident from this thesis that early fibrotic events are coupled with serotonergic signalling where treatment with 5-HT_{2B} receptor antagonist exhibits several anti-fibrotic effects. However, these effects were generated from prophylactic treatments, where the initiation of treatment and disease coincide with each other. The *in vivo* treatment in animal models with 5-HT_2 receptor antagonists would be of more clinical relevance if administered after the formation of fibrotic tissue, as the diagnosis and treatment of IPF patients occur in the late stages of the disease with IPF symptoms commonly mistaken for other lung diseases. At diagnosis, IPF patients display manifest remodelling and fibrosis, which is irreversible with current available treatments. This raises the question whether the therapeutic window is closed at the time of pharmacological interventions, emphasizing the importance of early diagnosis and the discovery of prognostic biomarkers.

The 3D-*ex vivo* model based on decellularized lung tissue derived from healthy individuals and IPF patients, utilizes the complexity of the lung ECM along with structural maintenance of the tissue. Adding cyclic stretch to the scaffolds during culture, mimicking the mechanical stretch during breathing, would further enhance the translational properties of the model. A system is currently being explored within the research group with the construction of a bioreactor that simulates physiological relevant mechanical loading of lung tissue performed in an air-liquid interface. Developing a model system that replicates the healthy and diseased matrix, opens up for fundamental insights in the progression of IPF and the identification and evaluation of new therapeutic targets, as animal models do not recapitulate all disease features and

clinical responses in humans. Initial results of secreted mediators from our scaffold model indicate translational properties of this system as the fibroblasts produce biomarkers associated with IPF, warranting further examination.

Additionally, combining the matrisome profile of site specific lung tissue with gene expression profiles of fibroblasts grown on scaffolds may together decipher key mediators and important signalling pathways associated with IPF, such as 5-HT_2 receptor signalling.

Populärvetenskaplig sammanfattning

(Summary in Swedish)

Lungfibros är en allvarlig kronisk lungsjukdom som karakteriseras av stora ansamlingar av ärrvävnad i lungan, vilket leder till försämrad syreupptagning. Man tror att en obalans i sårläkningsprocessen bidrar till en överproduktion och lagring av vävnad, vilket orsakar en patologisk omstrukturering av lungans arkitektur. Ett viktigt strukturellt stöd för celler med en betydande roll i sårläkningsprocessen är det extracellulära matrixet (ECM), ett komplext nätverk av sammanlänkande proteiner som omger varje cell. Denna mikromiljö bestående av kollagener, proteoglykaner och glykoproteiner är involverad i ett flertal cellulära processer såsom celltillväxt, cellaktivering och frisättning av matrixproteiner och tillväxtfaktorer, såsom TGF-β1. Allvarliga kroniska lungsjukdomar så som idiopatisk lungfibros (IPF) är associerade med ökning av matrixproducerande lungceller, såsom fibroblaster och myofibroblaster. Patologin av lungfibros är ännu inte kartlagd, men monoaminen serotonin (5-HT) har associerats till sjukdomsutvecklingen. 5-HT lagras i cirkulerande blodplättar och frisätts vid patologiska tillstånd, vilket orsakar en ökad lokal och systemisk koncentration av 5-HT. 5-HT binder och aktiverar ett flertal 5-HT-receptorer och inhibition av 5-HT_{2A} och 5-HT_{2B} receptorer, med hjälp av specifika receptorantagonister, har visat sig förhindra utvecklingen av fibros. De underliggande patologiska mekanismerna för IPF har hittills inte kartlagts och är i behov av vidare studier för att vidareutveckla nya behandlingsstrategier mot denna hittills obotliga sjukdom.

Avhandlingens syfte är att utreda vilken roll 5-HT och 5-HT_{2B} receptorn har vid lungfibros, vilket undersöks i avhandlingens tre första arbeten. I avhandlingens sista arbete belyses samspelet mellan celler och ECM, genom att studera hur friska celler påverkas när de odlas på friskt respektive sjukt (IPF) matrix. För att efterlikna en fibrotisk miljö, behandlas lungfibroblaster med 5-HT och TGF-β1, vilket triggar en fibrotisk respons med ökad produktion av proteoglykaner samt differentiering av fibroblaster till myofibroblaster. Genom att inhibera inbindningen av 5-HT till 5-HT_{2B} receptorer med hjälp av 5-HT_{2B} receptor antagonister (EXT5 och EXT9) hämmas dessa cellulära processer. Vidare undersöktes potentiella anti-fibrotiska egenskaper hos EXT5 och EXT9 i en djurmodell som efterliknar det patologiska tillståndet vid lungfibros. Med subkutana injektioner av cytostatikan bleomycin genererar modellen en ökad vävnadsproduktion i lungan samt ökning av antalet myofibroblaster, vilket inhiberas med en daglig oral behandling av receptorantagonisterna. Ett annat intressant fynd visade att serum från EXT-behandlade möss innehöll en minskad halt av inflammatoriska signalmolekyler (TNF-α och IL-1β), fynd som tyder på anti-inflammatoriska egenskaper hos EXT5 och EXT9.

Studier talar för att upprepade skador på luftvägar i kombination med en ökad mottaglighet för sårläkning driver uppkomsten av IPF. Vid en skada i lungan påverkas närliggande kärl och deras endotelceller. Vår hypotes innefattar att en ökad blodplättsaggregering och aktivering vid endotelskada i lungan leder till en ökad frisättning av 5-HT och aktivering av 5-HT_{2A} and 5-HT_{2B} receptorer, vilket stimulerar ett fibrotiskt cellsvar. För att vidare förstå de 5-HT-medierade effekterna, studerades signaleringsvägar associerade med $5HT_{2B}$ receptorn. *In vivo* modellen med lungfibros påvisade förhöjda nivåer av genen som kodar för p21, ett intracellulärt protein kopplat till celldelning. Vidare studier på lungfibroblaster visade att 5-HT stimulerade celltillväxt och induktionen av p21, händelser som inhiberas av EXT-behandling. Studien indikerar på en möjlig sjukdomsmekanism kopplad till 5-HT, där en ökad celltillväxt är associerad med p21.

Den patologiska omstruktureringen i en fibrotisk lunga medför en förträngning av luftvägarna. I avhandlingens tredje arbete granskas rollerna av 5-HT_{2A} och 5-HT_{2B} receptorer vid luftvägskontraktilitet. Små, tunna snitt så kallade PCLS från muslunga behandlades med 5-HT för att stimulera en luftvägskontraktion, en respons som undersöktes i realtid under mikroskop och med ökad koncentration av 5-HT drog luftvägen successivt ihop sig. Behandling med 5-HT_2 receptor antagonister inhiberar kontraktionen, med 5-HT_{2A} receptor antagonisten som en tydlig potent inhibitor. De glatta muskelcellerna som omger luftvägarna styr dess kontraktilitet och är producenter av matrixproteiner. Behandling med 5-HT_{2B} receptor antagonister påvisade minskad tillväxt av bronkiella glatta muskelceller samt frisättningen av TGF-β1, en kraftfull profibrotisk mediator som stimulerar ECM produktion.

I avhandlingens sista arbete odlades friska fibroblaster på tunna lungsnitt med avsaknad av celler men med bibehållen ECM från friska individer eller patienter med IPF. Denna modell gör att samspelet mellan celler och ECM kan studeras samt vilken betydelse ECM har i IPF. De materiella egenskaperna hos ECM skiljer frisk vävnad från sjuk i både proteinkomposition, vävnadstäthet och styvhet. Studien visade att friska celler är direkt influerade av sin mikromiljö, där ett sjukt ECM aktiverar cellerna till att producera mer ärrvävnad som främjar sjukdomsprogression.

Baserat på dessa studier dras slutsatserna att 5-HT via 5-HT_{2B} receptorn aktiverar flera profibrotiska svar i lungan och att dessa bidrar till en patologisk omstrukturering av lungan vid lungfibros. Den antifibrotiska effekten av 5-HT_{2B} receptor antagonister föreslår 5-HT_{2B} receptorn som intressant målmolekyl i utvecklandet av farmakologisk behandling av lungfibros. Den ökade depositionen av ECM, bl.a. stimulerat av 5-HT_{2B} receptor aktivering, bildar ett unikt komplex av proteiner som har förmågan att initiera specifika cellulära svar. Likt en negativ feedback loop, driver interaktionen mellan celler och ECM progressionen av fibros, en sjukdomsmekanism som kan vara avgörande i IPF och som tillsammans understryker vikten av upptäckterna presenterade i denna avhandling.

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