Studies on TGF-β family signalling in the diseased lung tissue

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Academic dissertation

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ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to by their Roman numerals in the text.

I Leppäranta O, Pulkkinen V, Koli K, Vähätalo R, Salmenkivi K, Kinnula VL, Heikinheimo M, Myllärniemi M. Transcription factor GATA-6 is expressed in quiescent myofibroblasts in idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2010;42(5):626-632

II Leppäranta O, Myllärniemi M, Salmenkivi K, Kinnula VL, Keski-Oja J, Koli K. Reduced phosphorylation of the TGF-β signal transducer Smad2 in emphysematous human lung. *COPD* 2009;6(4):234-241

III Leppäranta O, Sens C, Salmenkivi K, Kinnula VL, Keski-Oja J, Myllärniemi M, Koli K. Regulation of TGF-β storage and activation in the human idiopathic pulmonary fibrosis lung. *Cell Tissue Res* 2012;348:491-503

IV Leppäranta O, Tikkanen JM, Bespalov MM, Koli K*, Myllärniemi M*. The BMP-inducer tilorone identified by high-throughput screening is antifibrotic *in vivo*. *Manuscript*

* These authors contributed equally to the study.

ABBREVIATIONS

AEC	alveolar epithelial cell
α-SMA	alpha-smooth muscle actin
BMP	bone morphogenetic protein
cDNA	complementary deoxyribonucleic acid
COPD	chronic obstructive pulmonary disease
CPFE	combined pulmonary fibrosis and emphysema
CTGF	connective tissue growth factor
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EDA	extra type III domain A (fibronectin)
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
FEV1	forced expiratory volume in one second
FVC	forced expiratory vital capacity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA-6	transcription factor binding to the (A/T)GATA(A/G) consensus sequence
GDF	growth and differentiation factor
ICC	immunocytochemistry
IF	immunofluorescence
IFN-γ	interferon gamma
IHC	immunohistochemistry
IPF	idiopathic pulmonary fibrosis
JNK	c-Jun N-terminal kinase
miRNA	microRNA
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate-buffered saline
PLA	proximity ligation assay
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature

RT-PCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA; silencing RNA
Smad	vertebrate gene product; named after related *Caenorhabditis elegans* gene *Sma* and *Drosophila* gene *mothers against decapentaplegic (Mad)*
TGF	transforming growth factor
UIP	usual interstitial pneumonia
WB	western blot
Wnt	gene product; named after related *Drosophila* gene *wingless* and mouse mammary tumour virus *Int1*

ABSTRACT

This thesis focuses on transforming growth factor (TGF)-β signalling system in the human lung. Two lung parenchymal diseases, idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD), were studied. In IPF, persistent fibroblasts and extracellular matrix accumulate in the lung parenchyma, causing thickening of the parenchyma with subsequent difficulties in breathing. An opposing process in the lung parenchyma is seen in emphysematous lung in COPD, where alveolar wall damage causes loss of alveolar structure and shortness of breath.

The aim of the study was to examine alterations in TGF-β signalling activity in pathologically distinct areas of damaged lung tissue (fibrosis, emphysema). Furthermore, TGF-β signalling properties, i.e. storage and activation, as well as the role of transcription factor GATA-6 in the pathogenesis of IPF were studied. Finally, novel treatment options for IPF were sought.

The studied materials were tissue samples from patient lung biopsies along with a selection of commercial and primary lung cell lines. Gene expression levels and protein (co)localization as well as cell differentiation were studied with modern molecular biology tools. Reporter cells and high-throughput screening were used in the search for new candidate drugs for IPF. The results of the screen were validated in a mouse model of silica-induced pulmonary fibrosis.

The results showed that TGF-β signalling activity varies within pathologically distinct areas of the lung, with emphysematous areas showing decreased and fibrotic areas showing increased signalling activity. In fibrotic tissue, this is accompanied by a significant increase in the storage and activation of latent TGF-β.

It is a common belief that the fibroblastic foci (i.e. aggregates of (myo)fibroblasts) in IPF consist of actively proliferating cells. Here, this view was challenged as fibroblastic foci were found to consist of quiescent, non-proliferating and non-apoptotic cells. These cells, however, were still responsive to TGF-β signals, suggesting that they keep contributing to the extracellular matrix accumulation. Transcription factor GATA-6 was found to be a crucial mediator of the TGF-β-induced cell differentiation of epithelial cells and fibroblasts towards a myofibroblast-like phenotype.

For the first time in IPF research, high-throughput screening technology was used in the search for novel treatment options. The results were promising: primary *in vitro* data revealed one compound which affected TGF-β family signalling and showed biologically significant antifibrotic effects in a mouse model of fibrosis. The data obtained here offers a starting point for further studies.

TIIVISTELMÄ (FINNISH SUMMARY)

Idiopaattisessa keuhkofibroosissa (idiopathic pulmonary fibrosis, IPF) soluja ja soluväliainetta alkaa tuntemattomasta syystä kertyä keuhkokudokseen. Tämän seurauksena keuhkokudos paksuuntuu ja kaasujenvaihto vaikeutuu. Vastakkainen ilmiö on keuhkoahtaumataudille (chronic obstructive pulmonry disease, COPD) tyypillinen emfyseema eli keuhkojen laajentuma, jossa keuhkorakkularakenne on tuhoutunut.

Tämän tutkimuksen tavoitteena oli selvittää TGF-β-sytokiinin (transforming growth factor-beta) signaalinvälityksessä tapahtuvia muutoksia vaurioituneiden keuhkojen eri alueilla (fibroosi, emfyseema). Lisäksi tutkittiin TGF-β:n varastointia ja aktivaatiota sekä transkriptiofaktori GATA-6:n roolia keuhkofibroosin patogeneesissä. Lopuksi etsittiin uusia mahdollisuuksia keuhkofibroosin lääkehoitoon.

Tutkimusmateriaaleina käytettiin keuhkokudosnäytteitä, niistä eristettyjä primäärisiä solulinjoja sekä lisäksi kaupallisia solulinjoja. Geenien ilmentymistä, proteiinien sijaintia kudoksessa sekä solujen erilaistumista tutkittiin modernein ja monipuolisin molekyylibiologian menetelmin. Molekyylikirjastojen seulonnassa hyödynnettiin reportterisoluja ja tehoseulontateknologiaa.

Tutkimustulokset osoittivat, että TGF-β-signalointiaktiivisuus vaihtelee vaurioituneen keuhkokudoksen eri alueilla: se oli laskenut keuhkokudosnäytteiden emfysemaattisilla alueilla ja kohonnut fibroottisilla alueilla. Fibroottisessa keuhkokudoksessa havaittiin merkittävää nousua myös TGF-β:n varastointiin ja aktivaatioon liittyvän proteiinin ilmentymisessä.

Perinteisesti on uskottu, että fibroblastifokukset eli fibroosille tyypilliset (myo)fibroblastisolujen muodostamat pesäkkeet muodostuvat aktiivisesti jakautuvista soluista. Tutkimustulostemme mukaan näiden pesäkkeiden solut näyttäisivät kuitenkin olevan lepotilassa, ts. niillä ei havaittu aktiivista solunjakautumista tai ohjelmoitua solukuolemaa. Tästä huolimatta myofibroblastit olivat säilyttäneet kykynsä ottaa vastaan TGF-β-välitteisiä signaaleja, mikä viittaisi siihen, että ne edelleen ylläpitävät soluväliaineen tuottoa. Transkriptiofaktori GATA-6:n todettiin olevan oleellinen osa TGF-β:n indusoimaa, epiteeli- ja fibroblastisolujen erilaistumista ohjaavaa signaalinvälitysreittiä.

Tehoseulontateknologiaa käytettiin ensimmäistä kertaa IPF-tutkimuksessa. Tulokset olivat lupaavia: molekyylikirjastojen seulonnoissa löytyi yksi TGF-β-perheen signalointiin vaikuttava yhdiste, jolla pystyttiin osoittamaan biologisesti merkittäviä antifibroottisia vaikutuksia keuhkofibroosin hiirimallissa.

REVIEW OF THE LITERATURE

1 TGF-β & BMP SIGNALLING

1.1 The TGF-β superfamily

Thirty-three structurally related polypeptide growth factors are classified as members of the transforming growth factor (TGF)-β superfamily. These include TGF-βs, activins and inhibins, nodal, Leftys, bone morphogenetic proteins (BMPs), and growth/differentiation factors (GDFs) (de Caestecker 2004). They are phylogenetically divided into two main groups: the TGF-β/activin and BMP/GDF branches (de Caestecker 2004). These signalling molecules have wide-ranging and diverse roles in development, differentiation, and homeostasis.

1.2 Formation and activation of TGF-β

In mammals, there are three closely related isoforms of TGF-β, designated TGF-β1, -β2, and -β3. They have similar properties *in vitro* but distinct effects *in vivo* (Annes *et al.* 2003).

Thus far, TGF-β, GDF-8, and BMP-10 are the three unique members of this superfamily found to be synthesized as inactive precursors (de Caestecker 2004; Sengle *et al.* 2011). TGF-β is a 25 kD protein, composed of two 12.5 kD subunits held together by disulfide bonds (Assoian *et al.* 1983). The three TGF-βs are all synthesized as homodimeric proproteins (Annes *et al.* 2003). The TGF-β propeptides, also known as the latency-associated peptides (LAPs), are cleaved before the secretion from the cell (Gentry *et al.* 1988). However, they remain noncovalently bound to TGF-β, retaining TGF-β in a latent form incapable of binding to signalling receptors (Gentry *et al.* 1987).

A third component of the large latent TGF-β complex (Figure 1) is a disulfide-linked latent TGF-β binding protein (LTBP), which plays a critical role in the assembly and efficient secretion of the latent TGF-β. Through interactions with extracellular matrix proteins, LTBPs also regulate the storage and activation of TGF-β (Miyazono *et al.* 1991; Todorovic & Rifkin 2012). Thus far, four LTBPs have been identified (LTBP-1, -2, -3, and -4). Of these, LTBPs-1, -3, and -4 interact with latent TGF-β, whereas LTBP-2 does not (Saharinen & Keski-Oja 2000).

Latent TGF-β, bound to LTBPs, is targeted to fibrillin microfibrils and fibronectin in the extracellular matrix (ECM) (Dallas *et al.* 2005; Isogai *et al.* 2003; Massam-Wu *et al.* 2010; Taipale *et al.* 1996). For activation, the large latent TGF-β complex needs to be released from the ECM, and TGF-β liberated from the latent complex. TGF-β activation can be done via proteolytic or non-proteolytic mechanisms which result, respectively, in either cleavage or conformational changes of the latent TGF-β complex, allowing the active TGF-β peptide to interact with its receptors on cell surface. The proteolytic mechanisms include plasmin (Lyons *et al.* 1990), matrix metalloproteinase-9 and -2 (Yu & Stamenkovic 2000), and integrin αvβ8 (Mu *et al.* 2002); thrombospondin-1 (Crawford *et al.* 1998) and integrin αvβ6 (Munger *et al.* 1999) work in a non-proteolytic manner. In this context, fibrillins are

interesting because in addition to LTBP binding sites, fibrillins also contain binding sites for integrin receptors (Olivieri *et al.* 2010) and integrin-mediated TGF-β activation is suggested to be important in the pathogenesis of IPF (Chandramouli *et al.* 2011; Goodwin & Jenkins 2009). However, TGF-β activation in IPF is still not well understood.

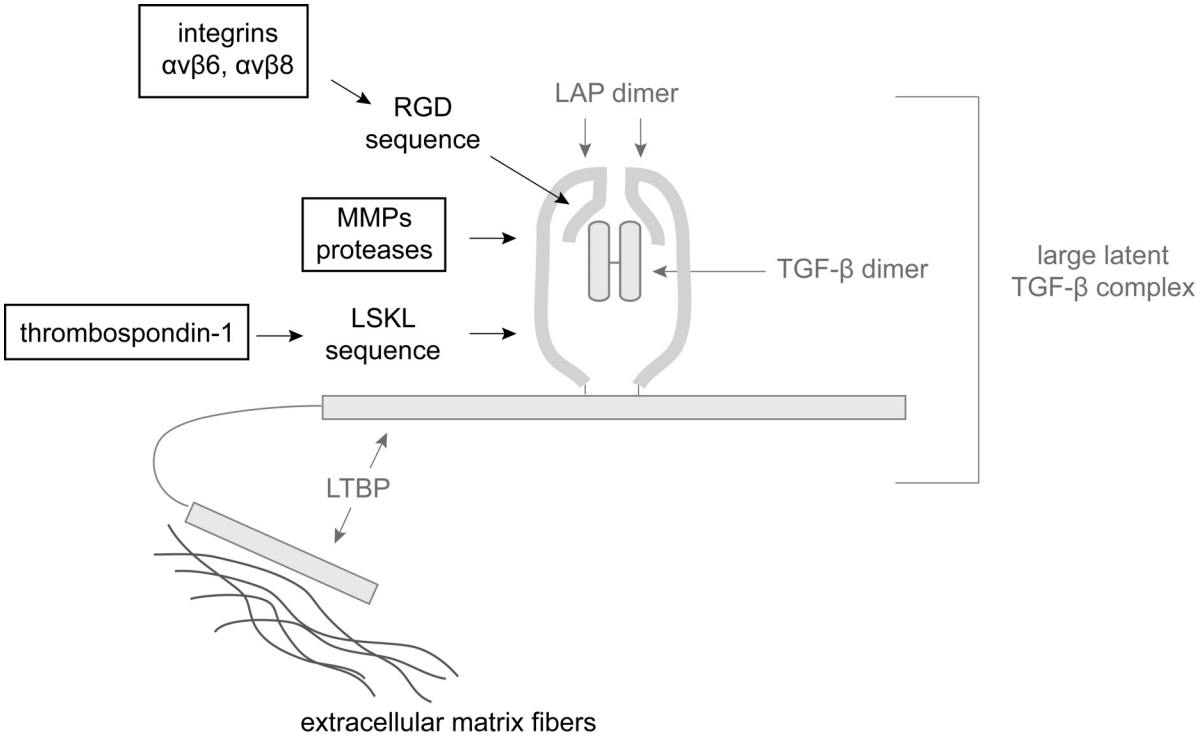

Figure 1.The large latent TGF-β complex consists of three components: TGF-β dimer, LAP dimer, and LTBP. TGF-β can be activated, i.e. liberated from the latent complex, *via* several mechanisms (marked with □). Integrins and thrombospondin-1 bind to recognition sequences in LAP (RGD and LSKL, respectively). Modified from Koli *et al.* 2008.

1.3 The expression and bioavailability of BMP

BMP-7 is secreted as a stable complex of processed growth factor dimer noncovalently associated with its two prodomain propeptide chains (Gregory *et al.* 2005). The possibility of forming similar complexes *in vivo* has also been suggested for BMP-2, -4, -9, and -10 (Brown *et al.* 2005; Sengle *et al.* 2008). Of these, BMP-10 complex needs activation like TGF-β and GDF-8 complexes (Sengle *et al.* 2011). The function of the propeptides is to target the

growth factors to ECM macromolecules, such as fibrillin microfibrils (Gregory *et al.* 2005; Sengle *et al.* 2008).

Contrary to TGF-β signalling activity, BMP signalling activity is regulated on the level of bioavailability. This is done by a number of BMP-binding proteins such as Noggin, Chordin, and Gremlin (Umulis *et al.* 2009). The binding proteins can either inhibit or promote BMP signalling by affecting BMP release, transport through tissues, and receptor binding (Umulis *et al.* 2009).

1.4 Receptors in TGF-β and BMP signalling

Active TGF-βs and BMPs signal through single-pass transmembrane serine/threonine kinase proteins, referred to as the TGF-β receptor family. Based on their structural and functional properties, the TGF-β receptor family is divided into two subfamilies: type I (55 kD) and type II (70 kD) receptors (Massague 1998).

A heteromeric receptor complex consists of two distinct transmembrane proteins (Attisano & Wrana 2002). Ligand binding induces the type I and type II receptors to associate, which leads to a unidirectional phosphorylation event in which the type II receptor phosphorylates the type I receptor, thereby activating its intracellular kinase domain (Attisano & Wrana 2002). The activated type I receptor then activates Smad family proteins or other intracellular mediators which carry the signal to the nucleus (Massague 1998).

1.5 Smad protein classification

After binding to their respective receptors, TGF-β and BMPs signal within the cell through the Smad family of transcription activators, which in mammals includes eight proteins. Smads have been named after related *Drosophila* gene *mothers against decapentaplegic (Mad)* and *Caenorhabditis elegans* gene *Sma*. Smads are divided into three classes: the receptor-regulated R-Smads, inhibitory I-Smads, and a co-Smad. A schematic diagram of TGF-β/BMP signalling through Smad proteins is presented in Figure 2.

R-Smads (Smad-1, -2, -3, -5, -8) are directly phosphorylated by type I receptors on two conserved serine residues at the C-terminus. Smad-2 and Smad-3 are phosphorylated in the presence of TGF-β ligand (Leask & Abraham 2004). Smad-1, Smad-5 and Smad-8 are substrates for bone morphogenetic protein receptor I (BMP RI) (Miyazono *et al.* 2010), and are thus mediators of BMP signals.

Phosphorylation stimulates R-Smads to accumulate in the nucleus as heteromeric complexes with Smad-4, which is the only member of the co-Smad class and shared by BMP and TGF-β/activin signalling pathways (Miyazono *et al.* 2010).

The third class, the I-Smads (Smad-6 and -7), antagonize TGF-β and BMP signalling by competing for receptor binding and thus preventing R-Smad phosphorylation, by competing with co-Smad for complex formation, and by inhibiting the Smad-DNA complex formation (Leask & Abraham 2004; Miyazono *et al.* 2010) (Figure 2). TGF-β rapidly induces

expression of Smad-7, suggesting that Smad-7 may participate in a negative feedback loop to control TGF-β responses (Nakao *et al.* 1997). In a similar fashion, the expression of Smad-6 is regulated by BMP-activated Smad1/5 (Ishida *et al.* 2000).

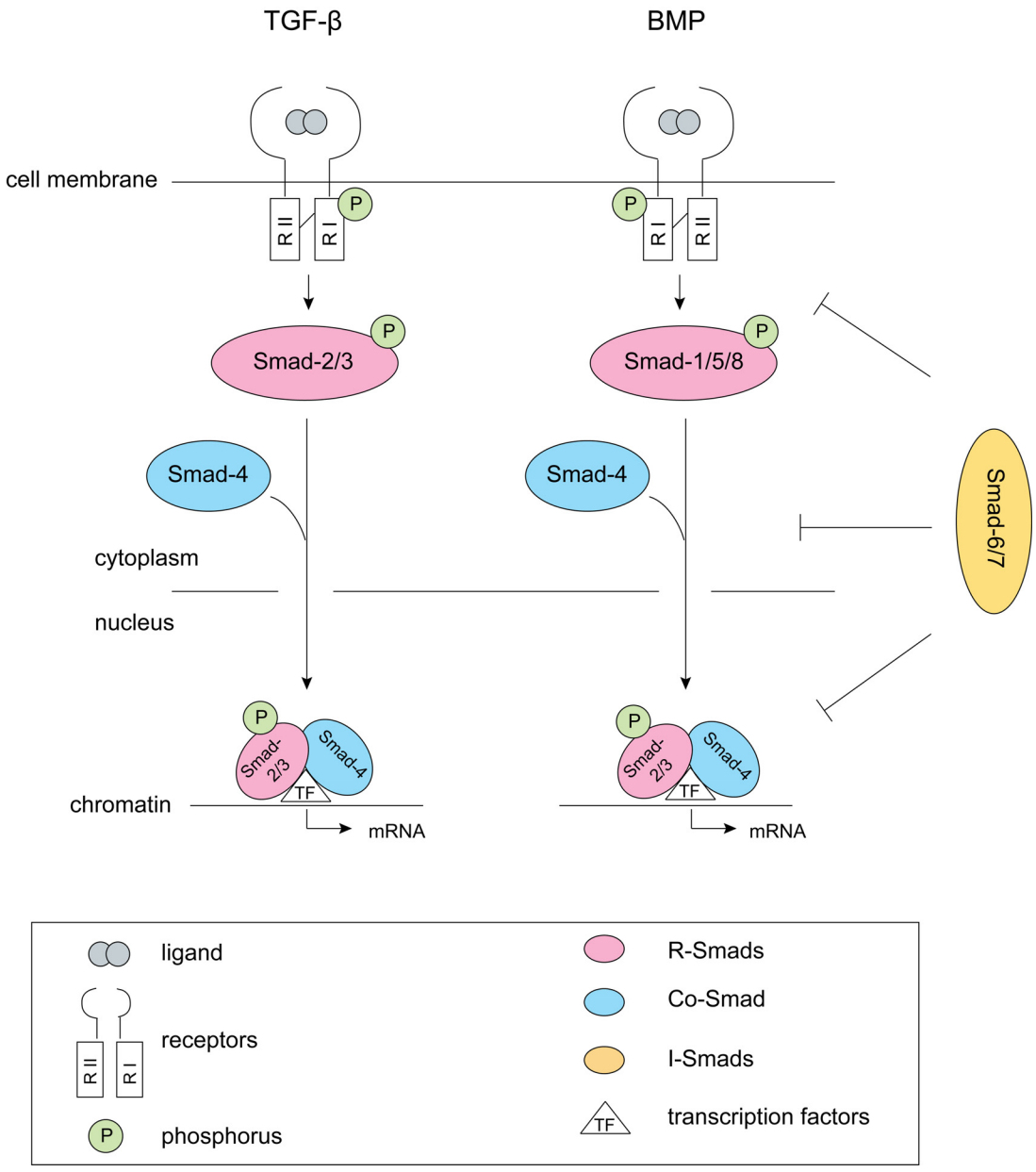

Figure 2. A simplified diagram of the TGF-β and BMP pathways with their corresponding Smad proteins. TGF-β and BMP receptors phosphorylate R-Smads, which form a complex with Smad-4. This complex is shuttled into the nucleus where it binds to chromatin and, together with other transcription factors, regulates target gene expression. Modified from Miyazono *et al.* 2010; Moustakas & Heldin 2009.

1.6 Function of Smads

Smads do not themselves activate transcription but are rather believed to assist in the formation of a functional transcriptional complex on target promoters with other transcription factors (Attisano & Wrana 2002). In the basal state, Smads exist as homo-oligomers in the cytoplasm (Massague 1998). Upon ligand activation of the receptor complex, the type I kinase phosphorylates specific Smads, which then form a complex with Smad-4 and move into the nucleus. In the nucleus, these complexes activate target genes by binding to specific promoter elements (Massague 1998). The cellular responses and transcriptional outcome is dependent on interactions with other transcription factors, co-activators, and co-repressors, whose identity varies depending on the promoter or cell type of interest (Leask & Abraham 2004; Mu *et al.* 2012).

Inman *et al*. (2002) have shown that during the active signalling, Smads are constantly shuttling between the nucleus and the cytoplasm. The bulk of nuclear Smad-2 and Smad-3 is not targeted for degradation, but are continuously being dephosphorylated which results in their dissociation from Smad-4. The Smads recycle back to the cytoplasm independently. If the receptors are still active in the cytoplasm, the R-Smads are rephosphorylated, form complexes with Smad-4, and return to the nucleus. If the receptors are no longer active, the R-Smads are retained in the cytoplasm.

1.7 Non-Smad signalling pathways

TGF-βs and BMPs have a complicated intracellular signalling network with both Smad and non-Smad signalling pathways. In Smad-dependent pathways, phosphorylated Smad proteins carry their signals to the nucleus. In addition to Smads, ligand binding to TGF-β receptors can activate other proteins such as the small GTPase Ras or mitogen-activated protein kinases ERK (extracellular signal-regulated kinase), p38 and JNK (c-Jun N-terminal kinases) (Moustakas & Heldin 2005). The activation of these alternative signalling pathways can result in either Smad phosphorylation or induce responses unrelated to Smads (Derynck & Zhang 2003; Nohe *et al.* 2002; Sovershaev *et al.* 2011; Tian *et al.* 2012). Non-Smad signalling proteins have three general mechanisms, which are presented in Figure 3.

Interestingly, these two signalling systems are parallel. TGF-β can activate e.g. JNK in two ways: rapid, Smad-independent JNK activation is followed by sustained, Smad-dependent JNK activity (Engel *et al.* 1999). It has also been shown that when BMP-2 binds to a preformed receptor complex, it activates the Smad pathway, whereas BMP-2-induced recruitment of receptors activates a Smad-independent p38 MAPK pathway (Nohe *et al.* 2002). Thus, the cellular responses to TGF-β and BMPs are often defined by the balance between direct activation of Smads and mitogen-activated protein kinase pathways (Derynck & Zhang 2003).

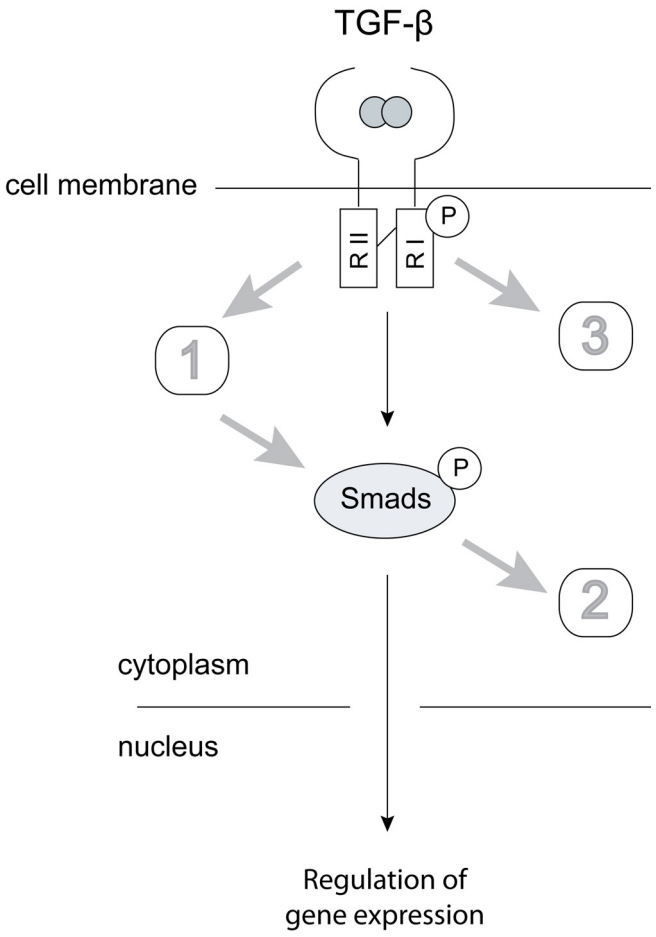

Figure 3. A schematic diagram of Smad and non-Smad signalling. The Smad pathway starting from the ligand-receptor complex and ending in the nucleus is illustrated by thin black arrows. Non-Smad signalling mechanisms are illustrated by thick grey arrows. There are three distinct but interrelated modes of action: **1**) non-Smad signalling pathways directly modify the Smads; **2**) Smads directly interact and modulate the activity of other signalling proteins and transmit signals to other pathways; and **3**) the TGF-β receptors directly interact with or phosphorylate non-Smad proteins, initiating parallel signalling that cooperates with the Smad pathway. Modified from Moustakas & Heldin 2005.

1.8 Transcription factors

Smad proteins form complexes with DNA-binding transcription factors (Attisano & Wrana 2002). Numerous transcription factors can mediate the signals of TGF-β family members but the repertoire of expressed transcription factors differs between tissue and cell type (Moustakas & Heldin 2009). This enables the complexity of transcriptional regulation of target genes (Ikushima & Miyazono 2012). Examples of transcription factors linked to TGF-β signalling in the lungs are presented in Table 1.

Table 1. Examples of transcription factors that are linked to TGF-β signalling in the lungs.

Transcription factor	Alternative name	Function	Reference
Early growth response factor (Egr)-1, -2	Egr-1 = Krox-20 Egr-2 = Krox-24	mediators of TGF-β-induced profibrotic responses, e.g. collagen expression	Bhattacharyya *et al.* 2008; Fang *et al.* 2011
Yin Yang 1 (YY1)		regulates fibrogenesis by increasing α-SMA and collagen expression	Lin *et al.* 2011
SNAI1, -2	SNAI1 = Snail SNAI2 = Slug	mediators of TGF-β-induced EMT	Jayachandran *et al.* 2009
Twist		contributes to EMT	Pozharskaya *et al.* 2009
Thyroid transcription factor (TTF)-1	Nkx2.1	inhibits TGF-β-mediated EMT in lung cancer	Saito *et al.* 2009
GATA-6		mediates TGF-β-induced cell differentiation	Leppäranta *et al.* 2010

α-SMA, alpha-smooth muscle actin; EMT, epithelial-to-mesenchymal transition (*see section 2.4.2*)

1.8.1 GATA-6

GATA transcription factors regulate the expression of various genes required for developmental processes and tissue-specific functions. Six GATA transcription factors have been identified in vertebrates. Based on their expression patterns, they have been divided into two subfamilies: GATA-1, -2, and -3 function in hematopoietic cells whereas GATA-4, -5, and -6 function in mesoderm- and endoderm-derived organs (Maeda *et al.* 2005).

Each of the GATA proteins contains a highly conserved DNA binding domain consisting of two zinc fingers, which bind to a consensus sequence (A/T)GATA(A/G) (Molkentin 2000). The GATA-6 gene has two transcriptional start sites, which enables the production of both the L-type (long) and the S-type (short) isoforms in the same tissue-specific and developmental stage-specific pattern (Brewer *et al.* 1999; Takeda *et al.* 2004).

GATA-6 plays an important role in the establishment of the endodermally derived bronchial epithelium in lungs, lung cell differentiation, and regulation of gene expression (Morrisey *et al.* 1998; Shaw-White *et al.* 1999). It is expressed in embryonic lung tissue with a precise temporal-spatial control which is required to enable normal lung morphogenesis (Liu *et al.* 2003; Suzuki *et al.* 1996).

GATA-6 is also expressed in the adult lungs (Suzuki *et al.* 1996). Within the respiratory epithelium, GATA-6 has been implicated in the transcriptional regulation of genes such as

surfactant protein (SP)-A, and thyroid transcription factor-1 (TTF-1) (Bruno *et al.* 2000; Shaw-White *et al.* 1999).

1.9 TGF-β family in development and disease

TGF-β family members play a key role in normal development, homeostasis, and tissue repair. Either gain or loss of these signalling processes underlies numerous disorders (Figure 4).

During development, various factors regulate cell fate, i.e. the commitment of stem and progenitor cells to differentiation pathways. TGF-β family members play a key role in this process, both in cell lineage selection and progression of differentiation (Derynck & Akhurst 2007). They are also able to induce transdifferentiation, which means redirecting the differentiation of cells in particular lineage. This kind of cell plasticity is an essential and well-orchestrated event in normal tissue and organ development (Derynck & Akhurst 2007). In adults, TGF-β helps to maintain tissue homeostasis by controlling the proliferation, apoptosis, and microenvironmental interactions of epithelial, endothelial, neuronal, and haematopoietic cell lineages (Siegel & Massague 2003).

However, problems arise when TGF-β signalling is disturbed. This can be the result of either mutations in TGF-β family ligands, receptors, and signalling proteins, or dysregulation of signalling in the TGF-β pathway itself (Horbelt *et al.* 2012; Santibanez *et al.* 2011). Aberrant TGF-β signalling contributes to a wide range of disorders, including cardiovascular, fibrotic, inflammatory, pulmonary, bone, and muscle diseases as well as cancer (Horbelt *et al.* 2012; Santibanez *et al.* 2011).

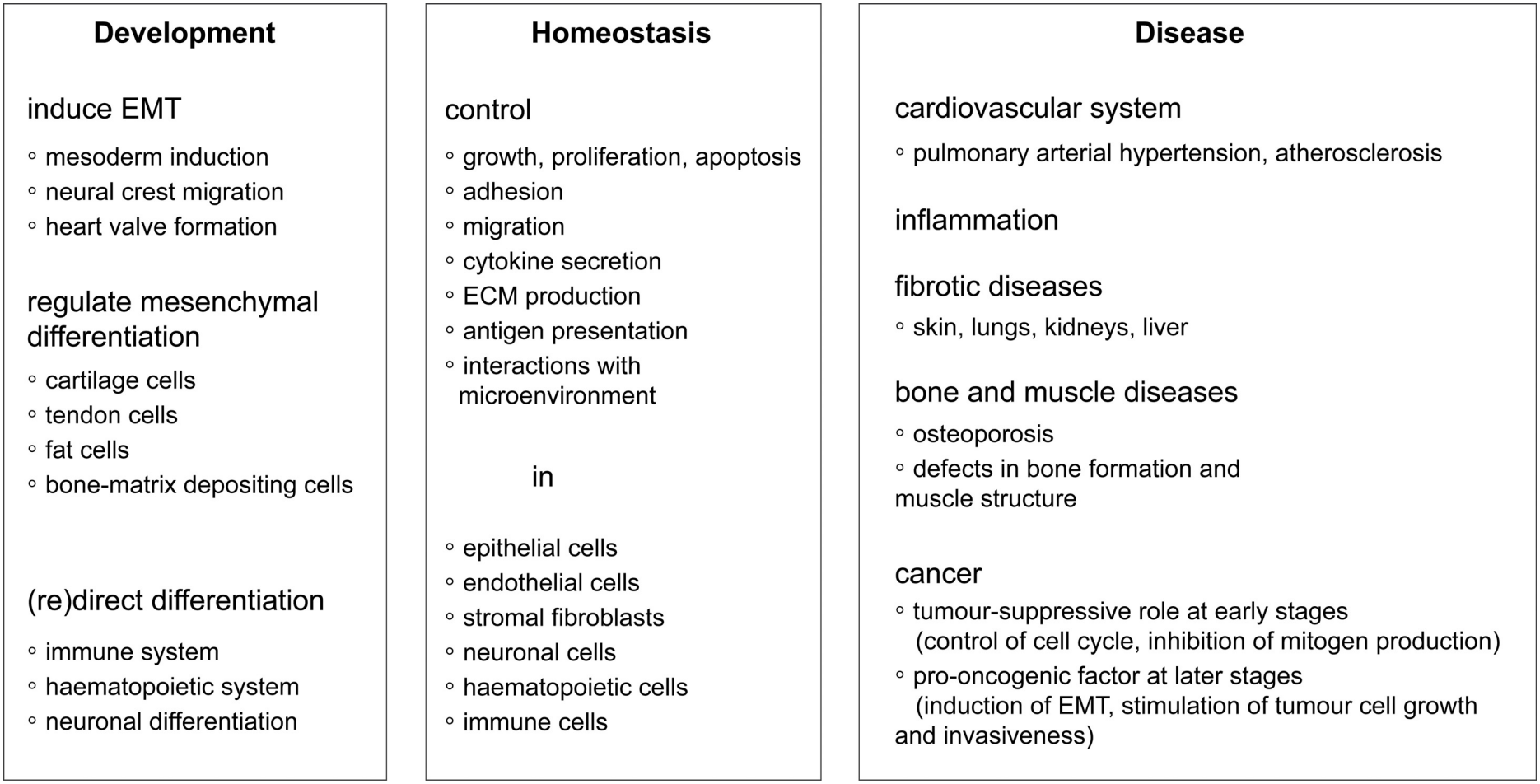

Figure 4. Examples of the roles of TGF-β family members in human development, homeostasis, and diseases (August & Suthanthiran 2006; Derynck & Akhurst 2007; Gordon & Blobe 2008; Santibanez *et al.* 2011; Siegel & Massague 2003). EMT, epithelial-to-mesenchymal transition (*see section 2.4.2*).

2 IDIOPATHIC PULMONARY FIBROSIS (IPF)

2.1 IPF and other interstitial lung diseases

The idiopathic interstitial pneumonias are a heterogeneous group of diffuse parenchymal lung diseases. *Idiopathic* indicates unknown cause and *interstitial pneumonia* refers to involvement of the lung parenchyma by varying combinations of fibrosis and inflammation (American Thoracic Society & European Respiratory Society 2002). The interstitium includes the space between the epithelial and endothelial basement membranes and it is the primary site of pathologic tissue accumulation in idiopathic interstitial pneumonias. However, these disorders frequently affect not only the interstitium, but also the airspaces, peripheral airways, and vessels along with their respective epithelial and endothelial linings (American Thoracic Society & European Respiratory Society 2002).

Idiopathic pulmonary fibrosis (IPF) is the most common form of idiopathic interstitial pneumonias (Meltzer & Noble 2008). It is characterized by the formation of scar tissue within the lungs in the absence of any known provocation (Meltzer & Noble 2008). IPF is a chronic, progressive disease with a median survival of 2-5 years following diagnosis (Meltzer & Noble 2008; Raghu *et al.* 2011; Scotton & Chambers 2007).

2.2 Clinical features and diagnosis of IPF

The patients' age at onset is usually greater than 60 years (Johnston *et al.* 1997). Breathlessness and chronic dry, non-productive cough are the most prominent symptoms (American Thoracic Society & European Respiratory Society 2002). Spirometry reveals decreased measures of forced vital capacity (FVC) and forced expiratory volume in one second (FEV1); the ratio of FEV1/FVC remains normal (or increased) in IPF (Meltzer & Noble 2008).

The accurate diagnosis of IPF requires a compatible clinical history, the exclusion of other known causes of interstitial lung diseases - such as drug toxicity, environmental exposures, or connective tissue disease - and the presence of a so-called UIP (usual interstitial pneumonia; *see section 2.4.1*) pattern on high-resolution computed tomography (Raghu *et al.* 2011). In typical cases of IPF, the diagnosis can be made without a surgical lung biopsy; however, it is often necessary to make a definitive diagnosis.

2.3 Incidence and prevalence of IPF

IPF is estimated to affect approximately 5 million people worldwide (Meltzer & Noble 2008). Both incidence and prevalence of IPF are generally higher among men than women (Johnston *et al.* 1997; Raghu *et al.* 2006). Data from around the world demonstrates that IPF favours no particular race, ethnic group or social environment (Meltzer & Noble 2008).

The world-wide incidence of IPF is estimated at 7-10 / 100 000 / year (Pardo & Selman 2002; Raghu *et al.* 2006). In the US, using narrow criteria, prevalence is estimated to be 14 / 100 000 (Raghu *et al.* 2006). In Finland prevalence is 16-18 /100 000 (Hodgson *et al.* 2002),

which corresponds to roughly 1000 patients. According to the European Union definition - any disease affecting fewer than 5 people in 10 000 - IPF is considered to be a rare disease (http://ec.europa.eu/health/rare_diseases/policy/index_en.htm).

Although sporadic cases constitute the majority of cases, familial cohorts of IPF are also reported (Meltzer & Noble 2008). For example in Finland, a cohort of familial cases was strongly clustered in a certain area in Eastern Finland, suggesting a founder effect among them (Hodgson *et al.* 2002).

2.4 Characteristic features of IPF

2.4.1 Usual interstitial pneumonia pattern

IPF is associated with the histopathologic pattern known as usual interstitial pneumonia (UIP), which consists of normal lung architecture alternating with patches of pulmonary parenchymal fibrosis, fibroblastic foci, and architectural distortion (Meltzer & Noble 2008; Raghu *et al.* 2011). In addition, histology of IPF/UIP lungs can show mild interstitial inflammation (Raghu *et al.* 2011; Scotton & Chambers 2007). The alveolar epithelium in UIP can undergo various morphological alterations. Especially over the fibrotic lesions, the normal epithelial cell layer is often replaced with either activated, hyperplastic epithelial cells or elongated and flattened fibroblast-like epithelial cells (Selman & Pardo 2006).

The pathological alterations in pulmonary tissue are reflected to radiological findings. In high resolution computed tomography, the UIP pattern is characterized by the presence of reticular opacities. Honeycombing, i.e. clustered cystic airspaces, is common and critical for making a definite diagnosis. The distribution of UIP on high resolution computed tomography is characteristically subpleural with basal predominance, and often patchy (Raghu *et al.* 2011).

2.4.2 Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a process whereby fully differentiated epithelial cells undergo transition to a mesenchymal phenotype giving rise to fibroblasts and myofibroblasts. It can be viewed as an extreme form of cell plasticity, which is characterized by loss of epithelial markers (e.g. E-cadherin and zonula occludens-1), cytoskeletal reorganization, transition to a spindle-shaped morphology concurrent with acquisition of mesenchymal markers (e.g. vimentin, α-smooth muscle actin (α-SMA), desmin, (pro)collagen, fibronectin, and fibroblast-specific protein-1), and an invasive phenotype (Willis & Borok 2007).

Nowadays, three different subtypes of EMT have been classified: type 1 EMT generates primary mesenchymal cells during embryogenesis and organ development and is thus associated with proper morphogenesis; type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis; and type 3 EMT occurs in neoplastic cells with genetic and epigenetic changes affecting oncogenes and tumor suppressor genes (Kalluri & Weinberg 2009).

In lungs, alveolar epithelial cells, and type II pneumocytes in particular, seem to be progenitors for mesenchymal cells (Kim *et al.* 2006). The contribution of EMT to the mesenchymal cell accumulation in fibrosis is suggested to be significant: genetic mapping of epithelial cells has shown that in experimental models of lung fibrosis, approximately one-third of fibroblasts are of epithelial origin (Kim *et al.* 2006; Tanjore *et al.* 2009).

The fibrotic EMT in response to TGF-β1 is mediated predominantly via Smad-dependent pathways (Gorowiec *et al.* 2012; Willis & Borok 2007) with a little help at the transcriptional level from a selection of transcription factors (e.g. members of the SNAI family and Twist, which is a member of the basic helix-loop-helix family; see Table 1) and other repressors and activators (Jayachandran *et al.* 2009; Pozharskaya *et al.* 2009; Xu *et al.* 2009).

2.4.3 Myofibroblasts

An essential and distinctive morphological feature in IPF is the development of the so-called fibroblastic foci, represented by widely scattered small aggregates of persistent subepithelial fibroblasts and myofibroblasts immersed within extracellular matrix (Pardo & Selman 2002).

Myofibroblasts are spindle-shaped cells that share features with smooth muscle cells in that they are contractile and contain α-SMA stress fibers (Scotton & Chambers 2007). They are the primary cell type responsible for the synthesis and deposition of ECM and the resultant structural remodelling that leads to the loss of alveolar function (Pardo & Selman 2002; Scotton & Chambers 2007).

The amount of fibroblastic foci is considered a prognostic factor in IPF patients, with a positive correlation between mortality and a high score of fibroblastic foci (King *et al.* 2001; Nicholson *et al.* 2002).

2.4.4 Extracellular matrix

ECM is a dynamic environment that interacts with cell surface receptors and soluble growth factors (Olivieri *et al.* 2010). It provides cells with support and guidance in essential cellular functions such as proliferation, migration, and differentiation (Olivieri *et al.* 2010; Todorovic & Rifkin 2012). Thus, it has an important role in normal tissue formation, homeostasis, and repair. This architectural matrix consists of multiple types of proteins, primarily fibronectin, collagenous and elastic fibers, fibrillin-containing microfibrils, adaptor proteins, and hydrophilic proteoglycans (Olivieri *et al.* 2010; Todorovic & Rifkin 2012). An intact fibronectin matrix is essential in the assembly of e.g. collagen types I and III and fibrillin-1 into the ECM, suggesting that fibronectin is the master orchestrator for the organization of various matrix components (McDonald *et al.* 1982; Sabatier *et al.* 2009; Sottile & Hocking 2002).

Characteristic for fibrotic lung tissue is the excessive accumulation of ECM, which eventually contributes to perturbation of normal tissue architecture and development of fibrosis (Eickelberg *et al.* 2001). Collagens are the most predominant ECM proteins in fibrotic lesions (Kuhn *et al.* 1989), and the increased collagen amount and concentration leads to increased tissue stiffness (Cox & Erler 2011). Another group of abnormally abundant

proteins in IPF lungs' ECM are fibronectins, especially a splice variant containing extra type III domain A (EDA) (Kuhn *et al.* 1989; Muro *et al.* 2008). EDA-containing fibronectin is necessary for the differentiation of fibroblasts into myofibroblasts, and it also participates in latent TGF-β activation (Muro *et al.* 2008; Serini *et al.* 1998).

2.4.5 Signalling molecules

One of the key factors in fibrogenesis is TGF-β, which is present in excess in fibrotic lungs (Khalil *et al.* 1991). TGF-β has many profibrotic effects (Figure 5): it contributes to mesenchymal cell accumulation by promoting EMT as well as fibroblast proliferation and transformation into myofibroblasts (Coward *et al.* 2010), and it also continually induces the synthesis of ECM components in fibroblasts and myofibroblasts (Leask & Abraham 2004). These profibrotic effects are mediated by both Smad and non-Smad signalling proteins (Eickelberg 2001).

In addition to TGF-β, Wnt is another signalling system related to developmental processes and homeostasis. TGF-β and Wnt pathways can interact through p38 and JNK (Akhmetshina *et al.* 2012; Herr *et al.* 2012). Recently, it has been found that in IPF, the canonical Wnt signalling is activated and its interaction with TGF-β is central in the pathogenesis of IPF (Akhmetshina *et al.* 2012; Chilosi *et al.* 2003).

Yet another profibrotic effect of TGF-β is its ability to induce gremlin production (Costello *et al.* 2010). Gremlin is a secreted protein which belongs to the DAN family of BMP antagonists (Hsu *et al.* 1998). It has a physiological role during lung development, as it balances the expression of BMP-4 and BMP-7 in lung branching morphogenesis (Bellusci *et al.* 1996; Shi *et al.* 2001). Gremlin is again activated in IPF patients whose mesenchymal cells secrete elevated levels of gremlin (Koli *et al.* 2006; Myllärniemi *et al.* 2008a; Myllärniemi *et al.* 2008b). It is also suggested to have a role in sarcoidosis, where variation in *GREM1* gene may predispose patients to pulmonary fibrosis via single nucleotide polymorphism in target sequences for miRNAs, which regulate the induction of gremlin expression (Heron *et al.* 2011).

Gremlin contributes to fibrosis progression by inhibiting BMP signalling (Figure 5) (Myllärniemi *et al.* 2008a). In normal lungs, BMP signalling has a role in basal airway homeostasis and it is activated in the adult airway epithelium during regeneration (Masterson *et al.* 2011). Due to reactivated expression of gremlin, BMP signalling is impaired and epithelial regeneration processes reduced in IPF patients (Koli *et al.* 2006).

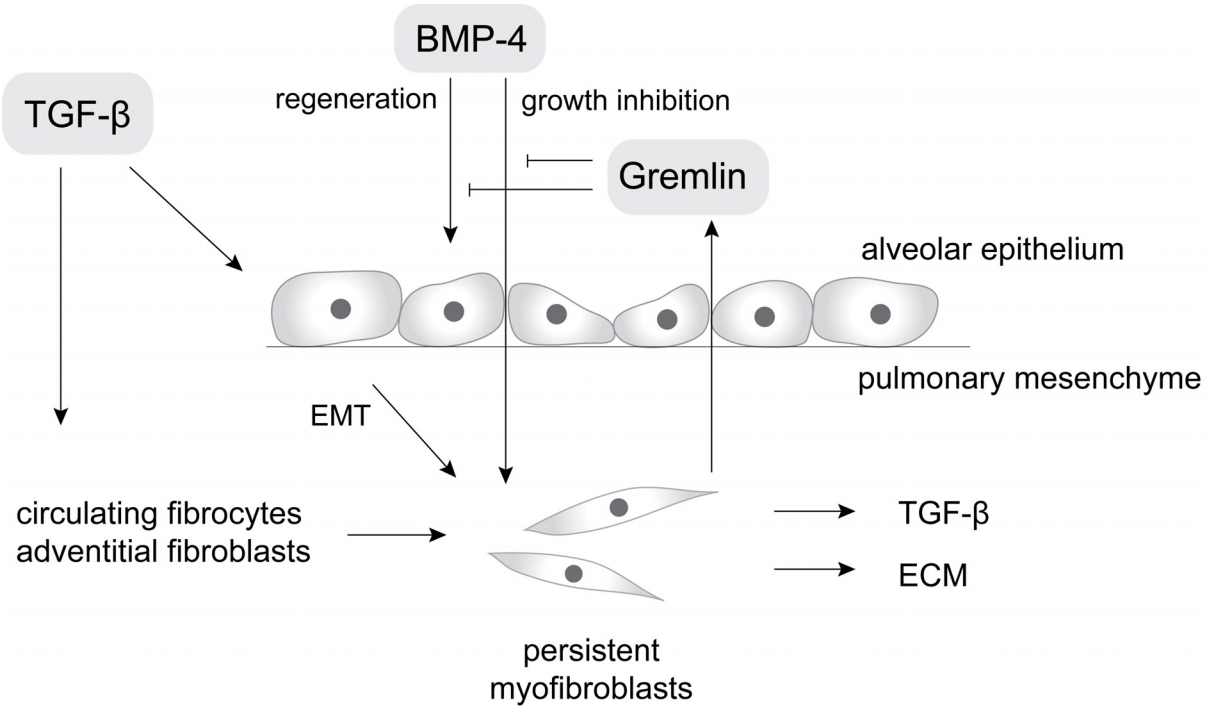

Figure 5. A schematic presentation of the potential roles of the TGF-β and BMP signalling pathways in IPF (modified from Koli *et al.* 2006). The expression of TGF-β is induced during early stages of IPF. TGF-β stimulates EMT of alveolar epithelial cells. It also recruits and activates fibroblasts which differentiate to myofibroblasts. The persistent myofibroblasts overexpress Gremlin, which inhibits BMP-induced regeneration of the epithelium and blocks BMP-mediated myofibroblast apoptosis.

2.5 Treatment options for IPF

There is still no therapy that has been shown to improve survival or otherwise significantly modify the clinical course of IPF in all patients. It is thus recommended that patients be considered for recruitment to high quality clinical trials of therapy and/or for lung transplantation if appropriate (Bradley *et al.* 2008; Raghu *et al.* 2011). Because of the lack of an effective therapy, new modalities are constantly being sought. Some of the most recent and promising clinical trials are presented below and in Table 2.

There has been some indication of a beneficial effect e.g. for combined triple therapy with prednisone (a corticosteroid), azathioprine (an immunosuppressant), and N-acetylcysteine (NAC) (an antioxidant) (Demedts *et al.* 2005). In late 2011, however**,** the US drug authorities released a clinical alert as the results of a NAC study (PANTHER-IPF) showed that this widely used three-drug regimen led to a significant increase in mortality and serious adverse effects compared to the nontreated group (www.IPFnet.org). Studies for the assessment of NAC monotherapy are ongoing (Table 2), and a recent study with early stage IPF patients has shown promising beneficial effects in some subgroups of patients (Homma *et al.* 2012; www.IPFnet.org).

Pirfenidone (Table 2), the first drug indicated for IPF therapy, was approved in Japan 2008, in India 2010, and it became available in Europe in 2011. It has been shown to decrease the number of IPF exacerbations, stabilize patient lung function, and the CAPACITY programme (Clinical Studies Assessing Pirfenidone in idiopathic pulmonary fibrosis: Research of Efficacy and Safety Outcomes) confirmed that pirfenidone has a favourable benefit-risk profile (Azuma *et al.* 2005; Noble *et al.* 2011; Raghu *et al.* 1999; Taniguchi *et al.* 2010). Additional clinical trials for evaluation of safety and efficacy of pirfenidone are currently recruiting patients (www.clinicaltrials.gov).

BIBF 1120 is a potent intracellular inhibitor of tyrosine kinase receptors. Its targets include platelet-derived growth factor receptors, vascular endothelial growth factor receptors, and fibroblast growth factor receptors, which all activate profibrotic signalling pathways (Richeldi *et al.* 2011). A randomized phase II trial showed promising results (Table 2) (Richeldi *et al.* 2011). Studies for long-term safety and efficacy are ongoing (www.clinicaltrials.gov).

Interferon gamma (IFN-γ) is a cytokine secreted by T-helper type I cells. It has been shown to inhibit fibroblast proliferation and collagen synthesis (Narayanan *et al.* 1992; Okada *et al.* 1993). Despite the promising results of *in vitro* and animal model studies, its efficacy remained controversial in clinical trials (Table 2) (Bajwa *et al.* 2005; King *et al.* 2009; Raghu *et al.* 2004). In these trials, the patients received IFN-γ subcutaneously. A new approach is inhaled IFN-γ aerosol, which enables higher concentration of drug delivered to the lung (Diaz *et al.* 2012).

Pulmonary hypertension is common in IPF patients awaiting lung transplant (Shorr *et al.* 2007). The rationale behind the use of sildenafil (Table 2) is to relief the increased vascular pressure and hence improve the patients' exercise tolerance (Jackson *et al.* 2010). Sildenafil inhibits phosphodiesterase type 5 (PDE5) (Corbin & Francis 1999). The pulmonary vasodilating effects of nitric oxide are mediated through cyclic guanosine monophosphate, which is rapidly degraded by PDE5 (Corbin & Francis 1999; Galie *et al.* 2005). Thus, inhibition of PDE5 by sildenafil enhances the cyclic guanosine monophosphate-mediated relaxation of vascular smooth muscle cells and causes pulmonary vasodilation (Ghofrani *et al.* 2002).

Table 2. Examples of ongoing clinical trials for finding new treatment options for IPF.

Drug	Mode of action	Clinical trials & Results	References
N-acetylcysteine (NAC)	○ stabilizes AEC intracellular redox state and preserves intracellular glutathione levels ○ inhibits EMT *in vitro*	○ in combination with standard therapy, NAC may slow down disease progression. However, this did not translate into survival benefit (IFIGENIA study) ○ one arm of PANTHER trial (conventional therapy + NAC) stopped because of increased mortality and serious adverse effects. The two remaining arms of PANTHER phase III trial (NAC, placebo) will continue. Results are expected in 2013. ○ NAC monotherapy may have some beneficial effect in early-stage IPF patients	Demedts *et al.* 2005; Felton *et al.* 2009; Homma *et al.* 2012; www.IPFnet.org
pirfenidone	○ inhibits collagen synthesis ○ reduces the number of myo-fibroblasts and the expression of TGF-β ○ suppresses Smads signalling	○ prevents acute exacerbation ○ approved in Japan, India, and Europe for the treatment of IPF ○ additional clinical trials recruiting patients	Azuma *et al.* 2005; Choi *et al.* 2012; Iyer *et al.* 2000; Kakugawa *et al.* 2004; Raghu *et al.* 1999
BIBF 1120	○ inhibits tyrosine kinase receptors	○ a trend toward a reduction in the decline in lung function, fewer acute exacerbations, and preserved quality of life ○ a trial for continuation of BIBF 1120 treatment for IPF patients is ongoing. Estimated study completion in 2015.	Richeldi *et al.* 2011; www.clinicaltrials.gov
Interferon-gamma (IFN-γ)	○ suppresses the level of procollagen ○ downregulates TGF-β over-expression	○ some patients might benefit from treatment with IFN-γ; individual susceptibility could determine clinical response. Efficacy remains controversial. ○ a pilot phase I study of aerosol IFN-γ showed that inhaled IFN-γ is safe and effectively delivered to the lung parenchyma; no therapeutic conclusions.	Bajwa *et al.* 2005; Diaz *et al.* 2012; Goldring *et al.* 1986; Gurujeyalakshmi & Giri 1995; Luppi *et al.* 2009; Raghu *et al.* 2004; www.clinicaltrials.gov
Sildenafil	○ inhibits phosphodiesterase-5 ○ causes pulmonary vasodilation	○ approved for use in pulmonary arterial hypertension ○ controversial results with IPF patients, further research needed ○ a phase IV study is ongoing, estimated study completion in 2013	Collard *et al.* 2007; Idiopathic Pulmonary Fibrosis Clinical Research Network *et al.* 2010; Jackson *et al.* 2010; www.clinicaltrials.gov

3 EMPHYSEMA

Chronic obstructive pulmonary disease (COPD) is a major global health problem, which is predicted to become the 4th most common cause of death in the world by 2030 (Mathers & Loncar 2006). It is characterized by airflow obstruction, for which three major mechanisms have been implicated: the first is the loss of elasticity and the destruction of the alveolar attachments of airways within the lung as a result of emphysema, which results in a loss of support and closure of small airways during expiration; the second is the narrowing of small airways as a result of inflammation and scarring; and the third is the blocking of the lumen of small airways with mucous secretions (Barnes 2004).

The characteristic lung parenchymal abnormality found in COPD patients is emphysema, which represents a reaction of the alveolar space distinct from fibrosis. Histopathologically, emphysema is characterized by loss of alveolar structure due to alveolar wall destruction, which leads directly to a reduction in total alveolar number and increased airspaces in the parenchyma (Fischer *et al.* 2011). This results in a reduction in the ratio of gas exchange surface area to lung volume, and respiratory insufficiency (Morty *et al.* 2009).

Emphysema is often accompanied by small airways disease, which includes airway inflammation with increased mucus production, airway remodelling, and peribronchiolar fibrosis, all contributing to the airflow limitation (Barnes *et al.* 2003).

3.1 Combined pulmonary fibrosis and emphysema

It is only recently that the two disorders, emphysema and fibrosis, have been recognized to exist in the same lung. This syndrome is called Combined Pulmonary Fibrosis and Emphysema (CPFE). There is still controversy about whether CPFE is a unique, distinct disease, or simply the coincidental coexistence of the two pathological alterations (Jankowich & Rounds 2012; Oliva *et al.* 2011). In either case, it typically consists of emphysema of the upper lung zones and fibrosis of the lower lung zones (Oliva *et al.* 2011).

CPFE patients usually have a relatively normal lung volume which is suggested to be based on the counterbalancing physiologic forces of hyperinflation in emphysema and restriction in fibrosis (Jankowich & Rounds 2012; Oliva *et al.* 2011). However, patients with CPFE demonstrate severe hypoxemia and severely reduced diffusing lung capacity for carbon monoxide (Oliva *et al.* 2011). There is a male predominance in CPFE patients, and a strong association with cigarette smoking (Cottin *et al.* 2005; Jankowich & Rounds 2012). Reported median survival has a range of 2-8 years (Jankowich & Rounds 2012).

PRESENT STUDY

4 AIMS OF THE STUDY

Fibrotic and emphysematous lung tissues are structurally very different: in fibrosis an excess of parenchymal cells and ECM is seen, and in emphysematous lung in COPD a marked tissue loss is observed. We hypothesised that these diseases represent opposing processes in the lung parenchyma, and that alterations in TGF-β signalling in opposite directions (upregulation in fibrosis, downregulation in emphysema) is a major contributing factor to either tissue loss or accumulation. In addition, we hypothesized that the restoration of TGF-β/BMP signalling imbalance could prevent the progression of fibrotic changes in IPF patients' lungs.

The aims of the study were to evaluate the mechanisms of the TGF-β signalling system in the human fibrotic lung and, in relation to TGF-β family signalling, to identify potential drug candidates for IPF.

In detail the aims were

- To study TGF-β storage, activation, and signalling properties in IPF.
- To assess the role of transcription factor GATA-6 in the pathogenesis of IPF, specifically as a mediator of TGF-β's profibrotic signals.
- To characterize TGF-β signalling in pathologically distinct areas of the human lung (production of excessive tissue in fibrosis *versus* tissue loss in emphysema).
- To search for novel treatment options for IPF by screening chemical compound libraries for molecules which could restore TGF-β and BMP signalling imbalance.

5 MATERIALS & METHODS

5.1 Patient material

Lung tissue samples were acquired from patients from the Helsinki University Central Hospital. UIP lung biopsies were either diagnostic biopsies or obtained from lung explants taken during lung transplantation. COPD was defined according to the GOLD criteria (FEV_1< 80% of reference, FEV_1/FVC < 70% and bronchodilatation effect < 12%). Control biopsies were obtained from healthy lung derived from operations in which benign tumours were removed.

All patients received written information and gave their written consent to use the samples. The Ethics Committee of the Helsinki University Central Hospital, Department of Internal Medicine, approved the study.

In total, the samples of 32 IPF/UIP patients and 16 COPD patients were used in this study. The details of patient age and gender are presented in Table 3.

Table 3. Age and gender of the patients studied.

	IPF/UIP	**COPD**
Age in years: range (mean ± SD)	34-79 (59 ± 11)	45-75 (58 ± 9)
Gender: M / F	22 / 10	6 / 10

5.2 Cell lines

The studies were conducted using commercial cell lines obtained from American Type Culture Collection (ATCC, Manassas, VA) or Lonza (Basel, Switzerland), primary cell lines isolated from patient lung tissue samples, and reporter cell lines (Table 4).

Table 4. Cell lines used in the studies.

Cell line	Characteristics
CCL-190, CCL-151	normal human lung fibroblasts (ATCC)
CCL-134	fibroblasts from a patient with IPF/UIP (ATCC)
CCL-185	A549 human lung carcinoma; epithelial cells (ATCC)
SAEC	human small airway epithelial cells (Lonza)
UIP-2, -3, -4, -8	primary lung fibroblast cell lines from patients with IPF/UIP
COPD	primary lung fibroblast cell line from a patient with COPD
BMP reporter cells	$(Bre)_2$-luc in pcDNA3 stably transfected to A549
TGF-β reporter cells	$(CAGA)_{12}$-luc in pcDNA3 stably transfected to A549

5.2.1 Isolation of primary cell lines

Lung tissue was washed with PBS and scraped with a scalpel into small pieces. They were placed on a cell culture dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine, and antibiotics. Upon follow-up, fibroblastic cells appeared growing either out the tissue pieces or as islets. Dead cells were removed and fresh medium added daily for 2-3 days and later twice a week. The average time to first passage was 3-4 weeks. For experiments, the cell lines were used between passages 4-10.

5.2.2 BMP and TGF-β reporter cells

$(Bre)_2$-luc and $(CAGA)_{12}$-luc in pGL3-Basic vector were provided by Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands). These are BMP- and TGF-β-responsive elements, respectively, linked to luciferase gene (Dennler *et al.* 1998; Korchynskyi & ten Dijke 2002). *Kpn*I-*Xba*I fragments from these constructs were cloned into pcDNA3 cloning vector's *Nru*I-*Xba*I sites.

For stable transfection, plated A549 cells were transfected o/n with plasmid DNA using FuGENE HD (Roche). Selection was started with 1.5 mg/ml of antibiotic G418 sulfate (Calbiochem, China) and fresh selection medium changed every 3-4 days. Cells were cultivated for 2-3 weeks before isolated clones were picked. For the maintenance of stably transfected reporter cells, 0.4 mg/ml G418 was used.

5.3 Immunological methods

5.3.1 Antibodies

Primary antibodies used in the studies are presented in Table 5.

Table 5. Antibodies used in the studies.

Antibody	Manufacturer	Studies
α-SMA	NeoMarkers Inc., Fremont, CA	ICC, IF, WB
Caspase-3	Cell Signalling Technology, Danvers, MA	IHC
Desmin	NeoMarkers Inc.	ICC
E-cadherin	Invitrogen, Carlsbad, CA	IF
Fibrillin-1	Prof. L. Sakai, Oregon Health and Science University, Portland, OR	IHC, PLA
Fibrillin-2	Millipore, Temecula, CA	IHC, PLA
Fibronectin	Sigma, St. Louis, MO	PLA
FOG-2	Santa Cruz Biotechnology, Santa Cruz, CA	IHC
GAPDH	Santa Cruz Biotechnology	WB
GATA-4	Santa Cruz Biotechnology	IHC
GATA-6	Santa Cruz Biotechnology	ICC, IHC, IF
Integrin αvβ6	Stromedix Inc., Cambrigde, MA	IHC
Integrin β8	Santa Cruz Biotechnology	IHC
Ki67	Thermo Fisher Scientific, Fremont, CA	IHC
LTBP-1	R&D Systems, Minneapolis, MN	IHC, IF, WB, PLA
LTBP-1	Santa Cruz Biotechnology	PLA
Phospho-Smad2 (Ser465/467)	Millipore	IHC, IF
P-Smad1/5/8	Millipore	IHC
P-Smad1/5/8	Cell Signalling Technology	WB

ICC, immunocytochemistry; IHC, Immunohistochemistry; IF, immunofluorescence; WB, western blotting; PLA, proximity ligation assay

5.3.2 Immunohistochemistry and immunofluorescence

Paraffin-embedded tissue sections from lung biopsies were deparaffinized in xylene and rehydrated in graded alcohol. Antigens were retrieved by heating the sections in 10 mM citrate buffer (pH 6.0) and endogenous peroxidase activity was neutralized with 0.3% hydrogen peroxide. Staining for bright field microscopy was performed with Histostain Plus Broad Spectrum Kit (Zymed, San Francisco, CA), Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA), Novolink Polymer Detection System (Novocastra, Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, United Kingdom), or ImmPRESS reagent kit (Vector Laboratories, Burlingame, CA) according to the manufacturers´ instructions. The sections were exposed to primary antibodies o/n at +4°C or 1 h, RT. Bound antibodies were visualized using peroxidase substrates 3-amino-9-ethylcarbazole (Zymed) or diaminobenzidine (Sigma or Leica). When needed, the cells and tissue sections were counterstained with Mayer's haematoxylin.

In immunocytochemical staining, cells were grown on chamber slides (NUNC Lab-Tek, Rochester, NY) and fixed with 4% paraformaldehyde (RT) or methanol (-20°C). The staining followed the same protocol, except antigen retrieval in RT by 0.1% Tween-20 for 10 min. Double staining was carried out using the Histostain Plus Broad Spectrum Kit (Zymed).

For fluorescence, bound antibodies were visualized using secondary antibodies with either green or red fluorescent labels. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

In all staining experiments, control samples were treated with goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), mouse isotype control (Zymed) or rabbit isotype control (Zymed). Also a negative control with primary antibody replaced by PBS was included in all series of stainings.

The samples were examined with an Olympus BX51 microscope (Olympus, Tokyo, Japan) connected to an Olympus U-RFL-T mercury lamp or Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

5.3.3 Digital image analysis and quantification of antigen-positive cells

Immunoreactivity was analyzed using digital image analyses. From each stained tissue section, representative images were taken with Olympus U-CMAD3 camera (Olympus, Japan) and QuickPHOTO CAMERA 2.1 software (Promicra, Czech Republic). If the area in the microscope field did not contain >50% tissue, the slide was moved vertically until a representative area was seen. The digital images were dissected to epithelial, parenchymal and fibroblastic foci pictures using a cutting knife tool from a digital imaging program (Photoshop). The areas of positive and negative staining were calculated using Image-Pro Plus 6.1 software (Media Cybernetics, Inc., Silver Spring, MD).

Alternatively, positive (stained reddish-brown) and negative (stained blue) nuclei were counted under the microscope. A hundred nuclei were counted from at least two different parts of each tissue specimen, and the average percentage of positive nuclei in each tissue sample was used for statistics.

5.3.4 Proximity Ligation Assay

Proximity ligation assay (Olink Bioscience, Uppsala, Sweden) provides a means for protein interaction studies *in vivo*. First, acetone-fixed fresh-frozen sections of human lung were incubated o/n at +4°C with two primary antibodies. Secondary antibodies are conjugated with DNA probes which, in close proximity, form a closed circle with the added oligonucleotides and ligase enzyme. During the rolling-circle amplification reaction, fluorescently labelled oligonucleotides are hybridized, resulting in spot-like positive signals indicating that the two primary antibodies are bound in close proximity.

5.3.5 Immunoblotting

Cells were lysed by either sonicating a PBS suspension or using ice-cold lysis buffer (120 mM NaCl, 0.5% NP-40, 100 mM NaF, 1 mM Na_3VO_4 in 20 mM Tris-HCl, pH 8.0; protease inhibitors added as Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany)). Whole-cell lysates were cleared by centrifugation and assayed for protein content using RC DC protein assay (Bio-Rad Laboratories, Hercules, CA). An equal amount of protein per lane was used in standard reducing SDS-PAGE (7% or 12%) and transferred to nitrocellulose membranes. The membranes were blocked with 10% milk in Tris-buffered saline containing 0.1% Tween-20, probed with antibody for 1 h, followed by horseradish peroxidase-conjugated secondary antibody treatment. The enhanced chemiluminescence system (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) was used for detection. Equal protein loading was ensured by reprobing the membrane for GAPDH.

5.4 Quantification of peribronchiolar fibrosis

Masson's trichrome stained paraffin sections were used for the quantification of peribronchiolar thickening in lung biopsies. Masson's trichrome is a standard histological staining protocol which stains collagen blue and cellular components red. Photomicrographs of bronchioles were taken with Olympus BX51 microscope and analyzed using Image-Pro digital image processing software (Media Cybernetics, USA). The proportion of blue staining from the total area of bronchiolar tissue was measured.

5.5 TGF-β activity assays

TGF-β activity was studied with two methods. In study (II), cells were transiently co-transfected with the $(CAGA)_{12}$-luciferase promoter construct together with pRL-TK (Renilla luciferase control, Promega, Madison, WI) plasmid using the FuGENE HD transfection reagent (Roche). After 48h incubation, the cells were lysed and subjected to luciferase activity measurements by a Dual Luciferase Kit (Promega) and Digene DCR-1 luminometer (MGM Instruments, Hamden, CT).

In study (III), the relative TGF-β activity of LTBP-1 and control siRNA-transfected cells was assayed by coculture with mink lung epithelial cells stably transfected with PAI-1 promoter fragment fused to luciferase reporter gene (Abe *et al.* 1994).

5.6 siRNA assays

The cells were transfected with siRNA for GATA-6 or LTBP-1 (Qiagen sciences, Maryland, USA) using Lipofectamine 2000 reagent (Invitrogen). AllStars siRNA (Qiagen) was used as a control. The transfection efficiency was analyzed in mRNA and protein expression levels.

5.7 Real-time reverse transcriptase PCR

Prior to RNA extraction, snap-frozen human lung biopsy samples were homogenized using Lysing Matrix D (MPBiomedicals, Solon, OH) and mouse lung tissue samples were crushed with a pestle. Total RNA was extracted from cultured cell lines and tissue samples using NucleoSpin RNA II kit (Macherey-Nagel GmbH, Düren, Germany) or RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA concentration as well as purity was determined using NanoDrop (Thermo Scientific, Wilmington, DE) or Agilent 2100 Bioanalyzer at the Biomedicum Biochip Center (Helsinki, Finland). RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) or using random hexamer primers (Roche Molecular Systems, Inc., or Invitrogen) and MuLV Reverse Transcriptase (Roche) or Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA). cDNA was used as a template for PCR reactions.

In real-time RT-PCR, a 64 bp fragment of human GATA-6 gene was amplified with primers 5′-AGAAACGCCGAGGGTGAAC-3′ (forward) and 5′-GCACCCCATGGAGTTTCATG-3′ (reverse) (Oligomer, Helsinki, Finland) using Power SYBRGreen PCR Master Mix (Applied Biosystems, Warrington, UK). α-SMA, Col1A1, Col3A1, CTGF, fibrillin-1, fibrillin-2, fibronectin, gremlin-1, Id1, Id3, LTBP-1, LTBP-3, LTBP-4, TGF-β1-3, and PAI-1 analyses were carried out using TaqMan Assays-On-Demand gene expression products (Applied Biosystems) and the ABI 7500 FAST System (Applied Biosystems) or Bio-Rad CFX96 system (Bio-Rad). Human 18S rRNA and human or mouse tata-binding protein (TBP) with TaqMan Universal PCR Master Mix (Applied Biosystems) were used as reference genes. The relative gene expression differences were calculated with the comparative ΔΔCT method.

5.8 High-throughput screening

BMP and TGF-β reporter cells were suspended in phenol red-free DMEM (Gibco, Grand Island, NY) with supplements, seeded on white 384-well plates and allowed to attach. The screening of chemical compound libraries (MicroSource Spectrum Collection (Microsource Discovery Systems, Inc., Gaylordsville, CT) and FDA approved drug library (Enzo Life Science, Farmingdale, NY)) was performed in high throughput center with the Institute for Molecular Medicine Finland (FIMM). First, reporter cells were treated with these compounds for 18 h at 37°C: for MicroSource library, 20 μM concentration was used; for the Enzo library, the compounds were tested over 10 nM-10 μM concentration range. Following the incubation, the cells were lysed and luciferase activity measured using SteadyLite Plus reagent (PerkinElmer, Waltham, MA). Positive controls (BMP-4 and TGF-β1) and a negative control (DMSO) were included in the experiments.

5.9 Mouse studies

Mouse studies were approved by the regional state administrative agency of Southern Finland (permission # ESAVI/871/04.10.03/2012). The animals were kept in animal facility with modern equipment, and received food, fresh water and humane care.

6-week-old male C57b6J mice were divided into three groups (8-12 mice per group). One experimental group of unexposed animals represented non-treated controls. For the other two experimental groups, silicon dioxide (SiO_2; 50 mg/ml in sterile PBS) was administered according to the protocol described previously (Lakatos *et al.* 2006). Two subsequent oropharyngeal aspiration doses were given to the animals on day 1 and day 8 of the experiment to ensure even exposure of all animals. The animals were sedated with isoflurane for the aspiration of silica suspension and euthanised with carbon dioxide at the time of sacrifice.

The effect of tilorone dihydrochloride (Sigma) was studied in one experimental group of mice exposed to SiO_2. The drug was administered intraperitoneally at a dose of 50 mg/kg (Golovenko & Borisyuk 2005; Mayer-Sonnenfeld *et al.* 2008; Zinkovsky *et al.* 2007) every three days for 30 days, starting from day 1 immediately after the first SiO_2-inhalation was received. The frequency of drug administration was chosen based on half-time of tilorone clearance [$t_{½}$ (lungs) = 65 h] (Golovenko & Borisyuk 2005).

Bronchoalveolar lavage fluid was obtained from the euthanised mice by cannulating the trachea and lavaging with 2x300 μl of PBS. The total cell number of bronchoalveolar lavage fluid was calculated using BIO-RAD TC10 automated cell counter (Singapore). Differential cell counts of macrophages, neutrophils, and lymphocytes were obtained by microscopy of May-Grünwald-Giemsa-stained cytocentrifuge preparates.

Lung tissue from the left-side lungs was fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. Histological evaluation fibrosis and inflammation was performed from haematoxylin and eosin stained sections. The frozen right lung was placed in liquid

nitrogen and crushed with a pestle until completely pulverized. The homogenized tissue powder was used for mRNA analyses and the hydroxyproline assay.

The hydroxyproline assay was performed in order to assess the collagen content of the lung tissue. Proteins were precipitated by incubating samples with trichloroacetic acid on ice. Hydrolysis of the precipitate was completed by addition of 37% HCl and heating at 110°C until the samples were charred and dry. The dried pellet was reconstituted with water and assayed for hydroxyproline content using chloramine-T as previously described (Reddy & Enwemeka 1996).

5.10 Statistical analyses

Data were analyzed with SPSS 15.0, PASW 18.0 (SPSS, Chicago, IL), and IBM SPSS statistics versions 19.0 and 20.0 (IBM, Armonk, NY) using the non-parametric Kruskal-Wallis and Mann-Whitney tests. A P value of <0.05 was considered to be statistically significant.

6 RESULTS

6.1 Protein localization in IPF/UIP tissue

Immunohistochemistry was performed to study the localization of proteins linked to cell differentiation (transcription factors GATA-4 and GATA-6, and their cofactor Friend Of GATAs (FOG)-2), cell cycle (proliferation marker Ki67, apoptosis marker Caspase-3), TGF-β storage (LTBP-1), TGF-β activation (integrins αvβ6 and β8), TGF-β signalling activity (signal transducer P-Smad2), and ECM components (fibrillin-1 and fibrillin-2) in control samples and different compartments (epithelium, parenchyma, fibroblastic foci) of the IPF/UIP samples (studies I, III). A summary of the results is presented in Table 6.

Table 6. Protein localization in IPF/UIP and control tissue samples.

		epithelium	**parenchyma**	**fibroblastic foci**	**controls**
transcription factors	**GATA-4**	+	+	-	N/A
	GATA-6	+	+	+	N/A
	FOG-2	+	+	-	N/A
proliferation & apoptosis	**Ki67**	+	+	-	+
	Caspase-3	+	+	-	+
TGF-β storage, activation, and signalling activity	**LTBP-1**	+	+	+	+
	integrin αvβ6	+	-	-	(+)
	integrin β8	(+)	(+)	(+)	(+)
	P-Smad2	+	+	+	+
ECM proteins	**fibrillin-1**	-	(+)	+	+
	fibrillin-2	-	(+)	+	+

+, protein expression; (+), very low level of protein expression; -, no protein expression; N/A, not analyzed

6.2 TGF-β signalling

6.2.1 The expression of TGF-β isoforms in IPF/UIP and COPD tissue

The mRNA expression levels of all TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) were analyzed from control, IPF/UIP, and COPD tissue samples (studies II, III). It was found that all TGF-β1-3 isoforms were expressed in lung tissue but in all study groups, TGF-β1 was clearly the most abundantly expressed isoform. There were no significant differences in expression levels when IPF/UIP and COPD samples were compared to normal lung samples.

6.2.2 LTBP-1 upregulation, interactions with ECM proteins, and functional role in IPF/UIP

Latent TGF-β binding proteins (LTBPs) play a critical role in the assembly, secretion, storage, and activation of TGF-β. When the mRNA expression levels of latent TGF-β-interacting LTBP isoforms (LTBP-1, -3, and -4) were analysed in IPF/UIP and control tissue samples (study III), it was discovered that all of these isoforms were expressed in lung tissue. However, LTBP-1 mRNA levels were significantly upregulated in IPF/UIP tissue compared to control samples. There was also a strong upregulation and accumulation of LTBP-1 protein in IPF/UIP tissue compartments.

The accumulation of extracellular matrix (ECM) is a characteristic feature of IPF. LTBP-1 binds to fibronectin and fibrillins, and these LTBP/ECM interactions play a role in TGF-β activation processes. Thus, the expression levels of fibronectin, fibrillin-1, and fibrillin-2 were studied in control and IPF/UIP tissue samples (study III). Compared to controls, the mRNA expression levels of all of these ECM-related genes were found to be significantly upregulated in IPF/UIP tissue samples (P(fibronectin)=0.009; P(fibrillin-1)=0.016; P(fibrillin-2)=0.014). These genes were expressed in the following order of abundance: fibronectin > fibrillin-1 > fibrillin-2.

Fibrillin-1 and fibrillin-2 proteins were found mainly in the areas on fibroblastic foci of IPF/UIP tissue samples (Table 6). LTBP-1 was shown to be in close proximity, i.e. associate, with fibronectin, fibrillin-1, and fibrillin-2 in IPF/UIP tissue samples.

The functional role of LTBP-1 was studied by siRNA-mediated silencing. The results of normal lung fibroblasts (CCL-190) and epithelial cells (SAEC) were, however, the opposite. In fibroblasts, reduced LTBP-1 expression led to increased TGF-β activity and also increased mRNA expression levels of TGF-β target genes connective tissue growth factor (*CTGF*), plasminogen activator inhibitor-1 (*PAI-1*), and collagen 1A1 (*Col1A1*). In epithelial cells, there was a decrease in TGF-β activity as well as mRNA expression levels of the above-mentioned TGF-β target genes.

6.2.3 Active TGF-β signalling in fibrotic and emphysematous lung tissue

As an indicator of active TGF-β signalling, the expression of the phosphorylated, i.e. activated, form of TGF-β signal transducer Smad2 (P-Smad2) was analyzed in both COPD and IPF/UIP tissue samples (studies II, III).

Compared to control samples, the emphysematous areas of the COPD samples showed a significant decrease in the number of P-Smad2 positive nuclei in alveolar epithelium (P = 0.006); there was also a small decrease in the bronchiolar epithelium. Furthermore, a weakened staining intensity was observed in the more severe stages of COPD. In contrast, the increased fibrotic tissue found outside the bronchioles in COPD samples showed significantly higher proportions of P-Smad2 positive nuclei.

In the fibrotic samples of IPF patients, P-Smad2 was expressed in the fibroblastic foci, in the thickened epithelium overlying the fibroblastic foci, and in the parenchyma (Table 6). There was more P-Smad2 positivity in IPF/UIP samples when compared to controls ($P = 0.008$).

In summary, emphysematous tissue showed decreased and fibrotic tissue increased TGF-β signalling activity.

6.2.4 GATA-6 mediates cell differentiation towards a myofibroblastic phenotype

Fibroblastic cell lines isolated from IPF/UIP patients' tissue samples were found to express mRNA for transcription factor GATA-6 and myofibroblast marker α-SMA, but there was no pattern in expression levels. On protein level, GATA-6 was shown to colocalize with α-SMA in the same cells in fibroblastic foci of IPF/UIP tissue samples.

A primary fibroblastic cell line with an abundant GATA-6 expression was selected for siRNA studies (study I). In non-treated fibroblasts, TGF-β caused a notable increase in the mRNA expression levels of GATA-6 and α-SMA, and also a minor increase in ECM components collagen and fibronectin expression. In contrast, in cells with a reduced GATA-6 expression due to siRNA treatment, TGF-β had virtually no effect on the expression of the genes mentioned above.

In IPF/UIP tissue samples, GATA-6 was expressed also in the epithelial cells (Table 6). The differentiation of epithelial cells towards a mesenchymal phenotype, i.e. epithelial-mesenchymal transition (EMT), is suggested to be a central mechanism in the pathogenesis of IPF/UIP (Willis & Borok 2007). Thus, the possible relationship of GATA-6 expression and EMT was examined *in vitro* in human A549 pulmonary epithelial cells, which do not spontaneously express GATA-6 (Vähätalo *et al.* 2011) but are known to undergo EMT when induced with TGF-β1 (Kasai *et al.* 2005).

A549 cells were transiently transfected with an expression plasmid containing human GATA-6 cDNA. These cells started to express GATA-6 and myofibroblast marker α-SMA. Control cells transfected with a corresponding empty plasmid, i.e. without GATA-6 gene, were negative for both GATA-6 and α-SMA. In comparison, A549 epithelial cells were stimulated with TGF-β1 for the induction of EMT, which was characterized by loss of expression of epithelial marker E-cadherin and simultaneous increase in the expression of α-SMA. It was also noted that during this process, GATA-6 was expressed.

6.3 High-throughput screening

6.3.1 In vitro

Since there is a known imbalance of BMP and TGF-β signalling in IPF (BMP decreased, TGF-β increased), we hypothesized that restoration of BMP signalling could be one strategy for the prevention of fibrosis progression or even treatment of fibrosis.

In study (IV), BMP- and TGF-β-responsive reporter cell lines were used in high-throughput screening of two commercial, partially overlapping, chemical compound libraries (MicroSource Spectrum Collection (ca. 2000 chemical compounds) and Enzo Life Science's FDA approved drug library (640 chemical compounds)).

Primary hits were selected based on pre-defined hit criteria: compounds inducing a minimum of 1.5-fold increase of BMP reporter cells; compounds causing a decrease or no effect on TGF-β reporter cells; compounds that are non-toxic; compounds that can be administered to experimental animals and humans. Based on these criteria, there were seven chemical compounds regarded as primary hits in MicroSource collection; screening of the smaller Enzo library revealed only one primary hit which had already been detected in the MicroSource collection.

Validation of the primary hit data revealed that only two of these compounds, tilorone (TIL) and 2',3-dihydroxy-4,4',6'-trimethoxychalcone (CHAL), showed consistent BMP reporter activation (Figure 6). In A549 pulmonary epithelial cells, tilorone was shown to cause a sustained induction in the expression of BMP target gene inhibitor of DNA binding/differentiation (*Id*)-3. It also induced the expression of BMP-7.

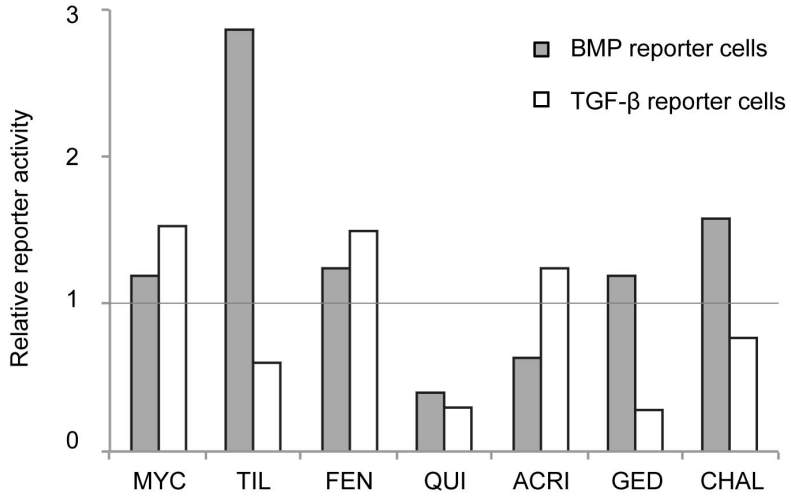

Figure 6. A representative figure of primary hit validation of chemical compound screening. The figure shows the relative activity of both reporter cell lines when exposed to chemicals at 10 μM concentration. Control is set to 1 (marked with a horizontal line). Tilorone (TIL) and 2',3-dihydroxy-4,4',6'-trimethoxychalcone (CHAL) show consistent activation of BMP reporter cell line and a decrease in TGF-β reporter cell activity.

6.3.2 Mouse studies

Because tilorone showed a favourable and sustained effect on BMP signalling *in vitro*, its biological effects were studied in a mouse model of SiO_2 (silica)-induced fibrosis.

On tissue level, the silica-induced fibrotic response was seen as an increase in the amount of lung hydroxyproline and mRNA expression levels of collagen and fibronectin. Compared to non-treated controls, silica-exposed mice also showed decreased P-Smad1 and increased P-Smad2 immunoreactivity, reflecting active BMP and TGF-β signalling, respectively. In tilorone-treated mice, all of these effects were partially reversed.

Histological evaluation of silica-exposed mice showed that there were less fibrotic changes and lung tissue infiltrating inflammatory cells in tilorone-treated mice. However, tilorone was not found to cause any statistically significant changes in total or differential cell counts of bronchoalveolar lavage fluid samples.

In summary, tilorone showed biologically significant antifibrotic properties in a mouse model of pulmonary fibrosis.

7 DISCUSSION

The aims of this thesis were to characterize TGF-β signalling activity in pathologically distinct areas of the human lung; to study TGF-β storage, activation, and signalling properties in IPF; and also to search for novel treatment options for IPF.

The results revealed several novel findings. Although numerous studies have inferred that emphysematous tissue may have inadequate TGF-β signalling, it was shown here that Smad-2 phosphorylation, an indication of active TGF-β signalling, is greatly decreased in emphysematous lung tissue. In fibrosis, we challenged the common belief that fibroblastic foci consist of actively proliferating cells, showed that TGF-β profibrotic signalling, at least in terms of cells' mesenchymal differentiation, is dependent on GATA-6 transcription factor and, for the first time, high-throughput screening was used in IPF research. The results serve as a platform for further studies on human fibrosis treatment options.

7.1 The cells in fibroblastic foci are in a quiescent state

It is a common belief that the fibroblastic foci in IPF consist of actively proliferating cells. Cool *et al*. (2006) have shown that these foci are not the result of malignant proliferation of a monoclonal population of fibroblasts, but rather suggested to be formed by heterogeneous group of fibroblasts. Here, we showed that even though the proliferation marker Ki67 and apoptosis marker Caspase-3 were abundantly expressed in lung parenchyma and epithelium, they were absent in fibroblastic foci (Table 6), suggesting that the cells in fibroblastic foci are actually in a quiescent state.

7.2 TGF-β storage in EMC structures and integrin-mediated activation

The deposition of latent TGF-β and BMP in the ECM regulates the local signals of these cytokines (Olivieri *et al.* 2010; Sengle *et al.* 2008). Numerous studies have reported that LTBP-1 binds to fibronectin and fibrillins *in vitro* and in some tissues also *in vivo* (Dallas *et al.* 2000; Dallas *et al.* 2005; Isogai *et al.* 2003; Ono *et al.* 2009; Raghunath *et al.* 1998; Taipale *et al.* 1996). Fetal lung tissue has shown co-distribution of LTBP-1 and fibrillin (Isogai *et al.* 2003) but the novel *in vivo* findings of this study showed that also in human fibrotic lung tissue, LTBP-1 is in close proximity, i.e. associated, with fibronectin, fibrillin-1, and fibrillin-2. In addition, we showed that LTBP-1 is targeted to different ECM structures in specific areas of the IPF/UIP lung: colocalization of LTBP-1 and fibrillin-1 was found throughout the tissue, including epithelial cells, whereas interactions of LTBP-1 and fibronectin were abundant only in the parenchymal tissue immediately below the epithelial cells. Similar results of LTBP-1 interactions with fibronectin and fibrillin-1 in mouse lung tissue have recently been published (Zilberberg *et al.* 2012).

Integrin-mediated TGF-β activation is suggested to be important in the pathogenesis of IPF (Goodwin & Jenkins 2009). In line with the results of Horan *et al*. (2008), we found intense

immunoreactivity of integrin αvβ6 in epithelial cells in IPF/UIP tissue, likely reflecting epithelial damage (Breuss *et al.* 1995) and contributing to TGF-β activation in the epithelial cells. The expression of integrin β8 was scarce throughout the tissue. Since the parenchymal areas were found to be rich with latent TGF-β, it is plausible that there are also local mechanisms for latent TGF-β activation. These mechanisms, however, are still to be discovered in future research.

7.3 TGF-β signalling in pathologically distinct areas of the human lung

P-Smad2 was studied as an indicator of active TGF-β signalling. In emphysema, alveolar structures are destroyed (lack of ECM and cells); in contrast, fibrotic areas represent thickening of parenchyma (an excess of ECM and cells). The present studies revealed decreased and increased P-Smad2 expression, respectively.

In a Smad-3 knockout mouse model, impaired TGF-β signalling leads spontaneously to increased airspace enlargement and development of emphysema (Bonniaud *et al.* 2004). In human COPD samples, single-nucleotide polymorphism in the TGF-β1 gene is associated with COPD (Celedon *et al.* 2004) and it has also been discovered that several factors related to TGF-β signalling (e.g. GDF-10, LTBP-1, inhibitory Smad proteins, and TGF-β receptors 1 and 2) are downregulated (Ezzie *et al.* 2012; Pons *et al.* 2005; Springer *et al.* 2004; Zandvoort *et al.* 2006). This results in the decreased production of ECM components and ineffective repair response to damage of the lung, ultimately resulting in an active emphysema process (Gauldie *et al.* 2006; Zandvoort *et al.* 2006). Our result of a decreased TGF-β signalling activity in human emphysematous lung tissue is in line with these studies. Opposite results have also been reported: Koenders *et al*. (2009) have found increased TGF-β staining in human emphysematous lung tissue. However, they used an antibody that recognizes both latent and active TGF-β, i.e. the ratio of these two forms remained unknown. The activity of TGF-β signalling is mainly regulated on the level of latent TGF-β activation, not mRNA/protein expression. The *in vivo* finding that fibrotic tissue areas showed accumulation of LTBP-1 and increased TGF-β signalling activity is in line with previous reports of excessive TGF-β expression in IPF and COPD fibroblasts (Koli *et al.* 2006; Togo *et al.* 2008).

7.4 TGF-β downstream signalling

In normal human lung tissue, TGF-β1 is expressed in a variety of cells, most profoundly in bronchiolar epithelium and alveolar macrophages, but also in mesenchymal and endothelial cells (Coker *et al.* 1996). In the fibrotic lung, TGF-β responsive cells were found throughout the tissue: in epithelium, parenchyma, and also in the highly differentiated myofibroblasts in fibroblastic foci. Myofibroblast are the primary cell type responsible for the synthesis and deposition of EMC and the resultant structural modelling that leads to the loss of alveolar function (Pardo & Selman 2002; Scotton & Chambers 2007). Even though the myofibroblasts in fibroblastic foci seem to be in a quiescent state, i.e. non-proliferating and non-apoptotic,

they still retain responsiveness to TGF-β signals, hence contributing to the ECM accumulation.

Zhang *et al*. (2008) have shown that the role of transcription factor GATA-6 in lung epithelium is to participate in the regeneration process through regulation of bronchioalveolar stem cell expansion and differentiation. In agreement with this, GATA-6 expression was observed in epithelial cells of IPF lung tissue, and it was expressed during EMT-related changes. EMT, in general, is dependent on TGF-β activation (Chapman 2011). However, there are numerous factors influencing this process, both on the level of Smad signalling as well as additional transcriptional regulators. Here, we added one more factor to this list: transcription factor GATA-6 mediates TGF-β signals in EMT.

It is virtually impossible to distinguish EMT-derived fibroblasts from other fibroblasts in humans *in vivo*. It has been documented that IPF/UIP lung biopsies contain cells which have both epithelial and mesenchymal features, suggesting that some degree of EMT occurs *in vivo* during fibrogenesis (Chapman 2011; Kim *et al.* 2006). This is supported by evidence of *in vivo* EMT in experimental animal models of pulmonary fibrosis (Kim *et al.* 2006; Tanjore *et al.* 2009).

7.5 High-throughput screening

High-throughput screening is a widely used tool in modern drug discovery. It refers to the possibility of conducting a large number of e.g. chemical or pharmacological tests in a very short period of time. This is done by the use of robotics, miniaturized assays, automated liquid handling, and sensitive detectors. Cell-based assays have the advantages of predictability and possibility of automation and the sensitivity of reporter gene assays also makes them ideal for miniaturization; the disadvantages of this set-up are long response time of the reporter system and the possible interference from other cellular pathways (Michelini *et al.* 2010). Thus, it is important to verify the effects of primary hit chemicals by other means, both *in vitro* and *in vivo*.

In this study, the chemical compound libraries used for high-throughput screening contained mostly Food and Drug Administration (FDA)-approved drugs. These are compounds whose safety has already been studied in humans. Hence, a potential alternative use of an existing and authority-approved drug molecule represents a fast track in the search of novel treatment options.

There are many different animal models of IPF in research use today: fibrosis can be experimentally induced by e.g. bleomycin, silica, asbestos, radiation, viral vector delivery of transgenes, or transgenic models can be used (Degryse & Lawson 2011). All of these models have slightly different features with strengths and weaknesses regarding the fibrotic response, which affects the choice of the model used. In this study, we used a mouse model of silica-induced fibrosis. It was chosen because it resembles human IPF in terms of gremlin upregulation which leads to perturbed BMP/TGF-β signalling balance. We did not choose the more commonly used bleomycin model due to the fact that this model represents a temporary

inflammatory response with a partially reversible fibrosis (Moeller *et al.* 2008) and thus the results may not be applicable to human disease. In fact, according to a review article published in 2008 (Moeller *et al.*), there are over 200 compounds which have shown beneficial antifibrotic effects in the bleomycin model of pulmonary fibrosis. To date, only one of them, pirfenidone, has been accepted to be used as a therapeutic agent in human IPF.

TGF-β's central role in fibrogenesis makes it an attractive target for drug therapy, and e.g. anti-TGF-β antibodies and TGF-β inhibitor peptides have been studied (Arribillaga *et al.* 2011; Datta *et al.* 2011). However, as TGF-β also regulates key homeostatic functions, interfering with TGF-β signalling may result in highly undesirable side effects (Datta *et al.* 2011). The other side of the coin is restoration of BMP signalling, which has shown to decrease experimentally-induced fibrosis (Myllärniemi *et al.* 2008a; Sugimoto *et al.* 2012). In this study, tilorone was shown to enhance BMP signalling, but it is also a known interferon inducer (Mayer & Krueger 1970). IFN-γ is suggested to have antifibrotic effects but the results of clinical trials have been controversial (Datta *et al.* 2011).

The retrospective power analysis (one-sided test, 0.05 significance) gave the animal experiment with tilorone ~ 70% power. It is possible that bigger study groups might have given more reliable results but, on the other hand, the data obtained here offers a promising starting point for further studies with e.g. tilorone analogues.

7.6 Sample selection and methodological aspects

The tissue samples were either diagnostic biopsies or obtained from lung explants taken during lung transplantation. The latter samples represent a selected population: due to transplantation criteria, these patients are e.g. generally younger than average IPF patients and they are non-smokers.

In a tissue sample, there are always pathologically distinct areas included. Even though gene expression levels can be roughly analyzed *in vivo* using tissue samples, *in vitro* analyses from selected cell lines can give more reliable results. Total mRNA levels in a tissue do not reveal any information about the localization of the protein expression, which is why parallel immunostaining was also performed.

Traditionally, the use of fresh-frozen tissue sections has been the standard for immunohistochemical analyses. However, it has been shown that formalin-fixed, paraffin-embedded tissue sections with antigen retrieval can give just as good – or even better – results (Shi *et al.* 2008). In this study, paraffin-embedded tissue sections were used for standard immunohistochemistry and immunofluorescence. Fresh-frozen tissue samples were used for proximity ligation assays.

The primary fibroblast cell lines were isolated from patient tissue samples. Originally these fibroblasts represent a heterogeneous population of cells, including myofibroblasts, but also non-differentiated 'normal' fibroblasts. As shown in study (I), the cells in fibroblastic foci do not seem to proliferate, but rather be in a quiescent state, and thus they are probably not the dominating subpopulation in the primary fibroblast cell cultures.

A549 cells are widely used and profoundly characterized lung cells that originate from type II cells. The use of A549 as a model of lung alveolar epithelial cells (AEC) has been criticized, due to their malignant origin of lung carcinoma. Commercial cell lines with better AEC II characteristics are available but unfortunately they are not human cell lines. Thus, in these studies (I, IV), A549 cells were chosen based on their human origin.

8 CONCLUSIONS

This thesis provides an overview on TGF-β signalling in two human lung parenchymal diseases - idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. The aim of the study was to examine alterations in active TGF-β signalling, characteristic to pathologically distinct areas of damaged lung tissue. Furthermore, the aim was to study TGF-β storage and activation as well as the role of transcription factor GATA-6 in the pathogenesis of IPF. Also, new possibilities for the treatment of IPF were sought.

In conclusion, this study showed that TGF-β signalling activity varies within pathologically distinct areas of the lung, with emphysematous areas showing decreased and fibrotic areas showing increased signalling activity. In IPF, the increased TGF-β signalling activity is accompanied by massive upregulation in the expression of latent TGF-β binding protein (LTBP)-1, which was found to be associated with fibronectin and fibrillins in the extracellular matrix.

It is generally believed that fibroblastic foci in IPF consist of actively proliferating cells. Here, this view was challenged as the areas of fibroblastic foci were found to be completely negative for markers of proliferation and apoptosis. This indicates that the cells are in a quiescent state and that an antiproliferative approach may not be a reasonable therapeutic target. However, the highly differentiated myofibroblasts in fibroblastic foci still retained responsiveness to TGF-β signals, suggesting that they keep contributing to the ECM accumulation.

Transcription factor GATA-6 was expressed in fibroblastic foci and it seems to act as a crucial mediator in the TGF-β-induced process of cellular differentiation of fibroblasts and epithelial cells towards a myofibroblast-like phenotype. As TGF-β is considered to be a ubiquitously expressed cytokine responsible for various tissue homeostatic events, downstream factors, such as transcription factors responsible for cells' mesenchymal differentiation, might serve as potential targets for drug therapy.

For the first time in IPF research, high-throughput screening technique was utilized in the search for new therapeutic chemical compounds. The idea was to find a molecule which targets the aberrant TGF-β family signalling found in IPF. The most promising candidate drug identified, tilorone, showed biologically significant antifibrotic properties in a mouse model of pulmonary fibrosis. The data obtained here provides a starting point for further studies with e.g. tilorone analogues.

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