Role of Bcl-xL in HGF-elicited epithelial protection in idiopathic pulmonary fibrosis

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AD	Alzheimer's disease
AECII	Alveolar epithelial type II cell
Akt	Rac-alpha serine/threonine-protein kinase
Apaf-1	Apoptotic protease activating factor 1
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
BALF	Broncho-alveolar lavage fluid
Bax	Bcl-2-associated x protein
Bcl-2	B cell CLL/lymphoma-2
Bcl-xL	Bcl-2 related gene, long isoform
BH	Bcl-2 homology region
Bid	Bcl-2-interacting domain death agonist
Bim	Bcl-2 interacting mediator of cell death
COX-2	Cyclooxygenase 2
CRD	Chronic renal disease
Cyt c	Cytochrome c
DISC	Death-inducing signaling complex
DMN	Dimethyl nitrosamine
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix
ED-A	Alternatively spliced domain of fibronectin
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
FADD	Fas-associated protein with a death domain
FasL	Fas ligand
FasR	Fas receptor
FEV_1	Forced expiratory volume in one second
FPF	Familial form of idiopathic pulmonary fibrosis
FVC	Forced vital capacity
Gab1	Grb2-associated binder 1
Grb2	Growth factor receptor-bound protein 2

HGF	Hepatocyte growth factor
HGFA	Hepatocyte growth factor activator
HtrA2	High temperature requirement protein A2
HVJ-HGF	Hemagglutinating-virus-of-Japan liposome containing HGF cDNA
IAPs	Inhibitor of apoptosis proteins
IIP	Idiopathic interstitial pneumonia
IM	Intramuscular
IP	Intraperitoneal
IPF	Idiopathic pulmonary fibrosis
IPT	Immunoglobulin-plexin-transcritpion domain
IT	Intratracheal
IV	Intravenous
MAPK	Ras-mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia 1
MMP	Matrix metallopreinase
MOMP	Mitochondrial outer membrane permeabilization
NSCLC	Non-small cell lung cancer
OMM	Outer mitochondrial membrane
PAI-1	Plasminogen activator inhibitor 1
PCD	Programmed cell death
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E 2
PI3K	Phosphatidylinositol 3 kinase
PLCγ	Phospholipase C γ
PP2A	Protein phosphatase 2A
PSI	Plexin-semaphorin-integrin domain
Puma	p53 up-regulated modulator of apoptosis
rHGF	Recombinant HGF
ROS	Reactive oxygen species
SC	Subcutaneous
SCLC	Small cell lung cancer
Sema	Region of homology to semaphorins
Ser	Serine residue
SFTPA	Gene encoding surfactant protein A
SFTPC	Gene encoding surfactant protein C

Shp2	SH2-containing protein tyrosine phosphatase 2
Smac/DIABLO	IAP binding protein with low pI
SP-A	Surfactant protein A
SP-C	Surfactant protein C
SRC	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinases
TM	Transmembrane domain
TNFR1	Tumor necrosis receptor 1
TNF-α	Tumor necrosis factor α
UIP	Usual interstitial pneumonia
uPA	Urokinase-type plasminogen activator
UPR	Unfolded protein response
UUO	Unilateral ureteral obstruction
α-SMA	α smooth muscle actin

IV Summary

Idiopathic pulmonary fibrosis (IPF) is a chronic and progressive diffuse parenchymal lung disease of unknown etiology (Ley *et al*., 2011). Existing evidence strongly suggests that the alveolar epithelial cell (AEC) is the key player in the pathogenesis of IPF. It is believed that repetitive cycles of epithelial cell injury, followed by impaired wound healing, lead to an excessive apoptosis of AECs, accompanied by aberrant activation of fibroblasts/myofibroblasts, deregulated remodeling and, finally, irreversible restructuring of the lung parenchyma (Selman *et al*., 2002, Zoz *et al.*, 2001). Hepatocyte growth factor (HGF) is a pleiotropic cytokine playing a major role in cellular repair processes, ensuring restoration of epithelial homeostasis in the damaged organ. Exogenous administration of HGF has been reported beneficial in experimental models of various organ fibrosis including the lung. Bcl-xL is an anti-apoptotic member of Bcl-2 family which consists of highly conserved proteins involved in the mitochondrial control of apoptosis. Since HGF signaling *via* c-Met receptor has been proposed to regulate the expression of Bcl-2 family members, the present study was performed to evaluate the potential role of Bcl-xL in HGF-mediated epithelial protection in IPF. We therefore aimed to characterize Bcl-xL expression and its cellular localization in lung tissues of IPF patients in comparison to donor lung tissues, to investigate if HGF mediates pro-survival effects on alveolar epithelial cells regardless of the kind of pro—apoptotic stimulus and to assess the potential role of Bcl-xL in this context.

Employing tissues from human IPF and donor lung resections, we observed that Bcl-xL protein was highly expressed in hyperplastic AECII found in regions of dense fibrosis in IPF. Donor lung tissues revealed a much weaker signal for Bcl-xL in the alveolar epithelium. These findings were confirmed by Western blot analysis which revealed a significant increase in the total Bcl-xL amount in IPF lung *versus* donor lung homogenates. Furthermore, staining for Bcl-xL in AECII in still regular imposing areas was less prominent than in hyperplastic AECII present in fibroblastic regions.

In vitro studies were performed on mouse (MLE12, MLE15) and rat (RLE-6TN) alveolar epithelial cell lines. Since it has been reported that human IPF is characterized by permanent oxidative stress, enhanced activation of ER stress and up-regulation of Fas ligand (FasL), we chose hydrogen peroxide, thapsigargin and FasL as apoptosis-

inducing factors in this study. We observed that simultaneous treatment with HGF and hydrogen peroxide or thapsigargin resulted in an improved survival of alveolar epithelial cells. In both cases, the HGF-mediated anti-apoptotic activity was associated with increased Bcl-xL expression and the beneficial effect of HGF could be abolished by using a c-Met specific inhibitor prior to HGF incubation. The siRNA-mediated knock-down of Bcl-xL caused an increased susceptibility of the epithelial cells to injury. However, although less efficient, HGF treatment still remained profitable and resulted in improved cell survival despite of the low level of Bcl-xL. Interestingly, FasL-triggered activation of Caspase 3 did not affect the expression level of Bcl-xL. In line with these results, we did not observe a beneficial effect of HGF on FasL-induced apoptotic cells.

Altogether, our findings demonstrate that i) Bcl-xL is up-regulated in human IPF, predominantly in AECII and especially in areas with dense fibrosis, ii) knock down of Bcl-xL makes alveolar epithelial cells much more susceptible to injury and cell death, iii) Bcl-xL accounts at least in part for the HGF-elicited epithelial protection against oxidative as well as ER stress. Bcl-xL therefore offers as interesting candidate for epithelial-protective therapies in IPF and other forms of lung fibrosis associated with epithelial apoptosis.

V Zusammenfassung

Die Idiopathische Pulmonale Fibrose (IPF) ist eine chronische, progressiv verlaufende Diffus Parenchymatöse Lungenerkrankung (DPLD), deren Ursache noch nicht vollständig bekannt ist (Ley et al., 2011). Es gibt vermehrte Hinweise, dass Alveolarepithel Typ II Zellen (AECII) eine zentrale Rolle bei der Pathogenese der IPF spielen. Man nimmt an, dass repetitive Schädigungen epithelialer Zellen, gefolgt von einer abnormalen Wundheilungsreaktion zu einer exzessiven Apoptose der AECII führen, die zusammen mit der Aktivierung von Fibroblasten/Myofibroblasten und einem dysregulierten *Remodeling* schließlich in einem irreversiblen Umbau des Lungenparenchyms münden (Selman et al., 2002, Zoz et al., 2001). Der Hepatozytenwachstumsfaktor (*Hepatocyte Growth Factor*, HGF) ist ein Zytokin mit pleiotropen Funktionen, der wesentlich für zelluläre Reparaturprozesse verantwortlich ist und in der Lage ist die epitheliale Homöostase in geschädigten Organen wiederherzustellen. Die exogene Verabreichung von HGF hat sich in tierexperimentellen Modellen verschiedener Organfibrosen, einschließlich der Lunge, als therapeutisch wirksam erwiesen. Bcl-xL ist ein anti-apoptotisch wirksamer Vertreter der Bcl-2 Familie, die aus hochkonservierten Proteinen besteht und an der mitochondrialen Kontrolle der Apoptose beteiligt ist. Da in früheren Studien gezeigt werden konnte, dass HGF über seinen Rezeptor cMet die Expression von Mitgliedern der Bcl-2 Proteinfamilie zu regulieren vermag, wurde in der vorliegenden Arbeit untersucht, inwieweit Bcl-xL an der HGF-vermittelten Protektion epithelialer Zellen bei der IPF beteiligt ist. Ziel war es die Bcl-xL Expression und deren zelluläre Lokalisation in IPF-Lungen im Vergleich zu gesunden Spenderlungen zu charakterisieren, und zu untersuchen, ob die HGF-vermittelten Epithelzell-protektiven Effekte unabhängig von der Art des apoptotischen Stimulus sind und welche Rolle Bcl-xL in diesem Zusammenhang spielt.

Im Vergleich zu nicht utilisierten Donorlungen konnte im Lungengewebe von IPF Patienten eine signifikant erhöhte Expression des Bcl-xL Proteins, vor allem in hyperplastischen AECII und Bereichen mit dichter Fibrose, nachgewiesen werden. Bcl-xL war ebenfalls in AECII von Donorlungengewebe nachweisbar, wurde dort allerdings deutlich schwächer exprimiert. Diese Befunde wurden durch Western Blot Analysen, die einen signifikanten Anstieg des Bcl-xL im Lungenhomogenat von IPF Lungen

versus Donorlungen zeigten, gestützt. In IPF Lungen war in Bereichen mit einer weitgehend normalen Lungenstruktur die immunhistochemische Anfärbung für Bcl-xL in AECII deutlich abgeschwächt, verglichen mit hyperplastische AECII in Bereichen mit einem starken Geweberemodeling.

An Maus (MLE12, MLE15) und Ratten (RLE-6TN) Epithelzelllinien wurden in vitro Versuche durchgeführt. Da bei der Pathogenese der IPF oxidativer Stress, Induktion von ER-Stress und eine erhöhte Expression von Fas Ligand (FasL) beschrieben ist, wurden für die Zellkulturversuche Wasserstoffperoxid, Thapsigargin und FasL als Apoptose-indizierende Stimuli eingesetzt. Eine gleichzeitige Behandlung der Zellen mit HGF und Wasserstoffperoxid bzw. HGF und Thapsigargin führte zu einer gesteigerten Überlebensrate der Zellen. In beiden Fällen war parallel zu den HGF-vermittelten anti-apoptotischen Effekten ein Anstieg der Bcl-xL Expression zu beobachten. Der protektive HGF Effekt konnte durch unter Verwendung eines cMet-spezifischen Inhibitors aufgehoben werden. Der siRNA-vermittelte *Knockdown* von Bcl-xL führte zu einer erhöhten Empfindlichkeit der Epithelzellen gegenüber den schädigenden Agenzien. Eine gleichzeitige Behandlung der Zellen mit HGF erwies sich –wenn auch in geringerem Umfang- als zellprotektiv und führte trotz geringerer Bcl-xL Spiegel zu einer verbesserten Überlebensrate der Zellen. Interessanterweise hatte die FasL vermittelte Aktivierung von Caspase 3 keinen Einfluß auf die Bcl-xL Spiegel, und ebenso hatte HGF keinen protektiven Einfluss auf die FasL-induzierte Apoptose von Epithelzellen.

Zusammenfassend zeigen unsere Ergebnisse, dass i) Bcl-xL bei der IPF erhöht ist, vornehmlich in AECII und speziell in Bereichen mit starker Fibrosierungsreaktion, ii) der *Knockdown* von Bcl-xL Alveolarepithelzellen anfälliger gegenüber einer Schädigung und Apoptoseinduktion macht , iii) Bcl-xL zumindest teilweise für den HGF-vermittelten Schutz von Epithelzellen gegenüber oxidativem Stress und ER-Stress verantwortlich ist. Bcl-xL bietet sich somit als ein potentieller Kandidat für Epithelzell-protektive Therapieregimen bei der IPF und anderen Formen von Lungenfibrose mit erhöhter epithelialer Apoptose an.

1 Introduction

1.1 Idiopathic pulmonary fibrosis

1.1.1 Epidemiology and clinical features of idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a fatal disease of unknown etiology, characterized by progressive and irreversible course. It is the most common and severe form of idiopathic interstitial pneumonia (IIP), a group of entities that belongs to the diffuse parenchymal lung diseases (Meltzer *et al*., 2008). IPF is a relatively rare condition and has a poor prognosis, with a median survival of 2,5 to 3,5 years from the time of diagnosis (Ley *et al*., 2011). The annual incidence fluctuates between 4,6 and 16,3 cases per 100000 and the prevalence is estimated to be 2 to 29 cases per 100000 people in general population, with higher frequency in men than women (Raghu *et al*., 2011). While some patients show a stable progression rate of the disease for extended time periods, the individual outcome is highly variable, as acute exacerbations may occur in an unpredictable manner (Meltzer *et al*., 2008).

Patients with IPF typically suffer from dry, non-productive cough and dyspnea upon excercise, which progresses into breathlessness at rest (White *et al*., 2003). On chest examination, inspiratory Velcro-like crackles can be auscultated in basilar lung regions. In up to half of all patients, finger clubbing is observed. In general, manifestation of IPF occurs in middle-aged and elderly adults, with a mean age at presentation of 66 years (King *et al*., 2011).

Due to the lack of specific symptoms, the clinical diagnosis of IPF requires an integrated approach. First of all, radiological and/or histological pattern characteristic for “usual interstitial pneumonia” (UIP) has to be evident and other forms of IIP caused by known factors, such as an environmental exposure to an inhalable irritant (*e.g.* asbestos), systemic disease (*e.g.* collagen vascular disease) or drug treatment (*e.g.* amiodaron), have to be excluded (Raghu *et al*., 2011). Secondly, restriction on pulmonary function should be observed, including reduced total lung capacity, decreased values of forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) alongside with impaired gas exchange (Martinez *et al*., 2006).

In principle, the disease is highly heterogeneous concerning its phenotype as well as the clinical course. The complexity of IPF still remains a challenge and only limited

treatment options are available. Only recently, next to the lung transplantation (Chan *et al*., 2013), pirfenidone has become available as anti-fibrotic treatment in Europe (Cottin, 2013). Given that, considerable progress towards the understanding and treatment of this devastating disease should be made within the next years.

1.1.2 Histopathology of idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis is associated with the histological pattern known as usual interstitial pneumonia (UIP). The key features of UIP comprise spatial heterogeneity, alveolar septal thickening, peripheral fibrosis with mild inflammation, presence of fibroblastic foci and microscopic honeycomb changes (Figure 1.1).

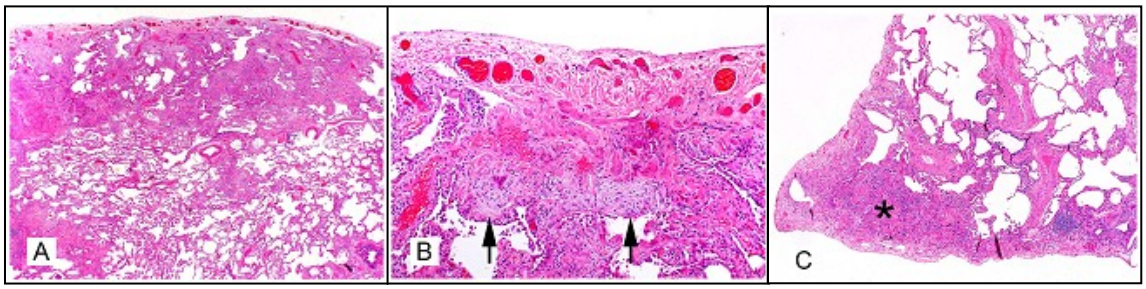

Figure 1.1: Histopathological features of usual interstitial pneumonia.
Characteristics of advanced fibrosis in usual interstitial pneumonia of idiopathic pulmonary fibrosis include (**A**) a subpleural distribution of fibrosis, 20x, (**B**) relative frequency of fibroblast foci (arrows) and relative absence of inflammatory cell infiltrate, 400x, (**C**) smooth muscle proliferation in the subpleural scars (asterisk), 40x, (adapted from Smith et al., 2013).

Heterogeneity is the most striking feature of UIP. In biopsies obtained from patients with IPF, regions with normal lung architecture alternate with patchy areas of histologically apparent parenchymal fibrosis (Meltzer *et al*., 2008). At the border between normal appearing and within the scar regions, a variable number of clusters of fibroblast/myofibroblast, termed fibroblastic foci, are found. They are believed to represent the active lesions of UIP (White *et al*., 2003). In those active regions, alveolar epithelial injury with hyperplastic alveolar epithelial type II cells (AECII) and alveolar septal thickening is often seen (King *et al*., 2011). Adjacent to pleural surface, enlarged cystic airspaces, termed honeycombing can be observed (White *et al*., 2003). The inflammation is mild and mostly associated with areas of collagen deposition or honeycombing. It seldom affects unaltered alveolar septa (Selman *et al*., 2001). In

patients with smoking history, additionally emphysema or respiratory bronchitis can occur next to the UIP pattern (Meltzer *et al*., 2008).

1.1.3 Pathogenesis of idiopathic pulmonary fibrosis

IPF is a chronic, progressive and irreversible disease of unknown origin. Despite extensive research, the mechanisms underlying the evolution of the disease remain poorly understood. According to a current concept, repetitive injury to alveolar epithelial cells (AECs) with consecutive aberrant wound healing process and disturbed crosstalk between epithelial cells and fibroblasts is thought to be the driving force for the development of pulmonary fibrosis (Jenkins *et al*., 2012). Indeed, a growing number of publications in the field suggests, that apoptosis of AEC may be the leading cause of the disease progression. Activation of oxidative and ER stress response pathways, telomere shortening and genetic factors such as surfactant protein C or other mutations and alterations in the cellular microenvironment maintained by activated myofibroblasts perpetually increase the susceptibility of alveolar type II cells to apoptosis (Jin *et al*., 2011; Hecker *et al*., 2011) (Figure 1.2).

In IPF, a severe imbalance between oxidants and antioxidants has been observed. Analysis of the epithelial lining fluid from IPF lungs showed increased levels of hydrogen peroxide, lipid oxidation products and oxidized proteins with carbonyl modifications. In contrast, there is a reduced antioxidant protection, for example decreased levels of glutathion in bronchoalveolar lavage fluid (BALF) and superoxide dismutase, especially in fibrotic regions of UIP lungs (Kliment *et al*., 2010). Excessive production of reactive oxygen species (ROS) may contribute to IPF pathogenesis *via* various pathways, such as altered cytokine expression, induction of apoptosis of epithelial and endothelial cells or activation of fibroblast (Waghray *et al*., 2005, Walters *et al*., 2008).

Another factor that may contribute to the development of pulmonary fibrosis is a genetic predisposition. Telomerase activity is crucial for the proliferation and proper repair of alveolar epithelial cells. Loss of function mutations in telomerase components has been observed in 8-15% of familial IPF cases (Armanios *et al*., 2007). Telomere shortening in various cell types, like type II cells or circulating leukocytes, has been described in patients with sporadic, familial and idiopathic pulmonary fibrosis (King *et*

al., 2011, Zoz *et al*., 2011). Telomere shortening leads to loss of AECII during re-epithalisation of injured alveoli, which in turn drives a fibrotic response (Whitsett *et al*. 2010). Moreover, genetic mutations in surfactant protein C (SP-C) and A (SP-A) have been linked to familial cases of IPF (Kropski *et al*., 2013). Accumulation of misfolded SP-C can lead to activation of the unfolded protein response (UPR) and ER (endoplasmic reticulum) stress induction (Korfei *et al*., 2008). Since SP-C is exclusively expressed by type II cells in the lung, the described mechanism may directly affect and promote AECII apoptosis, potentially leading to the progression of fibrotic process (Jin *et al*., 2011).

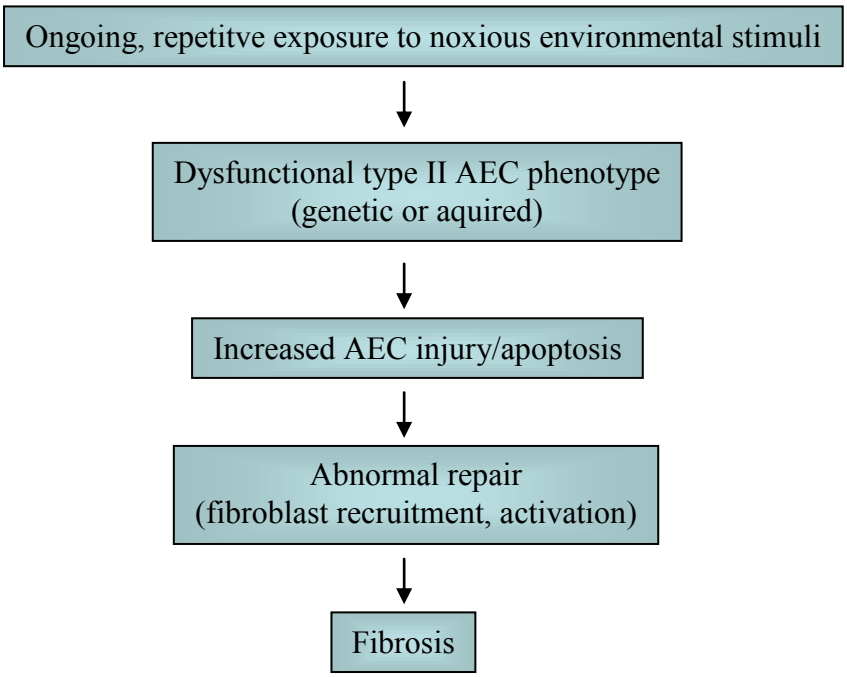

Figure 1.2: Hypothetical scheme for IPF pathogenesis (adapted from Zoz *et al*., 2011).

Apart from determining the etiology of the primary injury that triggers development of IPF, the mechanisms responsible for the progressive nature of fibrotic process, even without presence of the initial stimuli, need to be elucidated. Sustained deregulation of epithelial-fibroblast crosstalk, with constant deterioration of alveolar epithelial cells and expansion of activated fibroblasts/myofibroblasts, with excessive deposition of extracellular matrix, might contribute to pathogenesis of IPF (Selman *et al*., 2002). Strong evidence indicates that AECII are the primary source of chemotactic factors and mitogens for mesenchymal cells, *e.g.* platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) or tumor necrosis factor α (TNF-α). That in turn

promotes the expansion of fibroblasts, their trans-differentiation into α-smooth muscle actin-positive myofibroblasts and reinforce maintenance of a fibrotic phenotype characterized by high mechanical stress, local further increase of TGF-β synthesis or presence of specialized matrix proteins, like ED-A fibronectin (King *et al*., 2011). Additionally, TGF-β has also a negative effect on alveolar epithelial cells, for example *via* enhancing the Fas-mediated apoptosis of these cells (Hagimoto *et al*., 2001) (Figure 1.3).

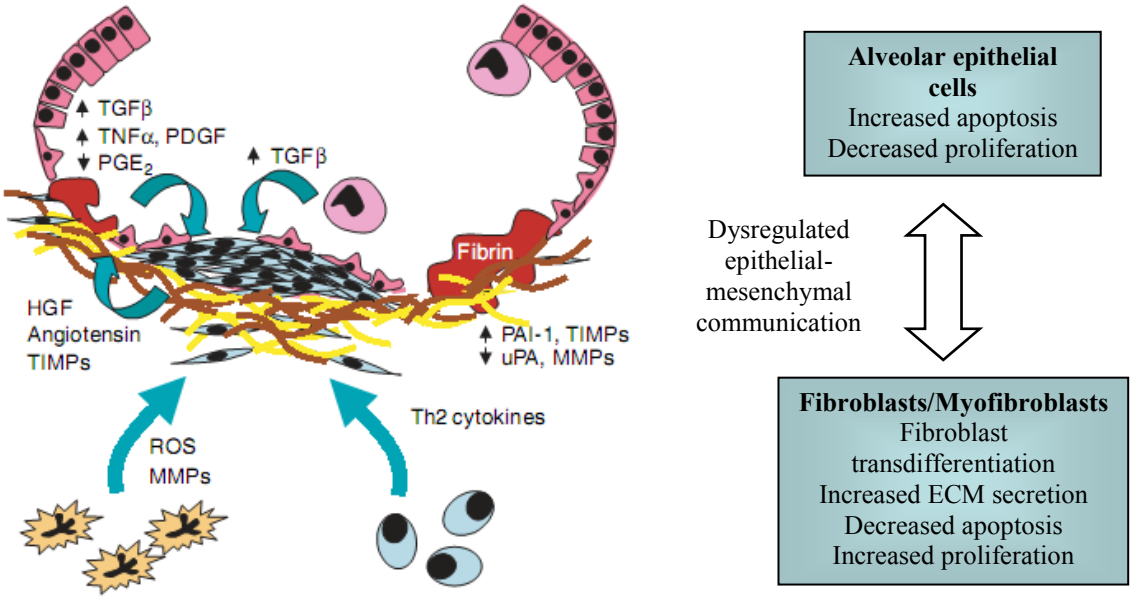

Figure 1.3: Overview of key pathogenic mechanisms in IPF.
Following unidentified insult, alveolar epithelial cells become injured and delayed re-epithelialization leads to a denuded, disrupted basement membrane. A fibrin clot forms early and serves as a provisional matrix for the migration and proliferation of reparative alveolar epithelial cells. Neutrophils secrete pro-inflammatory mediators, reactive oxygen species and MMPs, while recruited lymphocytes elaborate the Th2-type cytokines. Fibroblasts migrate into the wound and produce extracellular matrix proteins and mediators such as Angiotensin II which may further promote alveolar epithelial cell apoptosis. Alveolar macrophages and epithelial cells secrete TGF-β1, which promotes myofibroblast differentiation, increases extracellular matrix production, and inhibits apoptosis of fibroblasts/myofibroblasts. Reciprocal communication between alveolar epithelial cells and mesenchymal cells results in a positive feedback loop that promotes ongoing fibrosis and destruction of alveolar architecture (adapted from White *et al.*, 2003).

Despite the fact, that many elements of the innate and adaptive immune response participate in the differentiation and activation of fibroblasts (Wynn *et al*., 2012), it is still a controversial issue, if inflammation plays a significant role in the pathogenesis of IPF. In IPF lung tissue as well as in BAL fluid, some inflammatory cells known to produce various growth factors and cytokines exacerbating fibrosis can be found, including neutrophils, macrophages, plasma cells and lymphocytes. Based on the

evidence that inflammation itself is usually described as minimal to mild and combined immunosuppressive therapy with corticosteroids has been proven harmful to IPF patients (Jin *et al*., 2011, IPF Clinical Research Network *et al*., 2012), the current hypothesis postulates that inflammation is neither a triggering factor of IPF nor the major player in its pathogenesis (Bringardner *et al*., 2008)

Since idiopathic pulmonary fibrosis is a complex disease, the mechanisms underlying its pathogenesis may involve a number of molecular pathways that result in loss of cellular homeostasis within the alveolar wall and expansion of mesenchymal cells in the interstitium.

1.2 Hepatocyte growth factor

1.2.1 HGF/c-Met signaling pathway

Hepatocyte growth factor (HGF) is a pleiotropic cytokine playing major roles in the control of tissue homeostasis and regeneration, as well as during embryonic development. In mature organs, it promotes proliferation, survival, motility, differentiation and morphogenesis in diverse cell types. Besides, it is crucial for migration of skeletal muscle progenitor cells (Bladt *et al*., 1995) and essential for embryonic development of liver (Schmidt *et al*., 1995), placenta (Uehara *et al*., 1995), nervous system (Maina *et al*., 1999) and epithelial morphogenesis in different organs including the lung (Ohmichi *et al*., 1998).

HGF is mainly produced by cells of mesenchymal origin and secreted as a single-chain precursor. Specifically at the site of injury, HGF is converted by proteolytic cleavage into its biologically active form. Several proteases in the serum or cell membrane are responsible for the activation process, including HGF activator (HGFA), urokinase-type plasminogen activator (uPA), coagulation factors XI and XII and matriptase. The cleavage occurs between Arg 494 and Val 495 residues. The mature form of HGF is a heparin-binding, heterodimeric glycoprotein composed of α and β subunit linked by a disulphide bond. The 69 kDa α subunit consists of N-terminal hairpin loop and four kringle domains, whereas β subunit is smaller (34 kDa) and has serine protease-like structure (Nakamura *et al*., 2010, Nakamura *et al.*, 2011) (Figure 1.4 A). In the activated form, HGF is recognized by the specific cell surface receptor c-Met, expressed mainly in the epithelial cells of various organs, including the liver, kidney and lung. The mature

c-Met receptor is a heterodimeric protein composed of structural domains that include extracellular Sema, PSI and IPT domains, the transmembrane domain and the intracellular tyrosine kinase catalytic domain flanked by juxtamembrane and C-terminal sequences (Figure 1.4 B).

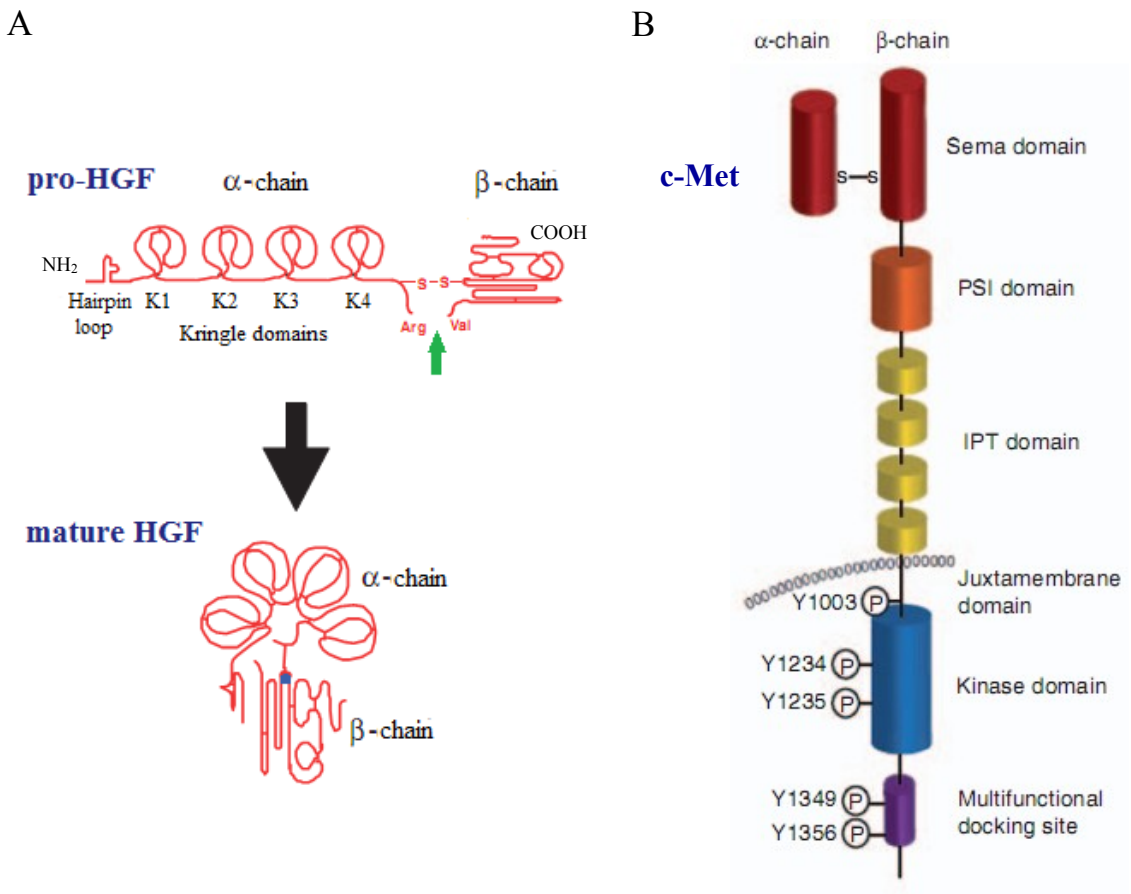

Figure 1.4: Structural characteristics of HGF and c-Met.
(**A**) HGF is secreted as a single-chain form and is converted into its biologically active form upon proteolytical cleavage between Arg and Val residues (green arrow). The mature form consists of α and β subunits linked by a disulphide bond. The α-chain contains N-terminal hairpin loop followed by four kringle domains (K 1-4). (**B**) c-Met receptor is a single-pass, disulphide linked heterodimer. The extracellular part is composed of three domain types: semaphorin domain (Sema), the plexin-semaphorin-integrin (PSI) domain and immunoglobulin-plexin-transcritpion (IPT) domains. The c-Met receptor contains tyrosine catalytic domain flanked by juxtamembrane domain and the multifunctional docking site in the C-terminal tail. (Adapted from Nakamura *et al.,* 2010, Organ and Tsao, 2011).

Direct interaction between the receptor and HGF occurs *via* high affinity binding of the HGF α subunit to the extracellular portion of the receptor, and the low affinity binding of the HGF β subunit to c-Met Sema domain which is necessary for inducing signal transduction. HGF association leads to homodimerization of the receptor and

phosphorylation of two tyrosine residues (Tyr 1234 and Tyr 1235) located within its catalytic loop. Subsequently the C-terminal Tyr 1349 and Tyr 1356 become phosphorylated which results in the recruitment of intracellular signaling molecules that include adaptor proteins, *e.g.* growth factor receptor-bound protein 2 (Grb2), Grb2-associated binder 1 (Gab1), SH2-containing protein tyrosine phosphatase 2 (Shp2) and the effector molecules, like phosphatidylinositol 3 kinase (PI3K), phospholipase C γ (PLCγ), signal transducer and activator of transcription 3 (STAT3) and the v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (Src). A large range of adaptor molecules, among which some function as additional platform for binding another proteins (*e.g.* Gab1), is the key to c-Met-mediated wide variety of cellular responses (Figure 1.5). Furthermore, the specific downstream response to c-Met activation can be affected by phosphorylation of additional tyrosine residues (Tyr 1313 and Tyr 1365) or be negatively regulated by phosphorylation of serine 985 and tyrosine 1003 (Nakamura *et al*., 2011, Organ *et al*. 2011). As a consequence, HGF/c-Met signaling pathway is able to regulate many distinct cellular processes in a controlled, accurate and well orchestrated manner.

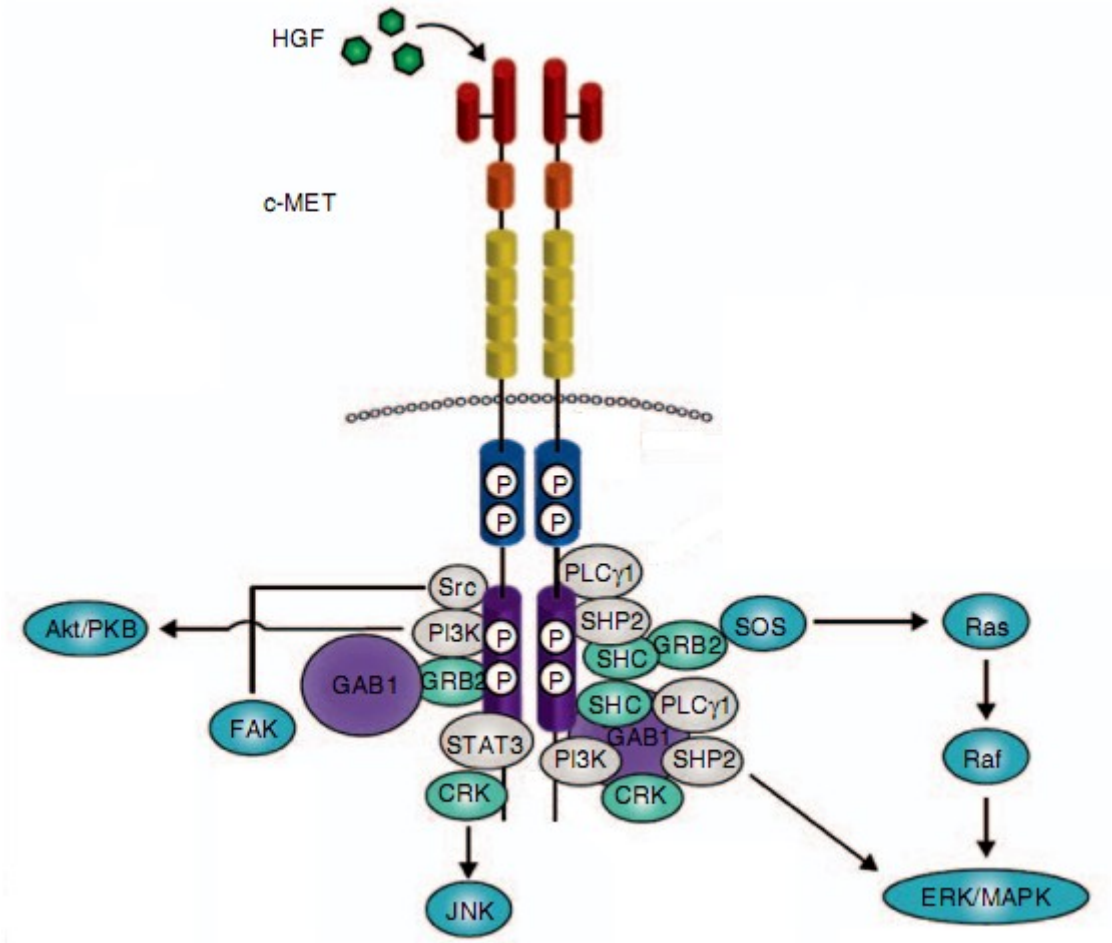

Figure 1.5: HGF-mediated c-Met signaling.
HGF binding to c-Met results in receptor homodimerization and tyrosine phosphorylation with multiple downstream effects. Each biological activity is elicited through recruitment of specific adaptor proteins. (Adapted from Organ and Tsao, 2011)

1.2.2 HGF as a fibrosis resolving factor

Under normal conditions, induction of endogenous HGF production following tissue injury is principally sufficient for proper regeneration and wound healing process leading to restoration of homeostasis in the damaged organ. However, during development and progression of fibrosis, the intrinsic production of HGF appears to be insufficient to promote full recovery and reduction of fibrotic changes (Crestani *et al*., 2012). Studies on animal models have provided strong evidence that supplementation of exogenous HGF has a beneficial role in a wide range of fibrotic disorders in various organs, including the lung, kidney, liver and heart (Table 1). In the rodent model of bleomycin-triggered pulmonary fibrosis, simultaneous or delayed administration of recombinant HGF protein or of HGF gene therapy, were both successful in ameliorating

fibrotic lesions, reducing the hydroxyproline content in the lung and improving survival rate of experimental animals. Administration of exogenous HGF has also been shown to restore kidney and liver function in corresponding models of liver cirrhosis and kidney fibrosis, suppress collagen deposition and finally resulting in resolution of fibrosis.

Organ	Model of disease (animal)	HGF application (apporach)	Outcomes	References
Heart	Genetic model of cardiomyopathy (hamster)	SC, rHGF (therapeutical)	↓ cardiac fibrosis, ↓ expression of TGF-β1 and collagen I, ↑ cardiac function	Nakamura *et al*., 2005
Liver	DMN model of cirrhosis (rat)	IV, rHGF (preventive)	↑ ECM degrading enzymes, ↓ ECM components, ↑ survival rate	Matsuda *et al.*, 1995
Liver	Bile duct ligation-induced cirrhosis (mouse)	IV, HGF cDNA (preventive)	↓ fibrotic lesions, ↓ α-SMA and TGF-β, ↓ hydroxyproline content	Xia *et al*., 2006
Liver	DMN model of cirrhosis (rat)	IP, rHGF (preventive)	↓ α-SMA, histological resolution of cirrhosis	Kim *et al*., 2005
Liver	DMN model of cirrhosis (rat)	IM, HVJ-HGF (therapeutical)	↓ TGF-β, resolution of fibrosis, ↑ survival rate	Ueki *et al*., 1999
Lung	Bleomycin model of fibrosis (mouse)	IP, rHGF (preventive and therapeutical)	↓ hydroxyproline content, ↓ pulmonary fibrosis	Yaekashiwa *et al*., 1997
Lung	Bleomycin model of fibrosis (mouse)	IT, rHGF (therapeutical)	↓ hydroxyproline content, ↓ fibrotic score	Dohi *et al*., 2000
Lung	Bleomycin model of fibrosis (mouse)	IM, HGF cDNA (preventive and therapeutical)	↓ lung and dermal fibrosis, ↓ collagen content, ↓ TGF-β	Wu *et al*., 2004
Lung	Bleomycin model of fibrosis (mouse)	IM, HGF cDNA (preventive)	↓ fibrotic score, ↓ hydroxyproline content, ↓ apoptosis of epithelial cells	Umeda *et al*., 2004
Lung	Bleomycin model of fibrosis (mouse)	SC, rHGF (preventive)	↓ hydroxyproline content, ↑ MMP-1 and MMP-9, ↑ myofibroblast apoptosis	Mizuno *et al*., 2005
Lung	Bleomycin model of fibrosis (mouse)	IV, HGF cDNA (preventive and therapeutical)	↑ IL-6 and TNF-α, ↑ endogenous HGF expression, ↓ hydroxyproline content, ↑ survival rate	Watanabe *et al*., 2005
Lung	Bleomycin model of fibrosis (rat)	IT, HGF cDNA (therapeutical)	↓ fibrotic score, ↓ hydroxyproline content, ↓ TGF-β, ↓ apoptosis of epithelial cells	Gazdhar *et al*., 2007
Lung	Bleomycin model of fibrosis (rat)	IM, HGF cDNA (preventive and therapeutical)	↓ fibrotic score, ↓ hydroxyproline content, ↑ COX-2	Long *et al*., 2007
Kidney	Spontaneous model of CRD (mouse)	SC, rHGF (preventive)	↑ tubular repair, ↑renal function, ↓ TGF-β and PDGF	Mizuno *et al*., 1998
Kidney	UUO-induced renal fibrosis (mouse)	IV, rHGF (therapeutical)	↓ fibrosis, ↓ collagen content, ↓ TGF-β and fibronectin	Yang and Liu, 2003

Kidney	UUO-induced renal fibrosis (mouse)	IV, HGF cDNA (preventive)	↑ endogenous HGF expression, ↓ collagen I, ↓ fibronectin, ↓ TGF-β, ↓ α-SMA	Yang *et al*., 2001

Table 1: Effects of exogenous HGF administration in animal models of different organ fibrosis. CRD – chronic renal disease, DMN – dimethyl nitrosamine, ECM – extracellular matrix, HVJ-HGF – hemagglutinating-virus-of-Japan liposome containing HGF cDNA, rHGF – recombinant HGF, UUO – unilateral ureteral obstruction, IP – intraperitoneal, IM – intramuscular, IT – intratracheal, IV – intravenous, SC – subcutaneous, (modified from Crestani *et al*., 2012, Nakamura *et al*., 2010)

The anti-fibrotic actions of HGF may be mediated *via* multiple direct and indirect mechanisms (Figure 1.6). As a regenerative factor, HGF is thought to block apoptosis and promote proliferation of epithelial and endothelial cells, thereby promoting injury-initiated repair. Three predominant pathways have been implicated in HGF pro-survival and pro-mitogenic signaling: ERK/MAPK, PI3K/Akt and STAT3 (Panganiban and Day, 2011). Although HGF was shown to stimulate proliferation through the ERK-STAT3 pathway and to have anti-apoptotic action through PI3K/Akt pathway in human aortic endothelial cells (Nakagami *et al*., 2001), not much is known up to date about its anti-apoptotic properties on alveolar epithelial cells. In analogy to other studies, it is assumed that it may occur through PI3K/Akt kinase signaling pathway. PI3K/Akt pathway has been reported to play a major role in HGF-mediated protection of hepatocytes (Moumen *et al*., 2007) and mouse lung endothelial cells (Wang *et al*., 2004). However, the exact mechanism remains unknown. Moreover, HGF has been demonstrated to induce DNA synthesis in primary rat alveolar type II cells *in vitro* (Shiratori *et al*., 1995) and *in vivo* (Panos *et al*., 1996).

Another important mechanism involved in HGF-driven resolution of fibrosis maybe the reduction of myofibroblast accumulation. In chronically injured organs, interstitial myofibroblasts are the major source of extracellular matrix deposition and the key mediators of pro-fibrotic remodeling that leads to distortion of normal tissue architecture (Wynn *et al*., 2012). In the lung, HGF has been identified to specifically elicit myofibroblast apoptosis *via* indirect mechanisms associated with increased activity of matrix metalloproteinases (MMPs), thus leading to the degradation of the ECM components (Mizuno *et al*., 2005). Additionally, HGF appears to be responsible for sustaining quiescent phenotype of fibroblasts and inhibiting fibroblast transdifferentiation into activated myofibroblasts (Panganiban and Day, 2011). This occurs through HGF-mediated up-regulation of epithelial and endothelial cyclooxygenase 2 (COX-2) expression that in turn promotes increased prostaglandin

E 2 (PGE_2) synthesis. PGE_2 acts as a potent inhibitor of TGF-β, the major inducer of fibroblast transdifferentiation (Thomas *et al*., 2007, Lee *et al*., 2008). Moreover, HGF has been described to directly counteract TGF-β actions through up-regulation of the endogenous TGF-β-signaling inhibitor, Smad 7. This leads to suppression of epithelial-to-mesenchymal transition of alveolar type II cells, thus antagonizing fibroblast phenotype and eliminating a potential source of fibroblasts in the diseased lung (Shukla *et al*., 2009). Above observations are not restricted to the lung and have been comprehensively described in experimental models of renal and hepatic fibrosis (Liu, 2004, Mizuno and Nakamura, 2007).

In conclusion, HGF has been described to affect various cell types in a specific manner that leads to improved function of different organs and reduction of fibrotic remodeling. In the lung, HGF is well known for its TGF-β-counteracting properties, being largely responsible for diminished fibroblast expansion and suppression of fibroblast/myofibroblast phenotype in fibrotic lesions. However, further understanding of mechanisms driving HGF protective activity on endothelial and especially alveolar epithelial cells is necessary for developing effective and multi-targeted cure. Based on the fact that AECs are the primary site of the initial injury, upon which they acquire hyperplastic phenotype and become source of important pro-fibrotic cytokines, AECs seem to be a crucial target for further investigation to create an integrated treatment options for IPF patients.

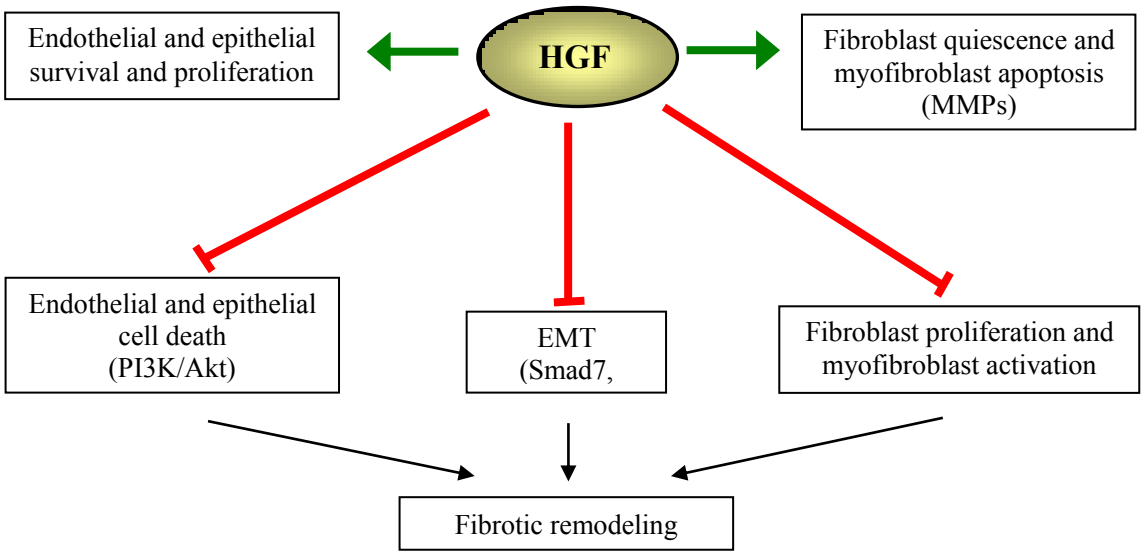

Figure 1.6: Mechanisms of the anti-fibrotic action of HGF in various organs (adapted from Panganiban *et al*., 2011)**.**

1.2.3 Role of HGF in lung cancer

Lung cancer is a multifaceted disease that can be divided in two major histological subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), which can be further subdivided in adenocarcinomas, squamous cell, bronchioalveolar and large cell carcinomas (Larsen *et al*., 2011). In general, advanced lung cancer is an aggressive malignancy with a poor prognosis. It develops *via* a multistep process involving tumor suppressors as well as oncogenes that trigger complex aberrations leading to the disruption of the normal balance of cellular life and death (Bai and Wang, 2013). The HGF/c-Met have been shown to stimulate a number of signaling molecules affecting cellular motility, growth, invasion, differentiation and angiogenesis (Sadiq and Salgia, 2013). A number of studies have reported *HGF* and/or *c-MET* over-expression as well as multiple *c-MET* activating mutations to be implicated in various oncogenic processes, in the lung inclusively (Cecchi *et al*., 2012, Feng *et al*., 2012). Amplification of the c-Met encoding gene have been found in several types of lung cancer, including NSCLC where it occurs in up to 20% of the patients and negatively correlates with survival (Cappuzzo *et al*., 2009). Enhanced c-Met activation and persistent HGF/c-Met signaling leads to increased transforming potential *via* STAT3-mediated anchorage independent cell growth, Ras-mediated mitogenesis and PI3K-mediated inhibition of apoptosis (Mizuno and Nakamura, 2013).

1.3 Cell death

1.3.1 Diversity of cell death processes

Cell death represents a key physiological process and is considered fundamental during development and aging as well as for maintaining tissue homeostasis in adult organism. Among others, cell death plays an important role during wound healing and repair *via* removal of activated inflammatory cells as well as myofibroblasts that are no longer essential at the site of injury. There are multiple factors determining cell fate, including the type and the intensity of the stimulus and the type of the affected cell.

The best understood and most common form of cell death is apoptosis, a coordinated and energy-dependent process that involves the activation of specific cysteine proteases called caspases and a complex cascade of events leading to cell removal.

Morphologically, apoptosis is characterized by cell shrinkage, the nuclear and cytoplasm condensation, DNA fragmentation and formation of apoptotic bodies containing cellular components with almost no concomitant inflammatory reaction (Favaloro *et al*., 2012). The apoptotic cascade can be initiated *via* two major molecular pathways: the extrinsic or death receptor-mediated pathway and the intrinsic or mitochondrion-mediated pathway (Figure 1.7). Triggering of either pathway leads to executive pathway activation *via* proteolytical cleavage of down-stream caspases (Caspase 3, 6 and 7) which in turn results in the activation of proteases responsible for degradation of chromatin, as well as nuclear and cytoskeletal components. The last phase of programmed cell death (PCD) is the phagocytosis of an apoptotic cell. Expression of surface markers, such as phosphatidylserines Annexin I and V enables early recognition by macrophages or other neighboring cells and facilitates cell degradation (Elmore, 2007).

Growing evidence indicates that the process of caspase activation is not the sole determinant of life and death decisions. Programmed cell death can be mediated *via* other executive proteases, *e.g.* calpains, cathepsins and endonucleases. It has been observed that upon excessive autophagy cells may be triggered into PCD without activation of caspases (Broeker *et al*., 2005).

Necrosis stands for another form of cell death that can be described in contrast to PCD as uncontrolled and passive. Necrosis is an unintended process caused by an external stimulus. It is characterized by increase in cell volume followed by enlargement of organelles and direct disruption of membrane integrity. This process is associated with a release of cellular components leading to inflammatory reaction in adjacent tissue (Rastogi *et* al., 2009).

1.3.2 Extrinsic pathway

Extrinsic pathway of apoptosis is activated by extracellular signals that result in the binding of specific ligands to the transmembrane receptors belonging to the tumor necrosis factor (TNF) receptor superfamily, such as TNF-α or Fas ligand (FasL) with their respective receptors, TNF receptor 1 (TNFR1) or Fas receptor (FasR). TNF-family members share conserved extracellular domains and a cytoplasmatic death domain which is responsible for the signal transduction. The cascade of events is similar for all

known death receptors. Upon ligand-receptor interaction, cytoplasmatic adapter proteins are recruited. Depending on the type of the receptor, a specific adapter protein is recruited. In the FasL/FasR-mediated signal transduction, binding of the adapter molecule Fas-associated protein with a death domain (FADD) occurs. This enables an assembly of a large multi-protein complex termed a death-inducing signaling complex (DISC) at the plasma membrane, which in turn results in the activation of initiator Pro-caspase 8. Additionally, FADD contains a highly conserved death effector domain that binds directly to a homologous region of Pro-caspase 8 leading to its cleavage. Once Caspase 8 is activated, the execution phase of apoptosis is triggered (Favaloro *et al*., 2012, Kaufmann *et al*., 2012).

1.3.3 Intrinsic pathway

Intrinsic pathway of apoptosis is associated with a receptor-independent cellular response to a wide variety of extracellular factors as well as internal stimuli, including reactive oxygen species (ROS), ER stress, radiation, DNA damage, viral infections and depletion of growth factors or cytokines. These multiple forms of cellular stress converge on the level of the mitochondria and lead to the mitochondrial outer membrane permeabilization (MOMP) and release of pro-apoptotic molecules, *e.g.* Cytochrome c (Cyt c), direct IAP binding protein with low pI (Smac/DIABLO) and serine protease high temperature requirement protein A2 (HtrA2) into cytosol. Cyt c, after being released from the intermembrane space of mitochondria, binds to an adaptor molecule termed apoptotic protease activating factor 1 (Apaf-1), which oligomerizes and recruits Pro-caspase 9, thus forming the apoptosome. Additionally, Smac/DIABLO further stimulates caspase activation by binding and thus neutralizing inhibitor of apoptosis proteins (IAPs). At the same time, nuclear translocation of endonucleases, including AIF and Endonuclease G, released from mitochondria leads to DNA fragmentation and advanced chromatin condensation. This results in activation of the executive phase through cleavage of Pro-caspase 3 (Elmore, 2007, Bai and Wang, 2013).

The Bcl-2 family proteins are essential regulators of the intrinsic pathway of apoptosis. Complex interactions between specific members of the family determine the integrity of the outer mitochondrial membrane and control of the cell fate. Additionally, Bcl-2

family member Bid is being proteolytically activated by Caspase 8, which constitutes an important link between the intrinsic and extrinsic apoptosis pathways (Cory and Adams, 2002).

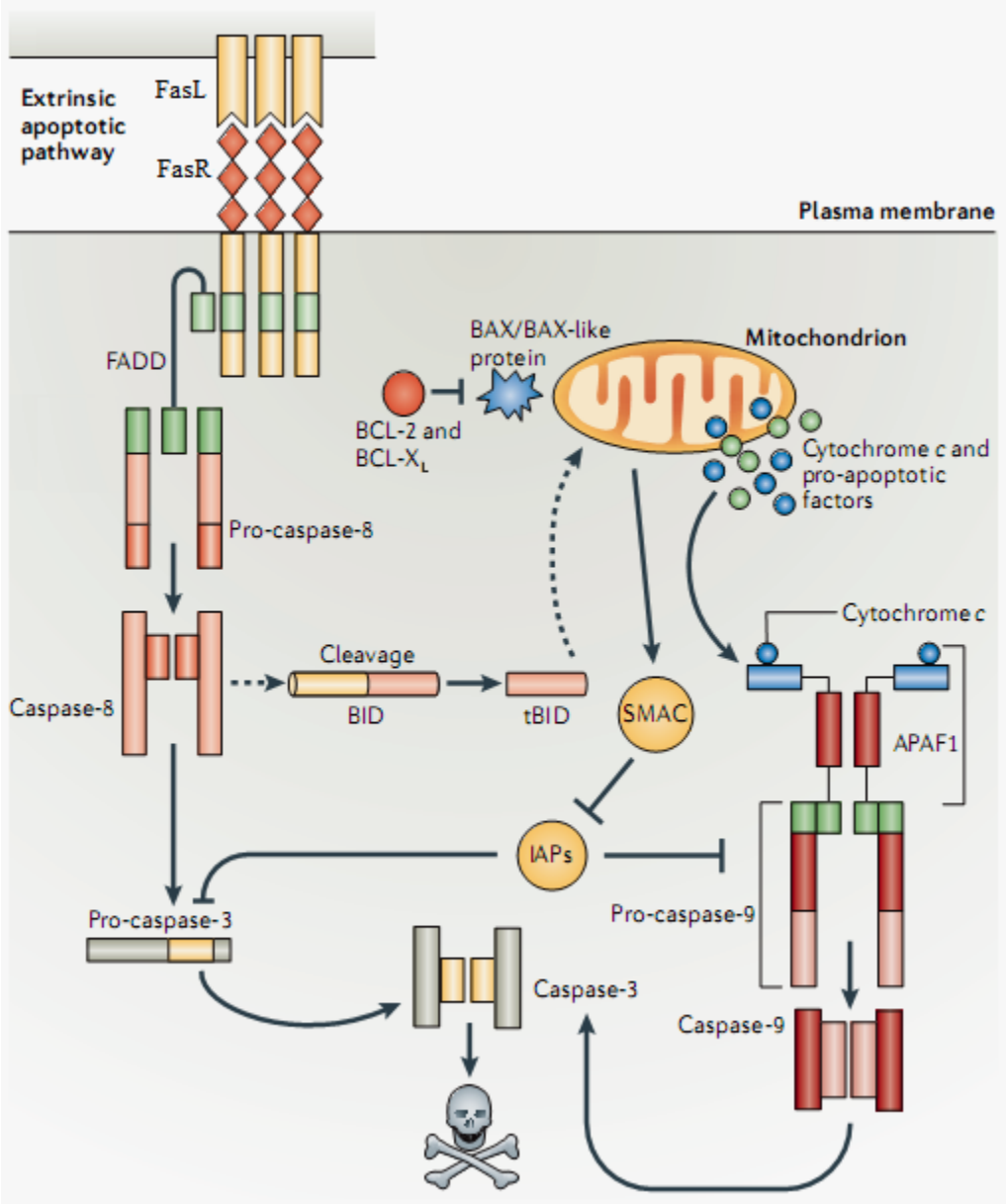

Figure 1.7: Schematic representation of extrinsic and intrinsic apoptotic pathways.
The extrinsic pathway is mediated by caspase-8 whereas the intrinsic pathway is mediated by caspase-9. FADD is an adaptor protein that couples death receptors, such as FasR, to Caspase 8. The two pathways are interconnected by truncated BID (tBID) cleaved by active Caspase 8. Bcl-2 and Bcl-xL inhibit the loss of mitochondrial membrane potential, whereas Bax/Bak mitochondrial membrane permeabilization. Cytochrome c is released from the mitochondria and together with Apaf-1 and Pro-caspase-9 form the apoptosome. SMAC/Diablo is also released from the mitochondria and blocks the effect of apoptosis inhibitory proteins, IAPs which promotes caspase activation. (Adapted from Hotchkiss and Nicholson, 2006)

1.4 Bcl-xL as a Bcl-2 family member

Bcl-xL (Bcl-2 related gene, long isoform) is a pro-survival protein that belongs to B cell CLL/lymphoma-2 (Bcl-2) family of proteins involved in mitochondrial control of apoptosis. The family comprises of proteins that share a three-dimensional structure and contain at least one Bcl-2 homology (BH) region. They can be functionally classified into three groups: anti-apoptotic, pro-apoptotic (also termed as effector proteins) and BH3-only proteins (Figure 1.8 A). Anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL, Bcl-w and myeloid cell leukemia-1 (Mcl-1), contain four BH domains (BH 1-4) and are mainly localized in the outer mitochondrial membrane (OMM). However, they may also be present in the cytosolic fraction or embedded in the ER. They are able to directly bind and sequester the pro-apoptotic proteins, thus preserving OMM integrity and preventing apoptosis. Bcl-2-associated x protein (Bax) and Bcl-2 antagonist killer (Bak) are two major representatives of pro-apoptotic multi-BH (BH 1-4) Bcl-2 related proteins. Whereas activation of Bax results from highly regulated, multistep process that requires its translocation into mitochondrial membrane, Bak is constitutively inserted into OMM. Oligomerization of Bax and Bak triggered by various mechanisms directly promotes MOMP, Cytochrome c release and apoptosis. The BH3-only proteins, *e.g.* (Bcl-2-interacting domain death agonist) Bid, (Bcl-2 interacting mediator of cell death) Bim, (Bcl-2 antagonist of cell death) Bad, p53 up-regulated modulator of apoptosis (Puma) and Noxa, are pro-apoptotic and function as initial sensors that integrate and transmit apoptotic signals to other Bcl-2 family members. Except Bid, the BH3-only proteins appear to lack a close evolutionary relationship to the multi-BH members of Bcl-2 family. However, they posses a highly conserved short motif called BH3, that allows them to bind and regulate both, the anti- and pro-apoptotic Bcl-2-related proteins and to promote cell death. The BH3-only proteins Bad, Noxa and Puma, which all have the ability to bind only the anti-apoptotic Bcl-2 family members, are referred to as "sensitizers/de-repressors", since they lower the threshold for Bax/Bak activation, but do not induce apoptosis in a direct manner. The BH3-only proteins Bid and Bim, that can as well as interact with the effector proteins and directly induce oligomerization of Bax/Bak, are termed "direct activators" (Adams and Cory, 1998, Chipuk *et al*., 2010, Youle and Strasser, 2008). The main event upon apoptotic stimuli is the proteolytical activation of Bid, predominantly by Caspase 8, and translocation to mitochondria of the

truncated form of Bid (tBid), where it's able to recruit and induce conformational change of Bax leading to Bax insertion and Bax/Bak oligomerization in the OMM (Martinou and Youle, 2011) (Figure 1.8 B).

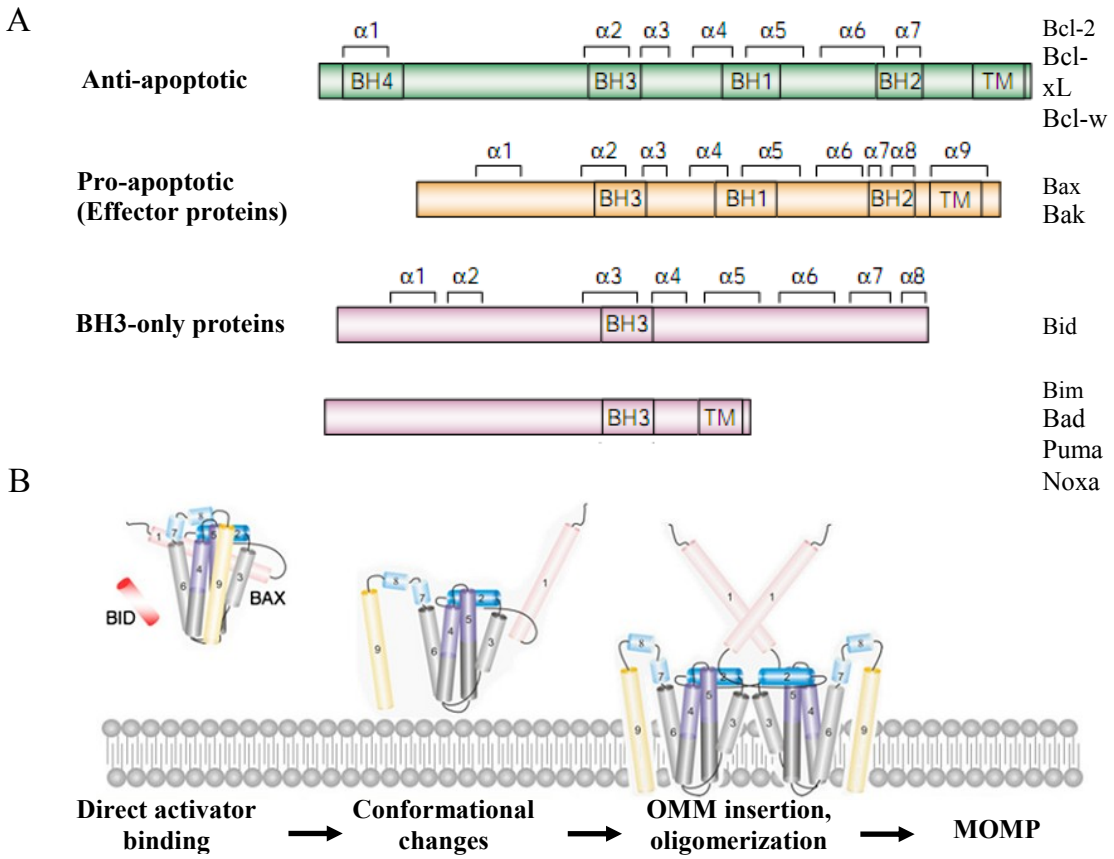

Figure 1.8: Bcl-2 family classification and membrane permeabilization.
(**A**) Bcl-2 family members can be divided into three groups: pro-apoptotic, anti-apoptotic and BH3-only proteins, based on their function and structural homology. BH regions, transmembrane (TM) domains as well as known α-helical structures are indicated. (**B**) Proposed model of Bax activation. Soluble Bax interacts directly with activated Bid and directly with OMM to promote MOMP and subsequent mitochondrial content release. (Adapted from Chipuk *et al.,* 2010, Cory and Adams, 2002).

Bcl-xL is a potent negative regulator of apoptosis. It promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria, and prevent Cyt c redistribution from the intermembrane space into the cytosol. Additionally, Bcl-xL has been shown to regulate these events also independently from caspases (Vander Heiden *et al*., 1997). Bcl-xL has been reported to inhibit MOMP by competing with Bax *via* direct and indirect mechanisms. Bcl-xL has been shown to directly bind to Bax by its C-terminal membrane anchor. Moreover, it has been described that Bcl-xL can be translocated from the cytosol to the mitochondria after Bid activation, where it is

capable of sequestering tBid into stable complexes. This prevents activation and further recruitment of Bax to OMM, which in turn suppresses MOMP and subsequent apoptosis at the relatively advanced stage (Billen *et al*., 2008).

1.5 Role of Bcl-xL and HGF in tissue fibrosis

Accumulating evidence suggests an important role of epithelial apoptosis in the development of tissue fibrosis. Current hypothesis states that chronic and deregulated apoptosis of alveolar epithelial cells triggered by repetitive injury may be the primary cause of IPF and the driving force of the disease progression (Jenkins *et al*., 2012). Since Bcl-xL is a potent regulatory protein involved in controlling mitochondrial pathway that can be activated by various stimuli, including oxidative damage and ER stress, it may play an important role in pathogenesis of pulmonary fibrosis.

It has been reported that spontaneous and continuous apoptosis of hepatocytes induced by specific knock-down of Bcl-xL in those cells, triggered liver fibrotic responses *in vivo*. Bcl-xL deficient mice showed increased production of TGF-β1 and collagen deposition. In addition, i*n vitro* exposure of macrophages as well as normal hepatocytes to apoptotic hepatocytes lacking Bcl-xL stimulated TGF-β1 production, resembling the situation during human liver fibrosis/cirrhosis (Baer *et al*., 1998, Takehara *et al*., 2004). Moreover, Zhang *et al*. observed that HGF promotes survival of renal tubular epithelial cells exposed to oxidative stress through increased Bcl-xL expression combined with Bad phosphorylation (Zhang *et al*., 2008). The role of Bcl-xL during development and/or progression of pulmonary fibrosis needs to be yet elucidated. In a recent study it was found that Bcl-xL is the predominant isoform expressed in the lung and the only isoform detected in alveolar epithelial cells. The loss of Bcl-xL in AECII shifted the lung towards a pro-apoptotic state defined by decrease of Mcl-1 and increase of Bak expression, as well as higher sensitivity of the respiratory epithelium to hyperoxia (Staversky *et al*., 2010). These observations suggest that Bcl-xL may be an important factor mediating protection of AECII during oxidative damage. Moreover, studies indicate that HGF signaling may be involved in the regulation of Bcl-2 family expression. In the rat model of ischemia/reperfusion injury, application of exogenous HGF improved survival of myocardiocytes, what was correlated with increased expression of Bcl-xL specifically in the ischemic areas (Nakamura *et al*., 2000).

However, HGF showed a protective effect on lung endothelial cells after oxidative damage, through blocking Bid and Bax translocation to mitochondria and inhibiting Caspase 8 activation (Wang *et al*., 2004).

Taken together, these data implicate that there might be a link between HGF and Bcl-xL, which could potentially lead to the suppression of fibrotic remodeling in the lung and result in improved pulmonary function. Thus, further understanding of the mechanism of apoptosis-induced fibrogenesis appears necessary for development of proper therapeutic options for controlling progression of pulmonary fibrosis and preventing complete organ failure.

2 Aim of the study

It is well established that HGF possesses anti-fibrotic properties. Studies on animal models have provided strong evidence that supplementation of exogenous HGF has a beneficial role in fibrotic disorders in various organs, including the lung. It has been reported that HGF can act *via* multiple direct and indirect mechanisms linked to improved cellular survival and reduced of myofibroblast accumulation. HGF-elicited, pro-survival pathways have yet not been investigated in detail in lung epithelial cells. In the liver and heart, Bcl-xL protein has been suggested to be a part of an important anti-apoptotic mechanism involved in resolution of fibrotic remodeling of these organs, however it remains to be elucidated in the lung. Since the HGF signaling via c-Met receptor has been proposed to regulate the expression of Bcl-2 family members, the present study was performed to evaluate the potential role of Bcl-xL in HGF-mediated epithelial protection in IPF.

In this context, the aim of this study was:

1. to characterize Bcl-xL expression and its cellular localization in lung tissues of IPF patients in comparison to organ donors
2. to assess the Bcl-xL expression pattern in highly remodeled areas in comparison to still normal-appearing regions of IPF lung tissue
3. to investigate whether HGF mediates pro-survival effect on alveolar epithelial cells driven into apoptosis by oxidative stress, ER stress and Fas ligand-triggered activation of cell death receptor
4. to assess the potential role of Bcl-xL in this regard.

3 Materials and methods

3.1 Materials

3.1.1 Reagents

Name	Company
2-(4,2-hydroxyethyl)-piperazinyl-1-ethansulfonate (HEPES)	Sigma Aldrich, Germany
2-amino-2-hydroxymethyl-1,3-propanediol (Tris)	Roth, Germany
2-Mercapto-ethanol	Sigma Aldrich, Germany
Acrylamide solution, Rotiphorese® Gel 30	Roth, Germany
Albumine, Bovine Serum (BSA)	Roth, Germany
Ammonium Persulfate (APS)	Roth, Germany
Bromophenol Blue	Sigma Aldrich, Germany
Citric Acid	Thermo Scientific, USA
c-Met Inhibitor, PHA-665752	Sigma Aldrich, Germany
Cytotoxicity Detection Kit (LDH)	Roche, Germany
DharmaFECT 1	Thermo Scientific, USA
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich, Germany
DMEM-F12 Medium	Gibco, Germany
Dulbecco's Phosphate Buffered Saline (PBS)	PAA, Austria
Ethanol 99,5%	Roth, Germany
Ethylenediamine-tetraacetic Acid (EDTA)	Sigma Aldrich, Germany
Fas Ligand (FasL)	Life Sciences, Germany
Fetal Calf Serum (FCS)	Roth, Germany
Glycergel® Mounting Medium	Dako, Denmark
Glycerole	Roth, Germany
Glycine 99%	Roth, Germany
Hepatocyte Growth Factor	R&D Systems, USA
Hydrobeta-estradiole	Sigma Aldrich, Germany
Hydrochloric Acid (HCl) 32%	Sigma Aldrich, Germany
Hydrocortisone	Sigma Aldrich, Germany
Hydrogen peroxide	Roth, Germany

Insulin, Transferrin, Sodium Selenite (ITS)	PAN Biotech, Germany
iQ™ SYBR® Green Supermix	Bio-Rad, USA
Isoflurane	Baxter, Germany
KH_2PO_4	Merck, Germany
L-Glutamine	Gibco, Germany
Methanol 99,9%	Roth, Germany
N,N,N',N'-tetramethyl-1,2-diaminomethane (TEMED)	Sigma Aldrich, Germany
$Na_2HPO_4x2H_2O$	Merck, Germany
Na-deoxycholate	Merck, Germany
Nucleotide Mix (dNTPs)	Qiagen, Germany
Oligo(dT) Primer	Roche, Germany
PageRuler™ Prestained Protein Ladder	Thermo Scientific, USA
Paraffin, Paraplast Plus®	Sigma Aldrich, Germany
Penicillin/Streptomycin	PAA, Austria
Pierce® BCA Protein Assay Kit	Thermo Scientific, USA
Pierce® ECL Plus Western Blotting Substrate	Thermo Scientific, USA
Potassium Chloride (KCl)	Merck, Germany
Protease Inhibitor Cocktail Complete™	Roche, USA
Restore™ Western Blot Stripping Buffer	Thermo Scientific, USA
RNase Inhibitor	Roche, Germany
Rnase-free Water	Qiagen, Germany
Roti®-Histofix 4%	Roth, Germany
Saccharose	Roth, Germany
siRNA, Scrambled RNA	Thermo Scientific, USA
Skim Milk Powder	Fluka, Germany
Sodium Chloride (NaCl)	Sigma Aldrich, Germany
Sodium Citrate Tribasic Dihydrate	Sigma Aldrich, Germany
Sodium Citrate Tribasic Dihydrate	Sigma Aldrich, Germany
Sodium Dodecyl Sulfate (SDS)	Sigma Aldrich, Germany
Sodium Hydroxide (NaOH)	Sigma Aldrich, Germany
Staurosporine	Calbiochem, Germany
Thapsigargin	Invitrogen, Germany

Triton-X-100	Sigma Aldrich, Germany
Trypan Blue	Sigma Aldrich, Germany
Trypsin/EDTA	PAA, Austria
Tween-20	Sigma Aldrich, Germany
ZytoChem HRP-DAB Kit	ZytoMed, Germany

3.1.2 Equipment

Name	**Company**
Analytical Balance	Mettler Toledo, Switzerland
Cell Culture 6-well Plates	Greiner Bio-One, Germany
Cell culture centrifuge, Universal 30RF	Hettich, Germany
Cell Culture Hood HERAsafe	Hereaus, Germany
Cell Culture Incubator, HERAcell 150i	Thermo Scientific, Germany
Cell Scrapers	Costar, USA
Centrifuge, Mikro 200R	Hettich, Germany
Cooling Plate, EG 1150C	Leica, Germany
Culture Slides	BD Falcon, USA
Dry Block Thermostat	Ditabis, Germany
Electrophoresis Chamber	Bio-Rad, USA
Falcon Roller	CAT, Germany
Falcon tubes	BD Falcon, USA
Freezer +4°	Bosch, Germany
Freezer -20°	Bosch, Germany
Freezer -80°C	Hereaus, Germany
Gel Blotting Paper	GE Healthcare, UK
Glass Slides, Automat Star	Langenbrinck, Germany
Glass slides, SuperFrost Plus	Langenbrinck, Germany
Heating Oven, FunctionLine	Hereaus, Germany
Heating Plate, HI 1220	Leica, Germany
iCycler IQ™ Thermocycler	Bio-Rad, USA
Light Microscope, Axiovert 25	Carl Zeiss, Germany

Magnetic Stirrer	Heidolph, Germany
Microsprayer	Penn-Century Inc, USA
Microtome	
Mirax Scan	Carl Zeiss, Germany
Multipipette	Eppendorf, Germany
NanoDrop	PeqLab, Germany
Neubauer Chamber	Optik Labor, Germany
Nitrile gloves	Ansell, Germany
Paraffin Embedding Module, EG 1140H	Leica, Germany
PCR Thermocycler	Bio-Rad, USA
Petri Dishes	Sarstedt, Germany
Pipette Tips	Biozym, Germany
Pipettes	Eppendorf, Germany
Power Supply, Consort	Roth, Germany
PVDF Transfer Membrane, Hybond™-P	GE Healthcare, UK
Scapels	Feather, Germany
Shaker, Duomax	Heidolph, Germany
SpectraFluor Plus	Tecan, Germany
Spin-down	VWR International, Germany
Syringe Filters 0,20um	Sarstedt, Germany
Syringes	Braun, Germany
Timer	Roth, Germany
Toploader Balance	Mettler Toledo, Switzerland
Trans-Blot® SD	Bio-Rad, USA
Vacuum Driven Bottle Filter, 33mm, 45mm	Millipore, USA
Vacuum-based Tissue Processor, ASP 300S	Leica, Germany
Vortex Machine	VWR International, Germany
Water Bath	Julabo, Germany

3.2 Methods

3.2.1 RNA isolation

Total RNA was isolated from cells using RNeasy® Plus Mini Kit (QIAGEN, Germany) according to manufacturer's instructions. In short, cells suspended in lysis buffer containing 1% 2-mercaptoethanol, were passed through a shredding column for lysis and homogenization. Ethanol was then added to promote binding of RNA to the membrane of the RNeasy spin column. During the next steps, contaminants were washed away and high-quality RNA was eluted with RNase-free water. Purity and concentration of RNA were measured based on its absorbance at 260 nm and 280 nm with a NanoDrop® spectrophotometer. If not used immediately for experiments, RNA samples were stored at -80°C.

3.2.2 Reverse transcription reaction

Reverse transcription (RT) was performed to obtain high yields of full length cDNA with RNA as a starting template. cDNA was synthesized from previously isolated RNA using Omniscript® Reverse Transcription Kit. 2 µg of total RNA was added to RNase-free water up to a volume of 14 µl and gently mixed. Then 6 µl of reaction mixture was added, each sample vortexed, spinned down and left standing at room temperature for 10 min for annealing to occur. Next, tubes were transferred to a heating block and kept for 65 min at 37°C. The newly obtained cDNA was used for further experiments, otherwise stored at -20°C.

RT reaction component	Volume	Final concentration
10xRT Buffer	2 µl	1x
5mM dNTP mix	2 µl	0,5 mM
50uM Oligo d(T) primers	0,5 µl	1,25 µM
RNase inhibitor (20U/ul)	0,5 µl	0,5 U
Omniscript™ RT (4U/ul)	1 µl	2 U
RNase-free water	up to total volume of 20 µl	-

3.2.3 Real-time polymerase chain reaction

Real-time PCR (qPCR) is an enzymatic method used for both qualitative and quantitative analysis of nucleic acid. The accumulation of amplified product is measured as the reaction progresses, after each cycle. This is achieved by the inclusion of a fluorescent reporter dye into amplicons in each reaction, which leads to increased fluorescence signal with increased amount of DNA product. Amplification of cDNA was performed according to the manufacturer's instructions provided with iQ™ SYBR® Green Supermix (Bio-Rad) that contains hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers and fluorescein. The qPCR reaction mix was prepared as follows:

qPCR reaction component	Volume	Final concentration
iQ™ SYBR® Green Supermix	10 µl	1x
Forward primer * (10pmol/ul)	0,5 µl	0,25 nM
Reverse primer * (10pmol/ul)	0,5 µl	0,25 nM
Water	7 µl	-
cDNA template (5ng/ul)	2 µl	10 ng per reaction

* Primer sequences:
Bcl-xL (NM_009743.4) forward primer 5' TGTCTGGTCACTTCCGACTG 3', reverse primer 5' GCTGGGACACTTTTGTGGAT 3'
β-actin (NM_007393.3) forward primer 5' CTACAGCTTCACCACCACAG 3', reverse primer 5' CTCGTTGCCAATAGTGATGAC 3'

Reaction components were mixed on ice in a total volume of 20 µl and transferred to a thermocycler. PCR was performed using a iCycler IQ™ Single Color Real-Time PCR Detection System (Bio-Rad) monitoring by an iQ™ 5 Optical System Software. Programmed steps are listed below:

Step		Time	Temperature
Polymerase activation and DNA denaturation		3 min	95°C
Amplification 35 cycles	Denaturation	15 sec	95°C
	Annealing/Elongation	30 sec	59°C
Melt curve analysis		10 sec/step	55-95°C 0,5°C increment

Before levels of nucleic acid targets could be quantified, the raw data was analyzed and baseline and threshold values set. The background component of the signal was removed by the software algorithm. The threshold was adjusted to a value above the background and below the plateau of the amplification curve, and threshold cycle (Ct) values were obtained. To normalize data for the amount of template used, qPCR on the identical template, but with primers specific for the endogenous reference gene (house-keeping gene: beta-actin) was performed simultaneously.

ΔCt = Ct (endogenous reference gene) – Ct (target gene)

The specificity of products was determined by their melting curves to get the highest specific amplification and the lowest background amplification.

3.2.4 Protein isolation

3.2.4.1 Protein isolation from cultured cells

Cells were lysed with protein extraction buffer containing protease inhibitor. Lysates were incubated on ice for 30 min and then centrifuged for 10 min at 13000xg at 4°C. Supernatants were transferred to new tubes and stored at -80°C.

Protein extraction buffer	**Final concentration**
Tris	50 mM
NaCl	150 mM
EDTA	5 mM
Triton-X-100	1%
Na-deoxycholate	0,5%
Protease inhibitor cocktail Complete™	4%

3.2.4.2 Human samples and patient data analysis

Tissue samples were obtained with the written consent of the patients. The study was approved by the local research ethics committee of Justus-Liebig-University School of Medicine (No. 31/93, 84/93, 29/01), provision of patients biospecimen was approved by University of Vienna Hospital ethics committee (EK-No. 076/2009) and European IPF Registry (No. 111/08, "eurIPFreg"). HGF expression in lung homogenates was studied in two groups of subjects: 18 patients with IPF (mean age 50,2 ± 12,2 years; 3 females, 15 males) and 10 control patients (mean age 45,4 ± 12,7 years; 3 females, 5 males, data not available for 2 control patients). Bcl-xL expression in lung homogenates was studied in two groups of subjects: 20 patients with IPF (mean age 50,9 ± 11,9 years; 3 females, 17 males) and 10 control patients (mean age 45,4 ± 12,7 years; 3 females, 5 males, data not available for 2 control patients). BAL fluid analysis was performed on 11 patients with IPF (mean age 63,8 ± 12,8 years; 3 females, 8 males) and 11 control subjects (mean age 36,9 ± 19,9 years; 6 females, 4 males). Diagnosis of sporadic IPF was made according to ATS/ERS International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias and a usual interstitial pneumonia (UIP) pattern was proven in all 20 explanted lungs. Non-utilized control lungs or lobes from donors fulfilled transplantation criteria.

3.2.4.3 Protein isolation from lung tissue

Lung tissues used for analysis were acquired from IPF patients after lung transplantation. Samples taken from peripheral regions of the lung were collected, shock-frozen in liquid nitrogen and stored at -80°C. As controls, donor lung tissues not utilized because of size incompatibility were used. About 70 mg of tissue was taken for protein isolation, put into micro packaging vials containing 1,4 mm and 2,8 mm zirconium oxide beads and protein extraction buffer with protease inhibitor. Tissues were homogenized at high speed in Precellys® (2 cycles of 20 sec at the speed of 5500 rpm) according to manufacturer's instructions. Samples were put on ice for 30 min and subsequently centrifuged for 10 min at 13000xg at 4°C to pellet the debris. Supernatants were transferred to new tubes and stored for further analysis at -80°C.

3.2.5 Protein quantification

Protein concentration was determined spectrophotometrically using Pierce® BCA Protein Assay Kit according to manufacturer's instructions. The assay is used for colorimetric detection and quantification of total protein compared to a protein standard. In the first step the biuret reaction takes place, where copper is chelated with protein in an alkaline environment, forming light blue complexes. In the second step the bicinchoninic acid (BCA) reacts with reduced cuprous ions, resulting in intense purple-colored reaction product which exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The absorbance was measured by ELISA-plate reader (SpectraFluor Plus, Tecan). BSA in ten different concentrations ranging from 1,5 mg/ml to 7,8 µg/ml was used as a standard.

3.2.6 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate extracted proteins according to their sizes in an electric field. 50 µg of protein was mixed with loading buffer containing 2-mercaptoehtanol (added freshly) and denaturated in heating block at 95°C for 10 min.

Loading buffer (4x)	**Concentration**
SDS	5% (w/v)
Tris/HCl, pH 6,8	156 mM
Glycerol	40% (v/v)
Bromophenol blue	0,01% (w/v)
2-mercaptoethanol	5% (v/v)
Water	-

Samples were shortly vortexed before loading into gel pockets. Depending on the size of the target protein, 8%, 12% or 15% gels were used.

Separating gel	Concentration
Acrylamide/Bisacrylamide (30%/0,8%)	8% / 12% / 15%
Tris/HCl, pH 8,8	375 mM
SDS	0,1% (w/v)
TEMED	0,1% (v/v)
APS	0,05% (w/v)
Water	-

Stacking gel	Concentration
Acrylamide/Bisacrylamide (30%/0,8%)	4%
Tris/HCl, pH 6,8	125 mM
SDS	0,1% (w/v)
TEMED	0,1% (v/v)
APS	0,1% (w/v)
Water	-

Electrophoresis was carried out in SDS-running buffer at 85V until the bands of the Prestained Protein Ladder were properly separated.

SDS-running buffer	Concentration
Tris	25 mM
Glycine	192 mM
SDS	0,1% (w/v)
Water	-

3.2.7 Immunoblotting

Immunoblotting is an analytical technique using antibodies to detect and identify target proteins among a number of unrelated protein species in a sample. Identification is based on a antigen-antibody specific interactions, which is further visualized using secondary antibodies labeled with enzymes or radioisotopes.

3.2.7.1 Protein blotting

Proteins resolved by SDS-PAGE were transferred onto a 0,45 µm polyvinylidene fluoride (PVDF) membrane in semi-dry blotting chamber. The membrane was activated before use in pure methanol. Transfer was performed in transfer buffer at 1mA/1cm^2 for 90 min.

Transfer buffer	**Concentration**
Tris	20 mM
Glycine	159 mM
Methanol	20% (v/v)
Water	-

3.2.7.2 Protein detection

To minimize background, membranes were blocked in 5% Skim Milk in wash buffer (TBS-T) for 1h 20 min at room temperature. Subsequently, they were transferred into falcon tubes with appropriate primary antibodies (see Appendix) dissolved in 5% Skim Milk and incubated overnight at 4°C.

After washing 3 times for 10 min in TBS-T buffer, membranes were incubated with horseradish peroxidase-labeled secondary antibodies for 50 min at room temperature and again washed 3 times for 10 min. Specific bands were visualized by chemiluminescence using a Pierce ECL Plus Western Blotting Substrate and a ChemoCam detection system. For re-probing, membranes were stripped in Restore™ Western Blot Stripping Buffer for 20 min at room temperature and subsequent protein detection was performed as described above.

TBS-T buffer, pH 7,5	**Concentration**
Tris	50 mM
NaCl	50 mM
Tween-20	0,1% (v/v)
Water	-

3.2.7.3 Densitometry

To quantify the amount of target protein in each sample, the intensity of bands was measured using AlphaEaseFC™ software. Beta-actin was used as loading control.

3.2.7.4 Coomassie Brilliant Blue staining

Coomassie Brilliant Blue permanently stains proteins bound to PVDF membrane with a detection limit of about 1,5 µg of protein. In order to obtain loading control for Western blot analysis of BAL fluid samples, coomassie staining was performed after detection of primary target. Membranes were briefly washed with distilled water and subsequently put into the staining solution (composition described below) for few seconds.

Staining solution	**Concentration**
Methanol	50% (v/v)
Acetic acid	5% (v/v)
Coomassie Brilliant Blue R-250	0,25% (v/v)
Water	-

Stained membranes were washed multiple times in destaining solution. When the background colour was effectively removed, membranes were rinsed with water and scanned.

Destaining solution	**Concentration**
Isopropanol	28% (v/v)
Acetic acid	5% (v/v)
Water	-

3.2.8 Immunohistochemistry

Immunohistochemistry (IHC) was performed to detect and localize the expression of specific antigens in lung tissue. Small pieces (~2 cm x 2 cm) of lung tissue from patients with IPF and donors were fixed with phosphate-buffered formaldehyde solution

(Roti®-Histofix 4%, pH 7,0) overnight at 4°C. Afterwards the tissue blocks were transferred into an embedding cassette and stored in phosphate-buffered saline (PBS) at 4°C.

PBS x10, pH 7,4	**Concentration**
NaCl	1,37 M
KCl	26,8 mM
$Na_2HPO_4x2H_2O$	64,6 mM
KH_2PO_4	14,7 mM
Water	-

Subsequently, dehydration in a vacuum-based tissue processor (ASP 300S, Leica) was performed overnight and tissue blocks were embedded in warmed to 65°C paraffin with use of Leica embedding module (EG 1140H, Leica). After cooling on a cooling plate (EG 150C, Leica), serial lung tissue sections (thickness of 3 μm) were cut with fully-automated rotation microtome and mounted on positively-charged glass slides. Finally, slides were dried on a pre-warmed heating plate (HI 1220, Leica), then incubated for 8-12h at 37°C in a heating oven and stored at room temperature. Directly before IHC procedure, slides were heated-up to 60°C in heating oven (FunctionLine, Hereaus) and subsequently deparaffinized in xylene for 10 min. Then they were rehydrated in descending ethanol concentrations (99,6% > 96% > 80% > 70% > 50%), each inembation step for 3 min. Slides were washed in PBS and permeabilized by boiling in citrate buffer, three times for 10 min.

Citrate buffer, pH 6,0	**Concentration**
Sodium citrate tribasic dihydrate	10 mM
Citric acid	109 mM
Water	-

After washing in PBS, further steps were performed with the use of ZytoChem HRP-DAB Kit according to manufacturer's instructions. Briefly, sections were first blocked in blocking solution for 5 min, then incubated with primary antibodies (see Appendix) overnight at 4°C. Following washing in PBS, sections were incubated for 15 min with

biotinylated secondary antibody provided with the kit. Streptavidin HRP-conjugate was put on sections for 15 min and DAB dissolved in substrate buffer was used as a substrate for HRP. Sections were stained in parallel. Counterstaining was performed with haemalaun for 35 sec, followed by washing the slides in running tap water. Such prepared sections were mounted with Glycergel® Mounting Medium and left to air-dry. Stained tissue slides were digitalized with the use of a Mirax slide scanning device (Zeiss) and analyzed with the Mirax Viewer software.

3.2.10 *In vitro* experiments

3.2.10.1 Cell culture condition

The mouse lung epithelial cell lines (MLE-12 and MLE-15) were obtained from ATCC, Manassas, USA. The rat lung epithelial cell line (RLE-6TN) was a kind gift of Dr. Istvan Vadász, Department of Internal Medicine, Justus Liebig University, Giessen, Germany.

All cells were grown on 10 cm Petri dishes in full medium based on growth medium DMEM-F12, at 37°C, 5% CO_2 and 95-100% humidity.

DMEM-F12 Full medium	Concentration
Hydrocortisone	10 nM
Hydrobeta estradiole	10 nM
ITS	5% (v/v)
HEPES	10 mM
L-Glutamine	2 mM
FCS	2%
Penicillin/streptomycin	1%

After reaching a confluence of 80-90%, cells were passaged to a new Petri dish. At first, cells were washed with PBS and incubated for 2-3 min with 3 ml of Trypsin/EDTA solution. The action of trypsin was stopped by adding 15 ml of medium containing FCS and cells were collected to a 50 ml falcon tube and centrifuged at 1100xg for 7 min. Next, the supernatant was removed and cells were resuspended in 10 ml of full medium, counted and plated on new culture dishes.

For the purpose of in vitro studies, cells were seeded on 6- or 12-well plates and kept in full medium for 24h to enable proper attachment of the cells to the plate. Stimulation with hepatocyte growth factor, as well as with cell death inducers (hydrogen peroxide, thapsigargin, Fas ligand) was performed in serum-free medium supplemented with 5% ITS.

3.2.10.2 Transfection with small interfering RNA

Transfection with small interfering RNA (siRNA) allows to specifically inhibit protein expression through selective silencing of gene expression by delivery of double-stranded RNA molecules into the cell. The siGENOME SMARTpool siRNA oligonucleotides specific for murine Bcl-xL mRNA were obtained from Thermo Scientific. Mouse lung epithelial cells (MLE-12) were transfected with 25 nM Bcl-xL-targeting siRNA or ON-TARGETplus non-targeting siRNA #1 as a negative control.

siGENOME mouse Bcl-xL	**Target sequence**
D-065142-01	UUAGUGAUGUCGAAGAGAA
D-065142-02	UGAGUCGGAUUGCAAGUUG
D-065142-03	GGAGAGCGUUCAGUGAUCU
D-065142-04	UGGAAAGCGUAGACAAGGA

Before transfection, 40000 cells were plated into each well of a 6-well plate and incubated overnight. Transfection procedure was performed according to manufacturer's instructions. Briefly, DharmaFECT1 and siRNAs were dissolved in antibiotic- and serum-free medium in separate tubes and incubated at RT. After 5 min both reagents were mixed and incubated for another 20 min. Subsequently, the siRNA mix was put onto the cells in serum-containing medium. After 24h, 48h and 72h cells were lysed and the gene knock-down level was assessed by the use of real-time PCR and Western blotting.

3.2.10.3 Cytotoxicity assay

Cytotoxicity Detection Kit (Roche) contains a colorimetric assay for the quantification of cell death and cell lysis, based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the cell-culture medium. The relative LDH release is directly proportional to the cell death level in an experimental setting. LDH assay was performed according to manufacturer's instructions. Experiments were performed on 12-well plates, each well of 1 ml volume. 500 µl of cell supernatant was transferred into a new tube containing 500 µl of fresh full medium and spinned down at 1000xg for 5 min. Cells left on wells were treated for 5 min with 500 µl of 2% Triton-X-100 in full medium. Subsequently, the reaction mix from the kit was added and the absorbance at 490 nm measured by an ELISA-plate reader (SpectraFluor Plus, Tecan). The raw data was analyzed according to the formula stated below and presented as a % of relative LDH release into the medium.

$$\frac{\text{Absorbance}_{\text{Medium}} - \text{Absorbance}_{\text{Blank}}}{\text{Absorbance}_{\text{Medium+Triton-x100}} - \text{Absorbance}_{\text{Blank}}} \text{x100\%} = \text{LDH relative release}$$

For each sample 5 biological replicates were used and 2 independent measurements have been performed.

3.2.12 Statistical analysis

Data are expressed as the mean value ± SEM. Statistical comparisons between two groups were done using unpaired Student t-tests. One-way ANOVA in combination with a Tukey post-hoc test was performed to compare differences between multiple groups. Probability value of $p<0,05$ was considered to be statistically significant.

4 Results

4.1 Analysis of human lung samples

4.1.1 Expression of Bcl-xL in lung homogenates and BALFs from fibrotic and healthy lungs

To investigate potential changes in the amount of Bcl-xL in human lungs during IPF, protein expression in homogenates and BALFs obtained from IPF patients and donors/healthy controls was examined. Significant increase in Bcl-xL level was observed in IPF homogenates compared to donor lungs (Figure 4.1 A). No detectable amount of Bcl-xL was found in lavage fluid samples for either IPF or controls containing 5 µg of total protein (Figure 4.1 B). MLE12 cell lysate has been used as a positive control.

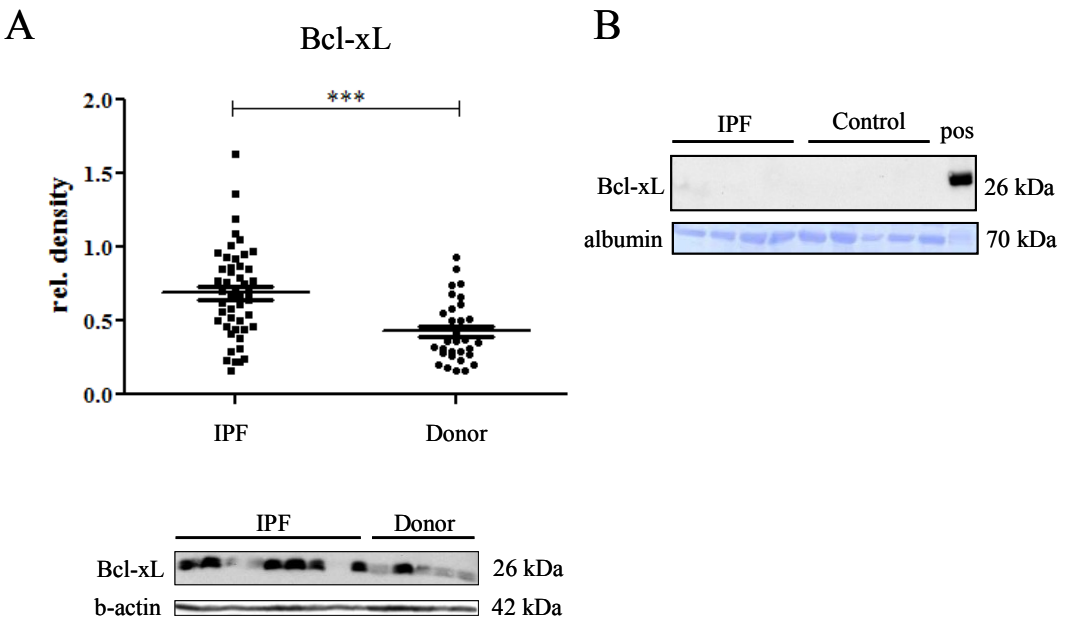

Figure 4.1: Expression of Bcl-xL in lung samples from IPF patients and healthy subjects.
(**A**) Western blot analysis of lung homogenates showing significantly increased levels of Bcl-xL in IPF patients compared to donors. IPFs n=20, donors n=11, N=3. (**B**) Western blot analysis of BAL fluids. IPFs n=11, controls n=11, N=2, pos: positive control, MLE-12 total cell lysate. *** p=<0,001.

4.1.2 Localization of Bcl-xL in lungs of IPF patients and organ donors

Since we observed an increased expression of Bcl-xL protein in total lung homogenates from IPF patients in comparison to healthy donors, immunohistochemical staining was performed on lung tissue obtained from organ donors and IPF patients in order to determine the Bcl-xL expression pattern (Figure 4.2).

In human IPF lung, Bcl-xL protein was highly expressed in hyperplastic alveolar type II cells found in the regions of dense fibrosis. It could also be detected in bronchial epithelial cells as indicated by co-staining with Cytokeratin 5 and cc-10, molecular markers for basal calls and Clara cells respectively. In donor lungs, Bcl-xL was similarly localized in bronchial and alveolar epithelial type II cells, however, the signal in control sections was much weaker when compared to fibrotic lungs, especially in type II cells, which is consistent with our data on the Bcl-xL protein content in lung homogenates (Figure 4.1 A, B).

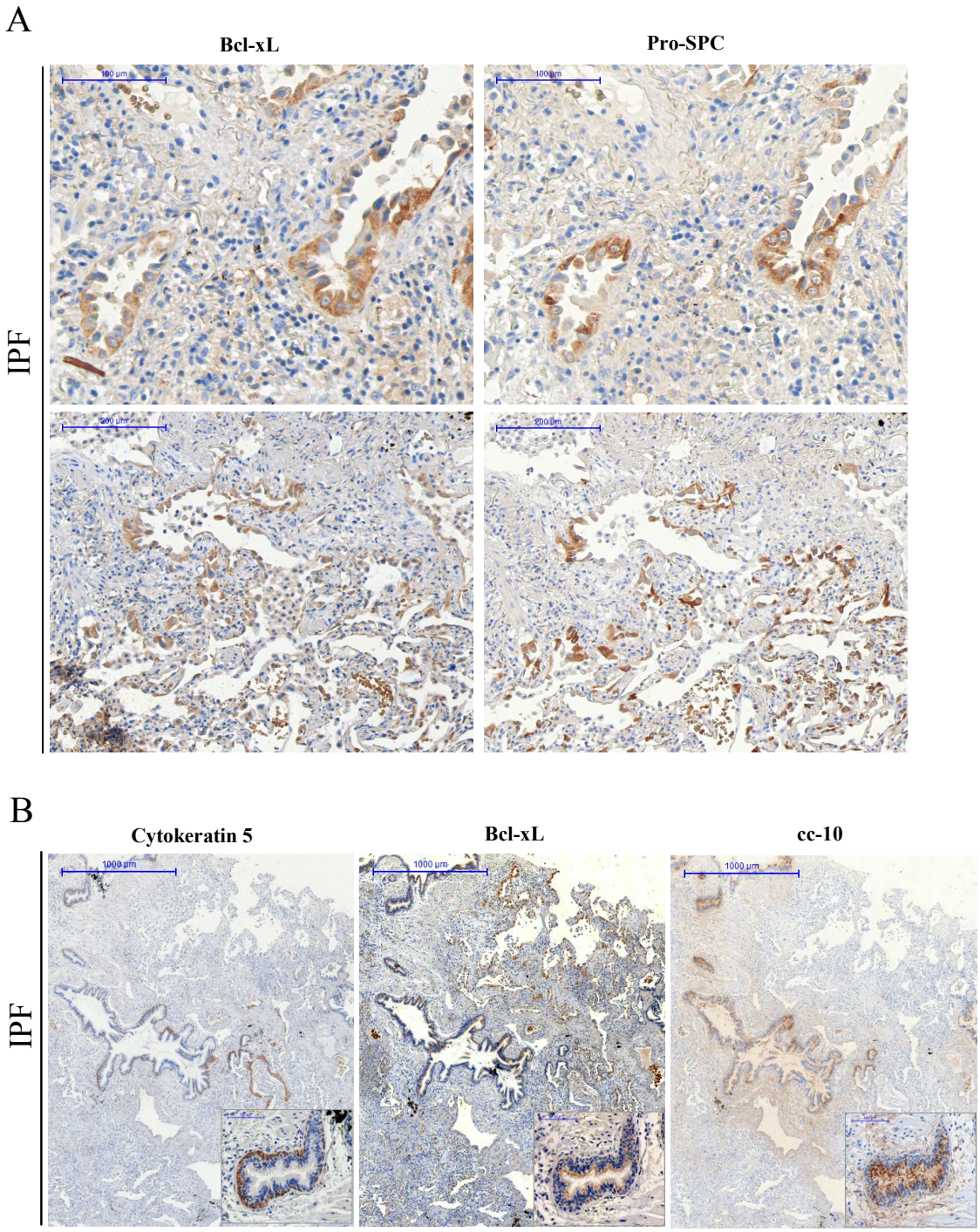

Figure 4.2: Localization of Bcl-xL in lungs of IPF patients and organ donors.
Paraffin-embedded lung section from IPF patients (**A, B**) and lung donors (**C, D**) were stained for Bcl-xL, Pro-SPC, cytokeratin 5 and cc-10, as indicated. Cell type markers were used as follows: pro-SPC for alveolar type II cell, cytokeratin 5 for basal cell, cc-10 for Clara cell. (**F**) Negative control. The pictures are representative of at least four IPF/donor subjects. Scale bars represent 100 µm and (B) 1000 µm at lower magnification.

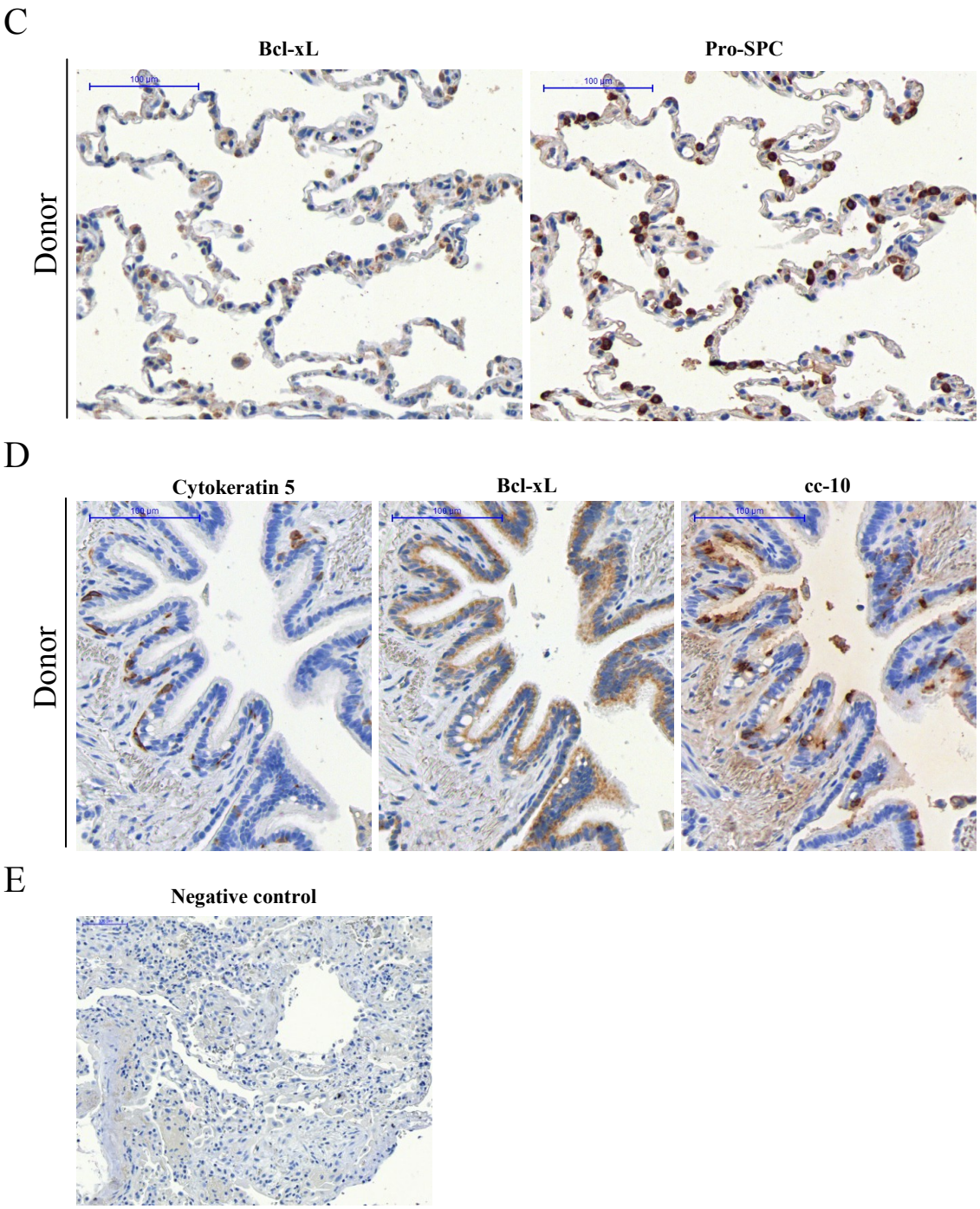

Figure 4.2: Localization of Bcl-xL in lungs of IPF patients and organ donors.
Paraffin-embedded lung section from IPF patients (**A, B**) and organ donors (**C, D**) were stained for Bcl-xL, Pro-SPC, cytokeratin 5 and cc-10, as indicated. Cell type markers were used as follows: pro-SPC for alveolar type II cell, cytokeratin 5 for basal cell, cc-10 for Clara cell. (**E**) Negative control. The pictures are representative of at least four IPF/donor subjects. Scale bars represent 100 µm and (B) 1000 µm at lower magnification.

4.1.3 Expression of Bcl-xL in fibrotic and non-fibrotic areas of IPF lungs

The histopathological UIP pattern is characterized by spatial heterogeneity with regions of normal lung architecture alternating with patchy areas of overt regions of fibrosis. Due to the fact that IPF is a slowly progressing, chronic disorder, we hypothesize that normal looking areas of a fibrotic lung may represent an early stage of the disease. Thus, we were interested in Bcl-xL expression pattern, especially its presence or absence in alveolar epithelial type II cells in remodeled *versus* not remodeled regions of the lung. To achieve that, immunohistochemical staining was performed on sections showing both, normal lung structure with adjacent areas of fibrosis (Figure 4.3).
In agreement with previous results, Bcl-xL was strongly expressed in hyperplastic epithelial type II cells of fibrotic areas. In contrast, staining for Bcl-xL in type II cells in still regular imposing areas of the IPF lung appeared to be much weaker and resembled the expression pattern observed for donor lung (Figure 4.2 and 4.3) rather than IPF.

4.1.4 Co-localization of Bcl-xL and c-Met in lungs of IPF patients

Since interdependence between Bcl-xL and HGF-mediated signaling pathway in alveolar epithelial type II cells was one of the major questions of this study, we were interested in co-localization of Bcl-xL and HGF receptor, c-Met. Therefore, we performed immunohistological staining for Bcl-xL and c-Met on serial sections obtained from IPF patients (Figure 4.4).
We observed that c-Met expression co-localized with Bcl-xL in the same bronchial and alveolar epithelial cell populations. Additionally, c-Met receptor was expressed by myofibroblasts present in fibrotic remodeled areas as assessed by α-smooth muscle actin co-staining (data not shown).

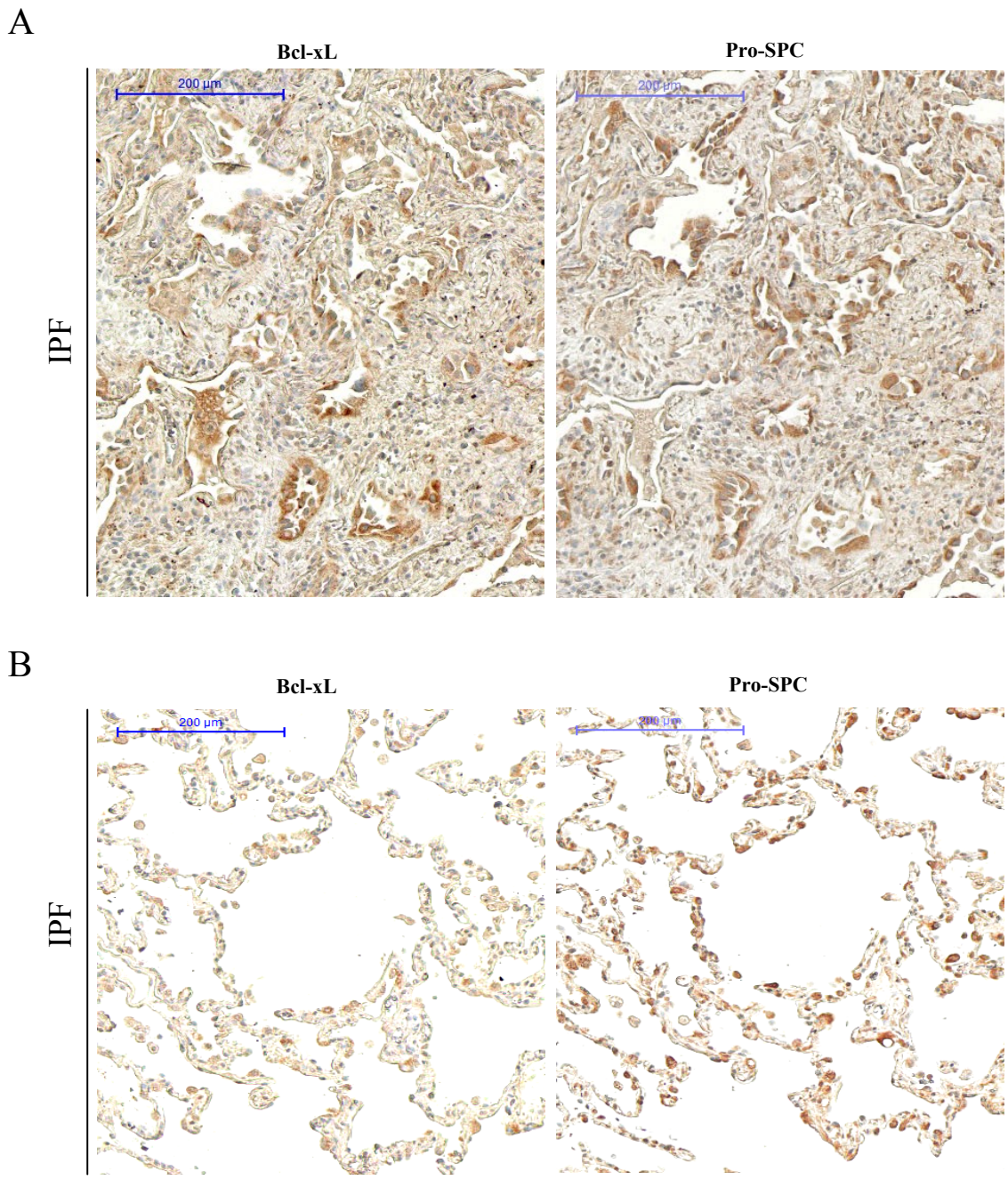

Figure 4.3: Expression of Bcl-xL in fibrotic and non-fibrotic areas of lungs of IPF patients.
Immunohistochemical staining of paraffin-embedded lung sections from IPF patients. Expression of Bcl-xL in alveolar type II cells (pro-SPC positive) in (**A**) fibrotic area representing end stage of disease *versus* (**B**), still regular apearing lung regions, potentially representing early stage of IPF. Pictures represent regions of the same section. Experiment was performed on samples from four different IPF patients. Scale bars represent 200 µm.

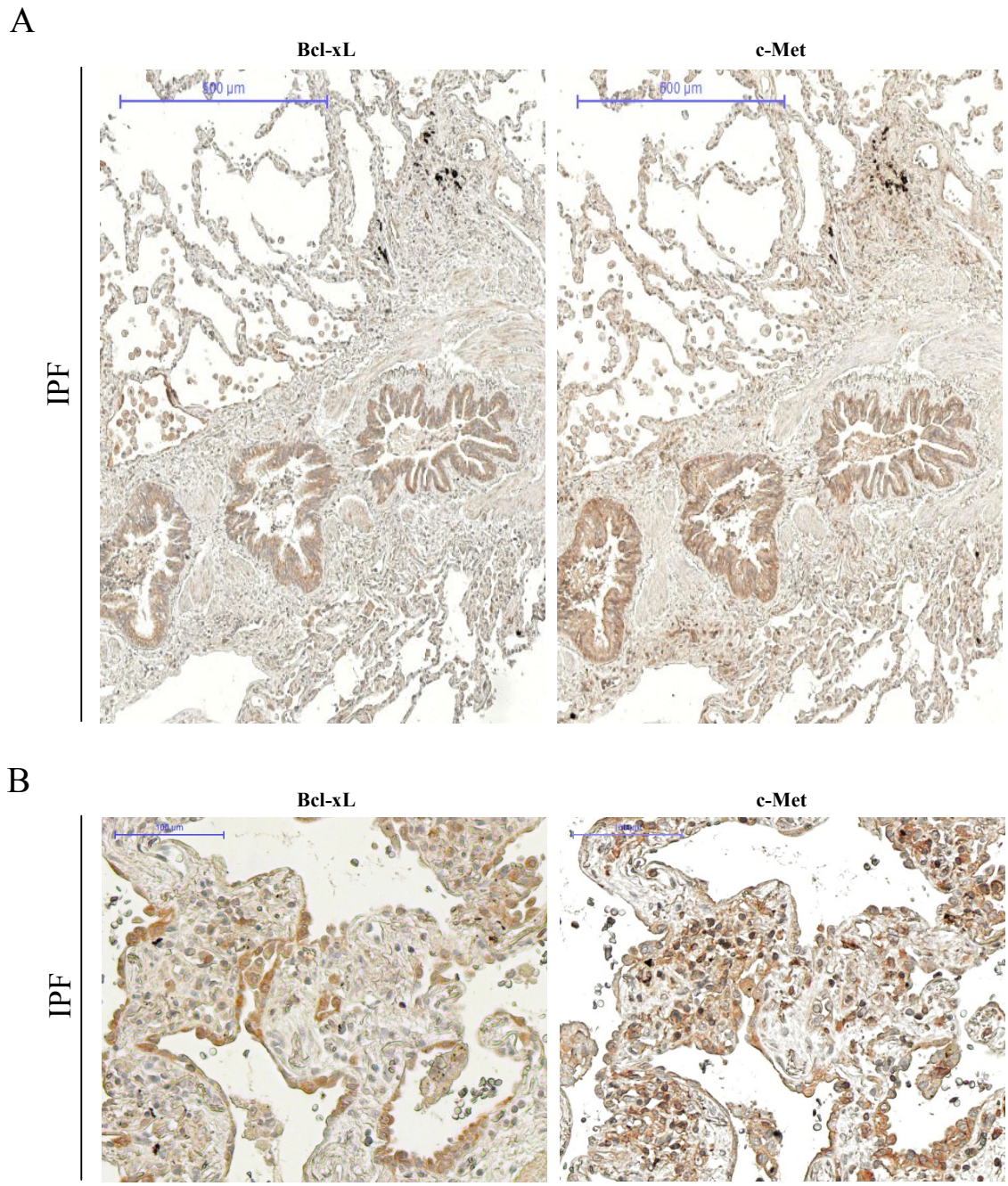

Figure 4.4: Co-localization of Bcl-xL and c-Met in lungs of IPF patients.
Representative staining results of paraffin-embedded lung sections from IPF patients showing co-localization of specific HGF receptor – c-Met and Bcl-xL in (**A**) bronchial and (**B**) alveolar epithelial cells. The pictures are representative of five IPF patients. Scale bars represent (A) 600 µm and (B) 100 µm.

4.1.5 Levels of HGF in BALFs and homogenates obtained from IPF and donor lungs

As immunohistochemical results showed co-localization of Bcl-xL and c-Met receptor, further studies were performed to assess the amount of HGF in lung homogenate and in BALF.

In a healthy lung HGF is produced in biologically inactive pro-form (~100 kDa) and stored as such within the extracellular matrix. It is then proteolytically activated specifically at sites of injury through several proteases. The active heterodimer consists of an α and a β subunit (69 kDa and 34 kDa respectively), and is recognized by all cells expressing the c-Met receptor. The anti-HGF antibody used for Western blot analysis recognized the cleaved (activated) and non-cleaved form of HGF. We observed, that in lung tissue of IPF subjects, the level of activated HGF is decreased when compared to donors. There was no statistically significant difference with regard to the expression of the non-cleaved form of HGF (Figure 4.5 A).

As expected, non-cleaved HGF could not be detected in BALFs, as it is bound and stored in extracellular matrix. No significant changes in active HGF levels were observed in BALFs from IPF subjects in comparison to healthy donors (Figure 4.5 B).

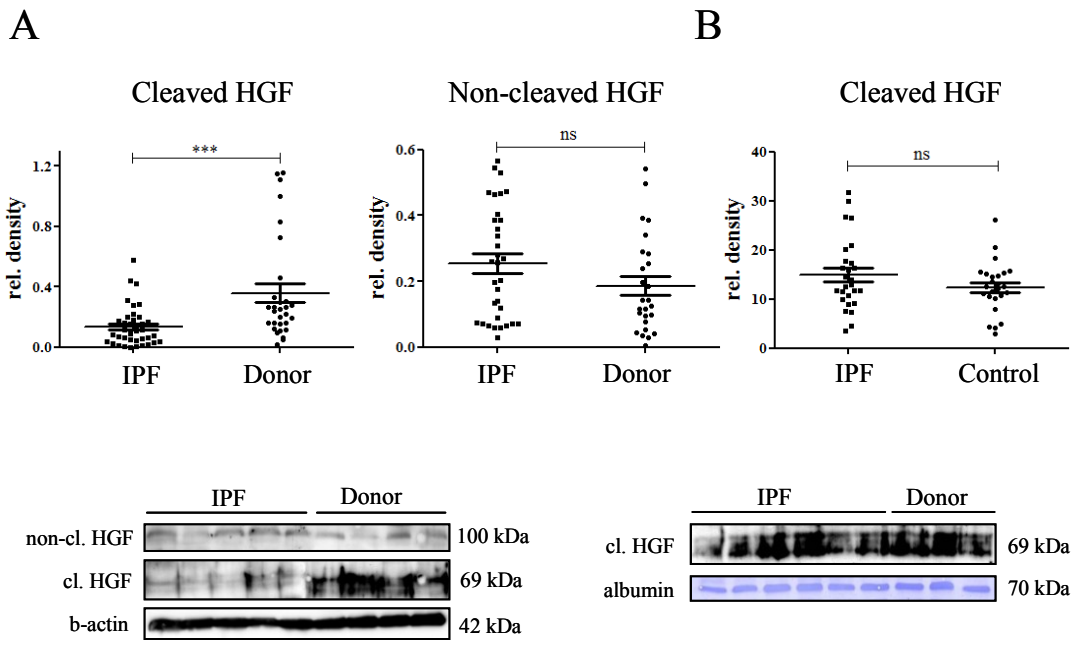

Figure 4.5: HGF levels in lung homogenates and BALFs from IPF patients and organ donors. Western blot analysis of HGF expression in (**A**) lung homogenates and (**B**) BALFs from patients and organ donors. Beta-actin and albumin (coomassie stained membrane) were used as loading controls in case of lung homogenates and BALFs respectively. (A) IPFs n=18, donors n=10, (B) IPFs n=11, donors n=11. Relative density and original blots are representative of two technical replicates. *** p=<0,001.

4.2 Role of Bcl-xL in HGF-mediated epithelial protection against oxidative stress

HGF is a pleiotropic cytokine playing a role in a wide spectrum of biological processes. It has been shown to activate a number of anti-apoptotic signaling pathways, such as ERK/MAPK, PI3K/Akt or STAT3, thus improving survival of some cell types. To test whether HGF has a pro-survival effect on alveolar epithelial type II cells, we performed a set of *in vitro* experiments using the lung epithelial cell lines, MLE-12, MLE-15 and RLE-6TN. Since it has been believed that repetitive and chronic micro-injuries affecting lung epithelial cells play an important role in the development and conceivably the progression of pulmonary fibrosis, we used three pro-apoptotic stimuli that have been reported to play a role in IPF, namely the oxidative stress inducer, hydrogen peroxide, ER-stress inducer, thapsigargin (Chapter 4.3) and Fas ligand (Chapter 4.4). Moreover, having observed that the Bcl-xL levels are significantly changed in IPF, we further investigated the role of Bcl-xL in HGF-mediated cytoprotection against above mentioned cell death inducers.

4.2.1 Loss of Bcl-xL expression caused by oxidative stress-induced cell death

Subconfluent cells were incubated for 24h with hydrogen peroxide in a concentration range of 200-2000 μM to provide a gradual increase of oxidative stress in the medium. Subsequently, LDH assay and Western blot analysis were performed to assess the level of cell death in each treatment variant. We observed, that increased apoptosis and total cell death level corresponded with decreased Bcl-xL expression in both, MLE-12 and MLE-15 cells (Figure 4.6). The highest Caspase 3 activation was detected at 600-1000 μM hydrogen peroxide concentration.

4.2.2 Pro-survival activity of HGF on cells treated with hydrogen peroxide

Epithelial cells were grown until reaching about 80% of confluence and then incubated for 24h with 650 μM hydrogen peroxide simultaneously with or without HGF under serum-free conditions. To validate an optimal dose, mouse recombinant HGF was used at two different concentrations: 50 ng/ml and 100 ng/ml, based on the literature. Non-treated cells served as a control. Consistent with the previous reports, we observed that HGF treatment significantly decreased Caspase 3 activation induced by H_2O_2 (Figure 4.7 A, C). Moreover, anti-apoptotic effect of HGF correlated with significant increase of Bcl-xL expression in cell lysates when compared to cells treated only with hydrogen peroxide (Figure 4.7 B, D). Both concentrations of HGF used in the study, led to a very similar readout, thus we decided to use the 50 ng/ml dose in further studies with thapsigargin and Fas ligand.

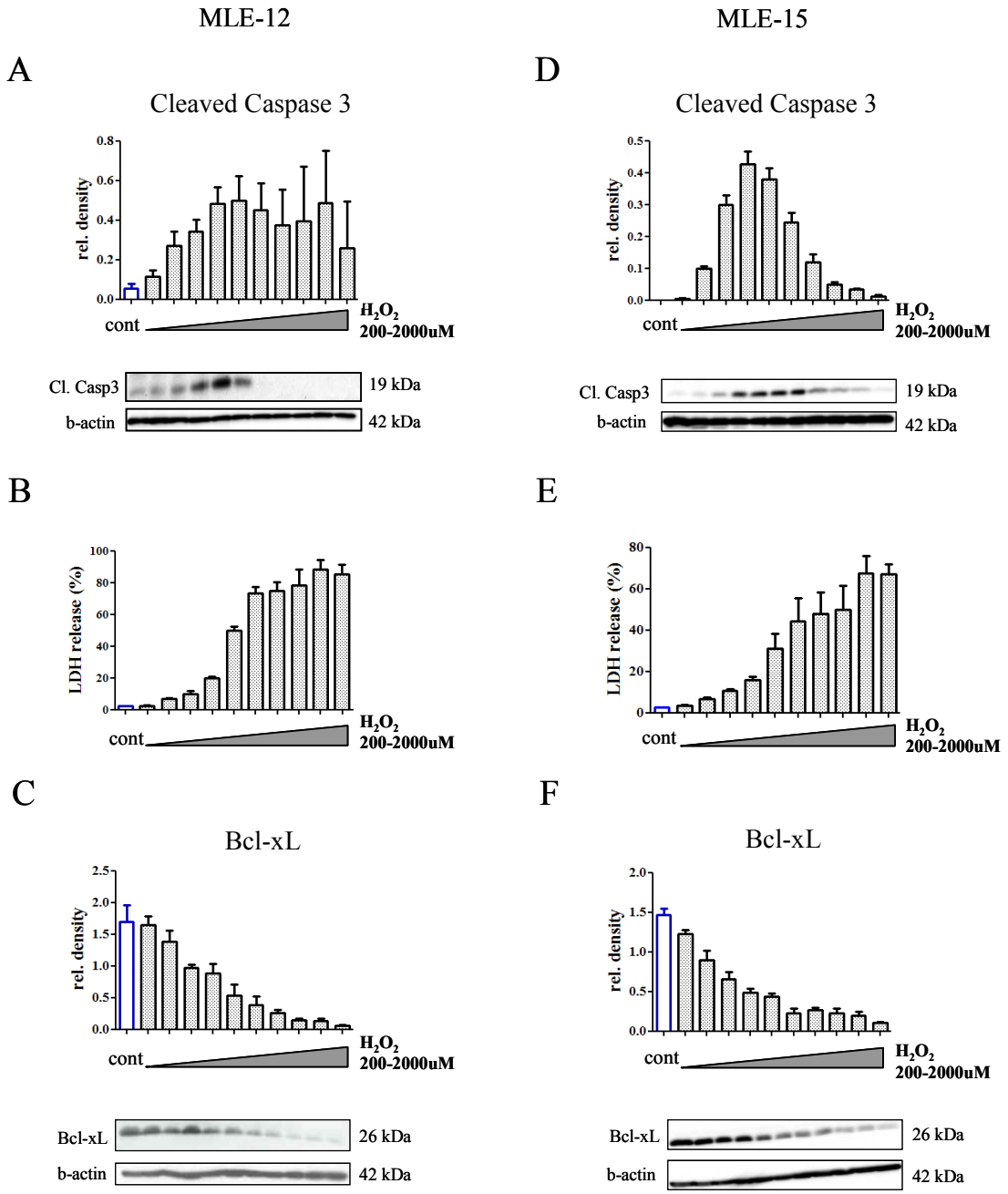

Figure 4.6: Loss of Bcl-xL expression caused by hydrogen peroxide-induced cell death.
Confluent MLE-12 (**A**, **B, C**) and MLE-15 (**D, E, F**) cells were treated with various H_2O_2 concentrations, for 24h. (**A, D**) Western blot analysis showed induction of apoptosis via activation of Caspase 3 with increasing H_2O_2 concentrations up to 1000 µM H_2O_2, **(B, D)** Constant increase of cell death detected by LDH activity assay after H_2O_2 treatment **(C, F)** The higher level of total cell death, the lower the expression of Bcl-xL protein in cell lysates. Values are represented as mean ± SEM, N=2.

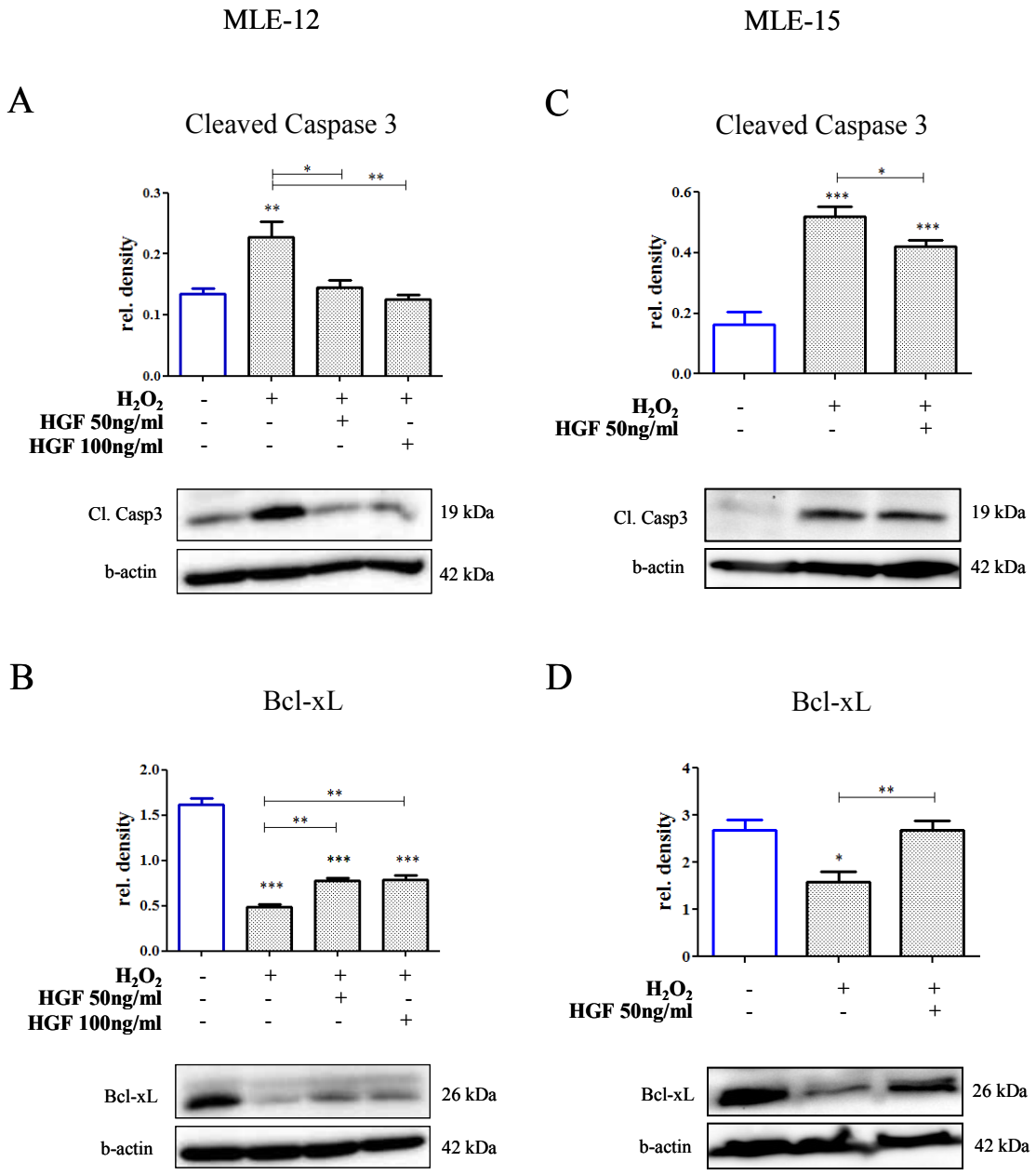

Figure 4.7: Effect of HGF on epithelial cells during oxidative stress-induced apoptosis.
Confluent MLE-12 (**A**, **B**) and MLE-15 (**C**, **D**) cells were treated with H_2O_2 and HGF simultaneously, for 24h under serum-free conditions. (**A**, **C**) Western blot analysis shows activation of Caspase 3 upon hydrogen peroxide and/or HGF treatment, N=3 (**B**, **D**) Bcl-xL expression in cell lysates, N=3. * p=<0,05, ** p=<0,01*** p=<0,001 *versus* control if not indicated otherwise.

4.2.3 Effect of c-Met inhibitor on HGF prosurvival activity

To confirm cell-protective effect of HGF on lung epithelial cells and to validate its specific signaling through c-Met receptor, we performed *in vitro* experiments with the use of PHA-66572 on rat epithelial (RLE-6TN) cells. RLE-6TN cells were used due to the lack of a good antibody against mouse c-Met receptor and availability of the antibody recognizing rat protein.

4.2.3.1 Dependency of c-Met inhibitor dose on phosphorylation of the receptor

To investigate the applicability of PHA-66572 as an effective c-Met signaling inhibitor, we examined the effect of different concentrations of this inhibitor on receptor phosphorylation. RLE-6TN cells confluent to 50-60% were treated for 24h with PHA-66572 at dose range of 0,02-2 μM. Subsequently, the cells were incubated with HGF (50 ng/ml) for 10 min and lysed for total protein isolation. Western blot analysis was performed to assess c-Met phosphorylation status at the tyrosine residues Y1234/Y1235, since they are involved in c-Met-mediated signal transduction (Figure 4.8). We observed a decrease in c-Met phosphorylation already at the lowest PHA-66572 concentration (0,02 μM), but complete inhibition of this process occurred at 0,1 μM or higher dose.

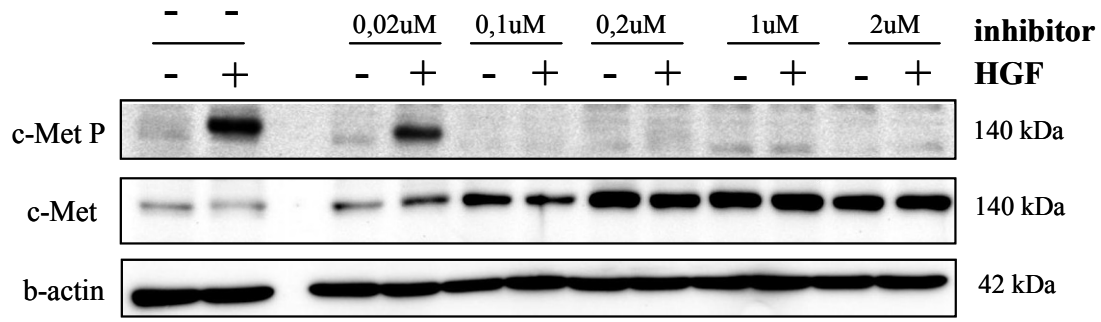

Figure 4.8: Dependency of PHA-66572 dose on c-Met phosphorylation.
RLE-6TN cells were treated with c-Met inhibitor at concentrations indicated on the graph. After 24h they were incubated with HGF of 50 ng/ml for 10 min. Western blot analysis shows phosphorylated c-Met (c-Met P) and total c-Met amount in cell lysate.

4.2.3.2 Increased Bcl-xL expression correlates with HGF-prosurvival activity

Prior to H_2O_2 treatment, RLE-6TN cells were incubated for 24h with PHA-66572 under serum-free conditions. Subsequently, the cells were treated for 48h with HGF at the dose of 20 ng/ml (HGF added freshly every 24h) in medium with or without a c-Met inhibitor. Control cells were kept in serum-free medium for the same time. Afterwards, 800 μM hydrogen peroxide in medium with or without inhibitor, but not containing HGF, was introduced to the cells. After 24h, LDH assay and Western blot analysis were performed to assess cell death level and Bcl-xL expression, respectively (Figure 4.9 A, B). We observed that treatment with HGF significantly reduced the level of cell death induced by hydrogen peroxide in RLE-6TN cells. As expected, incubation with PHA-66527 effectively abolished HGF prosurvival activity. Inhibitor treatment itself did not affect cell viability (Figure 4.9 A). Western blot analysis showed decreased level of Bcl-xL in control cells incubated with inhibitor in comparison to inhibitor-free conditions. The rescued cells treated with HGF showed higher Bcl-xL expression in comparison to the non-treated cells (Figure 4.9 B), again indicating that HGF-mediated pro-survival signaling may occur via altered Bcl-xL expression. Cell morphology was examined using phase contrast light microscopy. We observed that after H_2O_2 treatment with absence of HGF, the cells died extensively, while upon HGF treatment the condition of the cells was improved with markedly more cells attached to the plate (Figure 4.9 C).

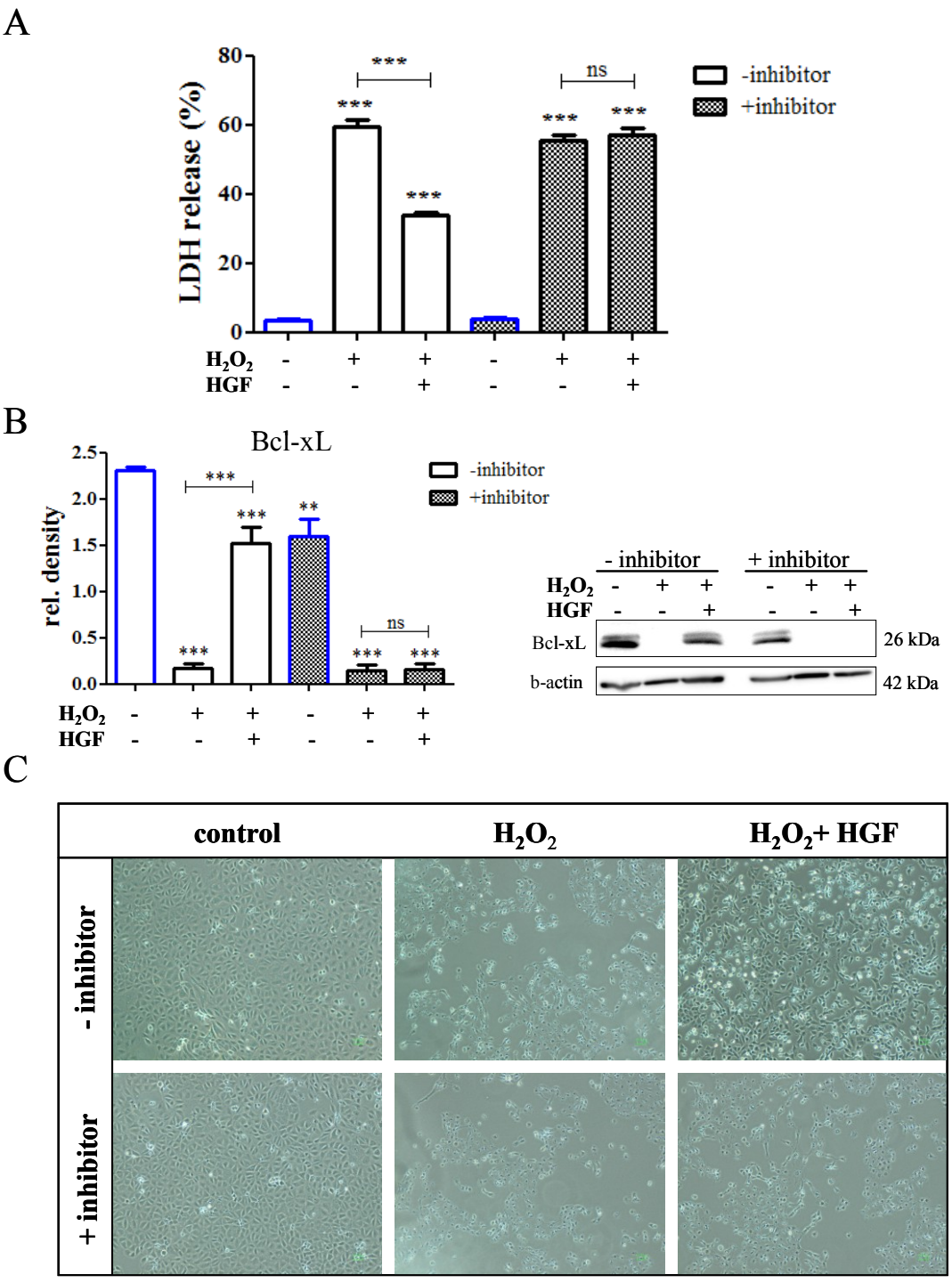

Figure 4.9: Increased Bcl-xL expression correlates with HGF prosurvival activity on cells incubated treated with H2O2.
RLE-6TN cells were treated with 800 µM hydrogen peroxide with or without HGF pre-treatment, in the presence or absence of c-Met inhibitor (as indicated on each graph). (**A**) LDH assay-assessed cell death quantification shows abolition of HGF prosurvival activity in the presence of PHA-66572, N=3, n=5 (**B**) Western blot analysis of Bcl-xL expression, N=3. ** p=<0,05, *** p=<p=0,001 *versus* control if not indicated otherwise. (**C**) Cell morphology. Pictures are taken under the same magnification (x20) and are representative of three independent experiments.

4.3 Role of Bcl-xL in HGF-mediated epithelial protection against ER-stress

4.3.1 Loss of Bcl-xL expression caused by ER-stress-induced apoptosis

To further investigate changes in Bcl-xL expression during pro-apoptotic stimulation, we treated confluent cells with another stress inducer - thapsigargin. Already at low concentrations, thapsigargin is a potent inhibitor of both endoplasmic and sarcoplasmic reticulum and Ca^{2+}-ATPases in various cell types. It has been described to cause severe ER stress leading to cell death.

MLE-12 and MLE-15 cells were incubated with increasing concentrations of thapsigargin for 24h and subsequently total protein was isolated and analyzed by Western blot. Both cell lines responded in the similar way to the treatment. We observed a strong induction of apoptosis via activation of Caspase 3 which correlated with decreased expression of Bcl-xL (Figure 4.10).

4.3.2 Prosurvival activity of HGF on cells treated with thapsigargin

MLE-12 and MLE-15 cells were treated for 24h with 5 nM thapsigargin and with or without HGF (50 ng/ml) in serum-free medium. HGF prosurvival effect was confirmed by Western blot analysis, which showed a decrease in Caspase 3 activation (Figure 4.11 A) after HGF treatment under ER stress conditions. In agreement with what we observed for oxidative stress-induced cell death, we observed that MLE-12 cells that were rescued from apoptosis by HGF showed increased expression of Bcl-xL in comparison to cells incubated only with thapsigargin (Figure 4.11 B). Interestingly, we did not observe prosurvival HGF activity in MLE-15 cells upon the same experimental settings. We could detect a slight increase of Bcl-xL expression in HGF-treated cells, however it was not a statistically significant trend (Figure 4.11 C, D).

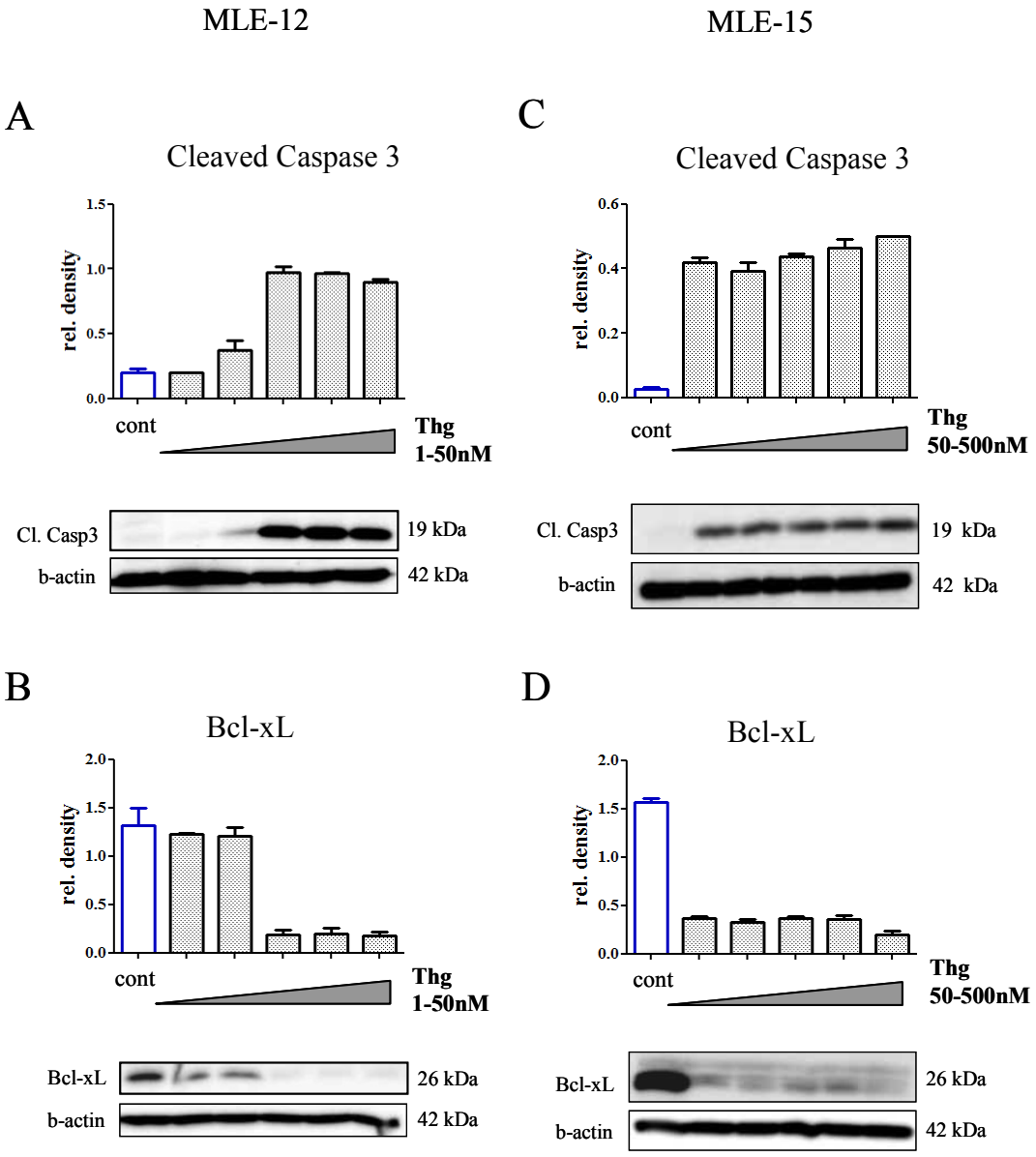

Figure 4.10: Loss of Bcl-xL expression during apoptosis induced by thapsigargin treatment. Confluent MLE-12 (**A**, **B**) and MLE-15 (**C**, **D**) cells were treated with different thapsigargin (Thg) concentrations, as indicated, for 24h. (**A**, **C**) Western blot analysis activation of Caspase 3 with increasing thapsigargin concentrations, N=2. (**B**, **D**) Bcl-xL protein level in cell lysates, N=2.

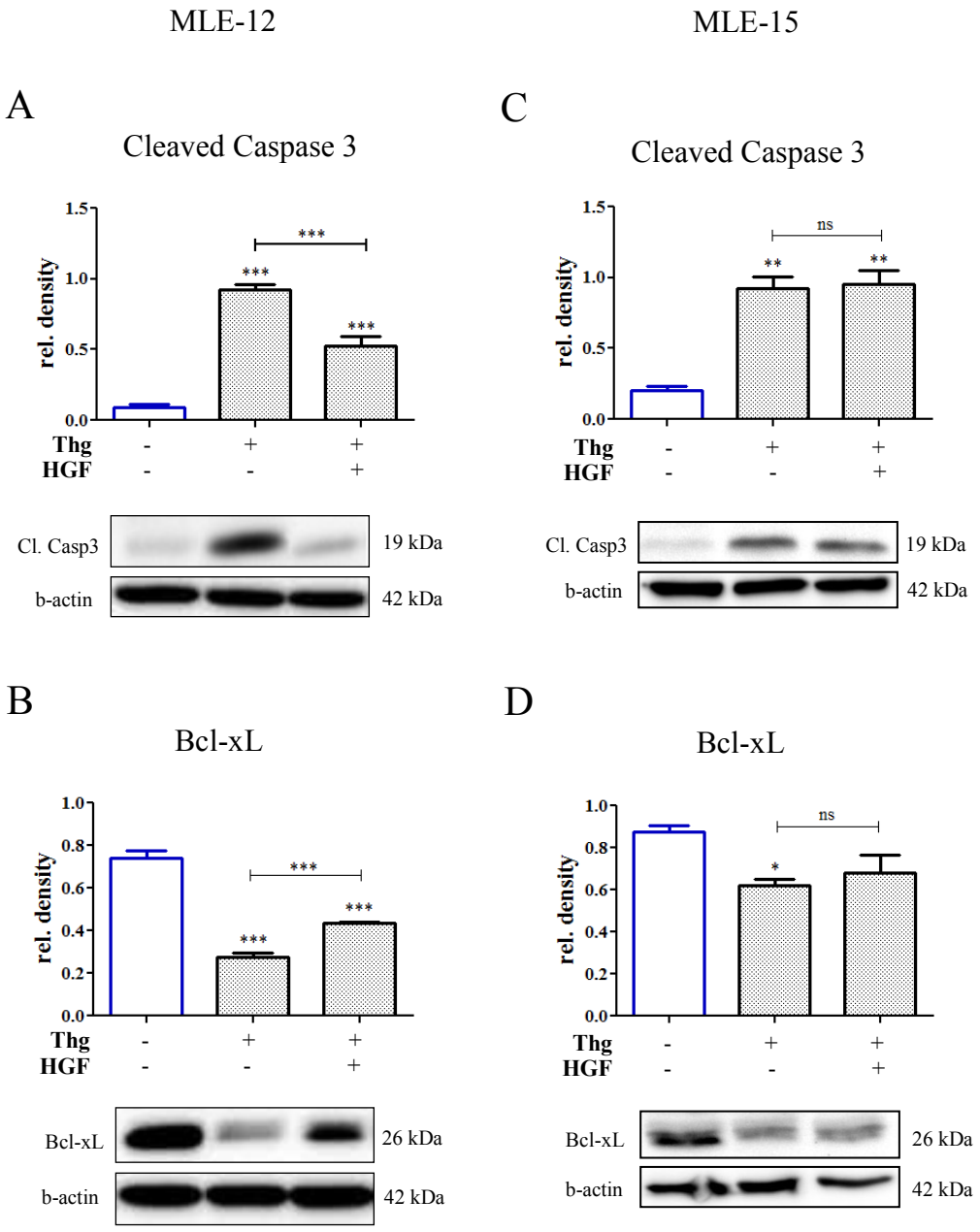

Figure 4.11: Prosurvival activity of HGF on cells treated with thapsigargin.
Confluent MLE-12 (**A**, **B**) and MLE-15 (**C**, **D**) cells were treated with thapsigargin and HGF simultaneously, for 24h under serum-free conditions. (**A**, **C**) Western blot analysis shows Caspase 3 cleavage upon thapsigargin and/or HGF treatment, N=3, (**B**, **D**) Bcl-xL expression in cell lysates, N=3. * p=<0,05, ** p=<0,01*** p=<0,001 *versus* control if not indicated otherwise.

4.3.3 Elevated level of Bcl-xL correlates with pro-survival activity of HGF

RLE-6TN cells were incubated with 0,25 μM of the c-Met inhibitor PHA-66572 for 24 h, prior to 48h long HGF (20 ng/ml) pre-treatment and subsequent 4 μM thapsigargin treatment for 24h. In agreement with previous results, we detected a reduction of cell death upon HGF treatment only in cells not incubated with c-Met inhibitor (Figure 4.12 A). We did not observe the influence of c-Met inhibition on viability of the cells not stimulated with thapsigargin, however, the stimulated cells treated with PHA-66572 seemed to be more sensitive to ER stress than control cells (Figure 4.12 A). Western blot analysis determined that increase of Bcl-xL expression was correlated with improved survival of epithelial cells and was only observed in HGF-treated cells with absence of inhibitor (Figure 4.12 B). The morphology of the RLE-6TN cells under control conditions remained unaltered after incubation with PHA-66572. Upon stimulation with thapsigargin cells in both variants (with or without inhibitor) died extensively and the majority detached from the culture plate. In contrast, HGF treatment resulted in markedly higher amount of surviving cells attached to Petri dish, which was not observed upon c-Met inhibitor pre-incubation (Figure 4.12 C).

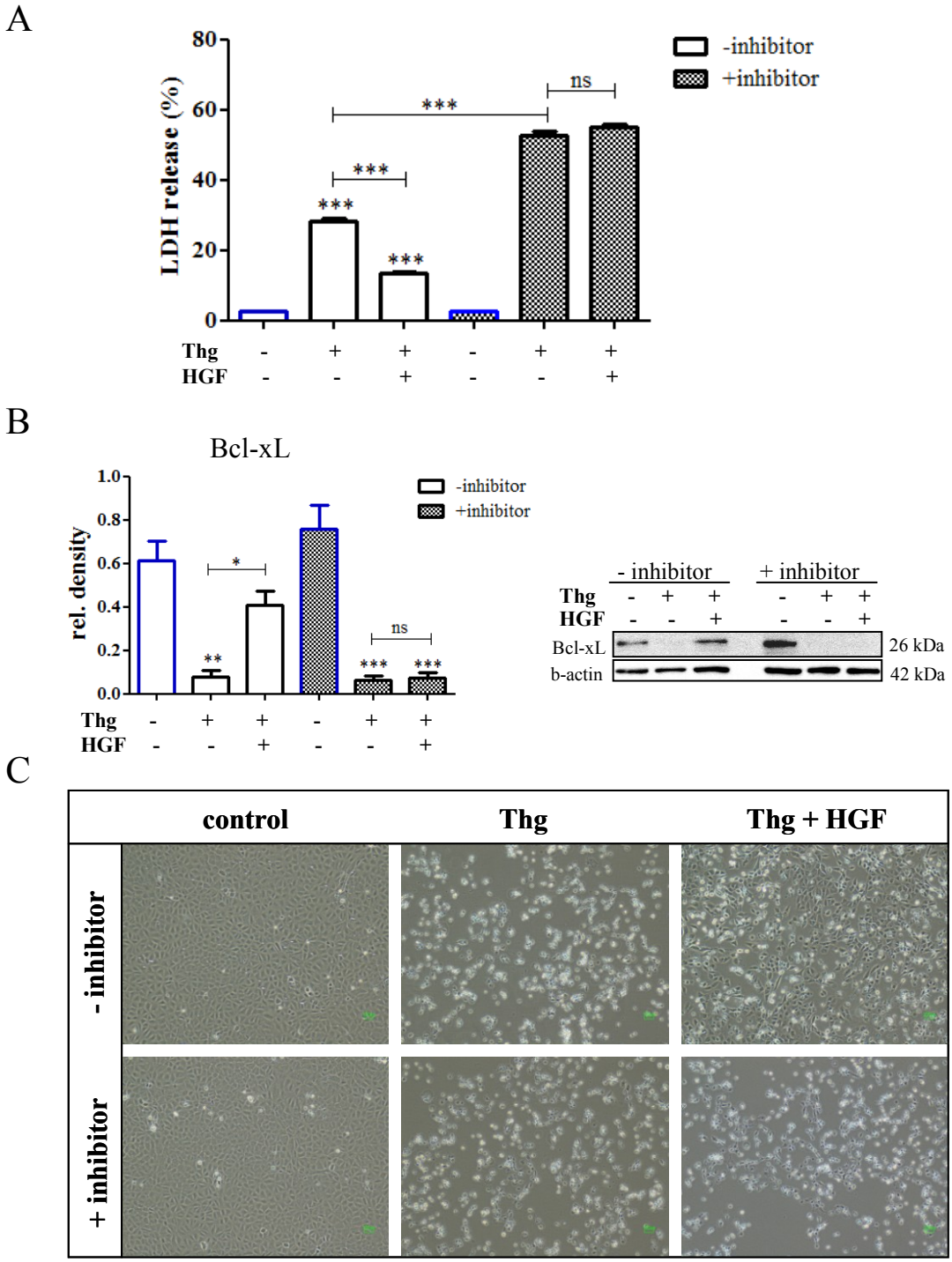

Figure 4.12: Elevated level of Bcl-xL correlates with pro-survival activity of HGF.
RLE-6TN cells were treated with thapsigargin with or without HGF pre-treatment, in the presence or absence of c-Met inhibitor (as indicated on each graph). (**A**) LDH assay shows cell death level under different cell culture conditions, N=3 (**B**) Western blot analysis of Bcl-xL expression in cell lysates, N=2, n=4. Values are represented as mean ±SEM. * p=<0,05, *** p=<0,001 *versus* control if not indicated otherwise. (**C**) Cell morphology. Pictures are taken under the same magnification (x20) and are representative of three independent experiments.

4.4 Expression level of Bcl-xL upon Fas ligand treatment

4.4.1 No effect of FasL-induced apoptosis on Bcl-xL expression level

Fas ligand belongs to tumor necrosis factor family. Its binding to cell death surface receptor Fas results in apoptotic cell death mediated by caspase activation. In order to induce apoptosis, subconfluent MLE-12 and MLE-15 cells were incubated different concentrations of FasL for 24h. We observed that in FasL dose of 50 ng/ml or higher effectively induced Caspase 3 cleavage (Figure 4.13 A, C). However, in contrast to our previous results, no changes in Bcl-xL expression were observed in response to apoptotic response mediated by FasL treatment (Figure 4.13 B, D).

4.4.2 No protective effect of HGF on cells treated with FasL

MLE-12 and MLE-15 cells were treated simultaneously with 50 ng/ml FasL in absence or presence of HGF at dose of 50 ng/ml, as indicated on the graphs. As previously, FasL treatment resulted in Caspase 3 activation with no concomitant alteration in Bcl-xL level. Under these conditions, HGF treatment did not result either in reduction of apoptosis or up-regulation of Bcl-xL expression (Figure 4.14).

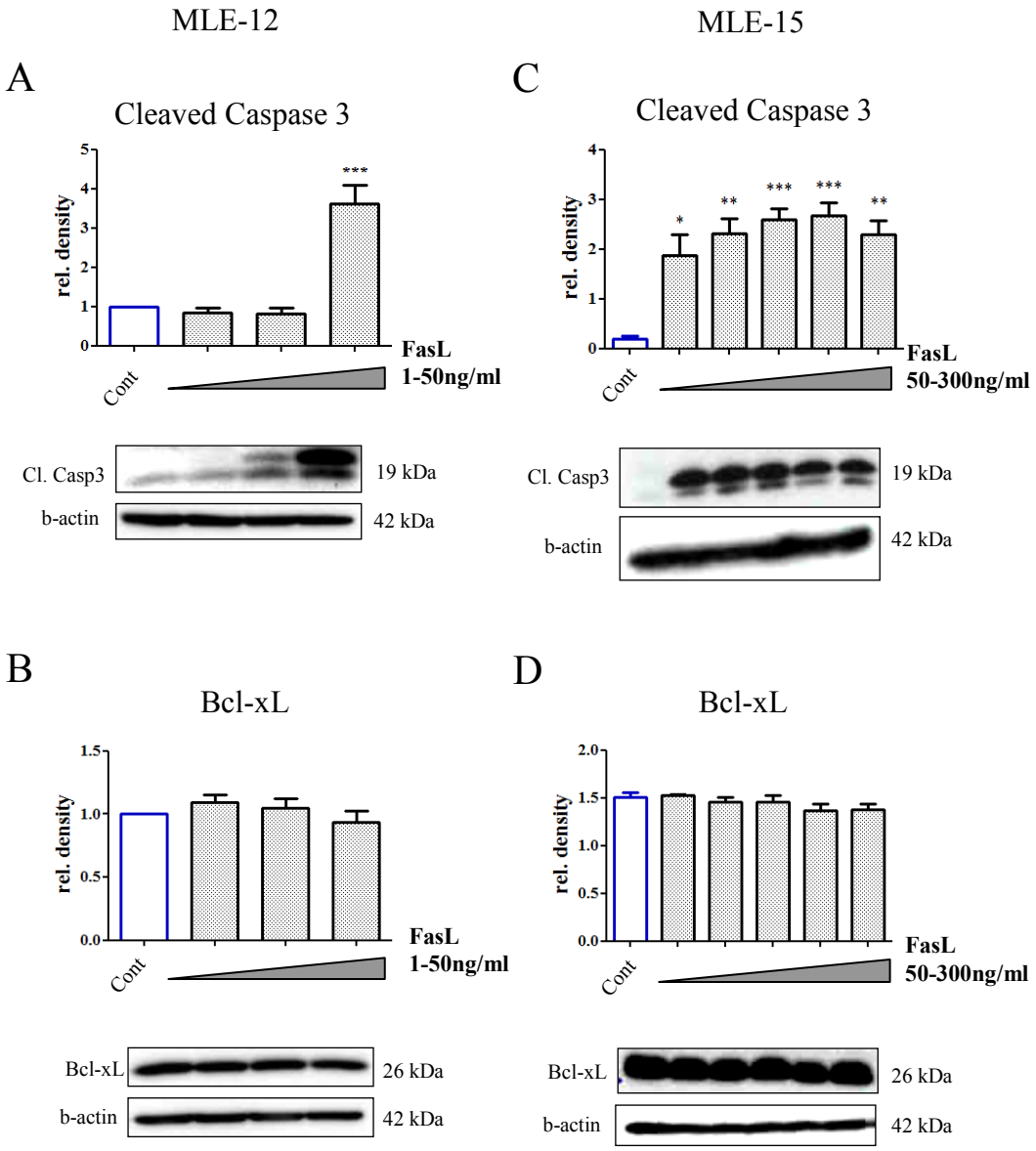

Figure 4.13: No effect of FasL-induced apoptosis on Bcl-xL expression level.
Confluent MLE-12 (**A**, **B**) and MLE-15 (**C**, **D**) cells were treated for 24 h with different concentrations of FasL, as indicated on the graphs. (**A**, **C**) Western blot analysis shows activation of Caspase 3 after FasL treatment, N=3, (**B**, **D**) Bcl-xL protein level in cell lysates, N=3. ** p=<0,01; *** p=<0,001 *versus* control.

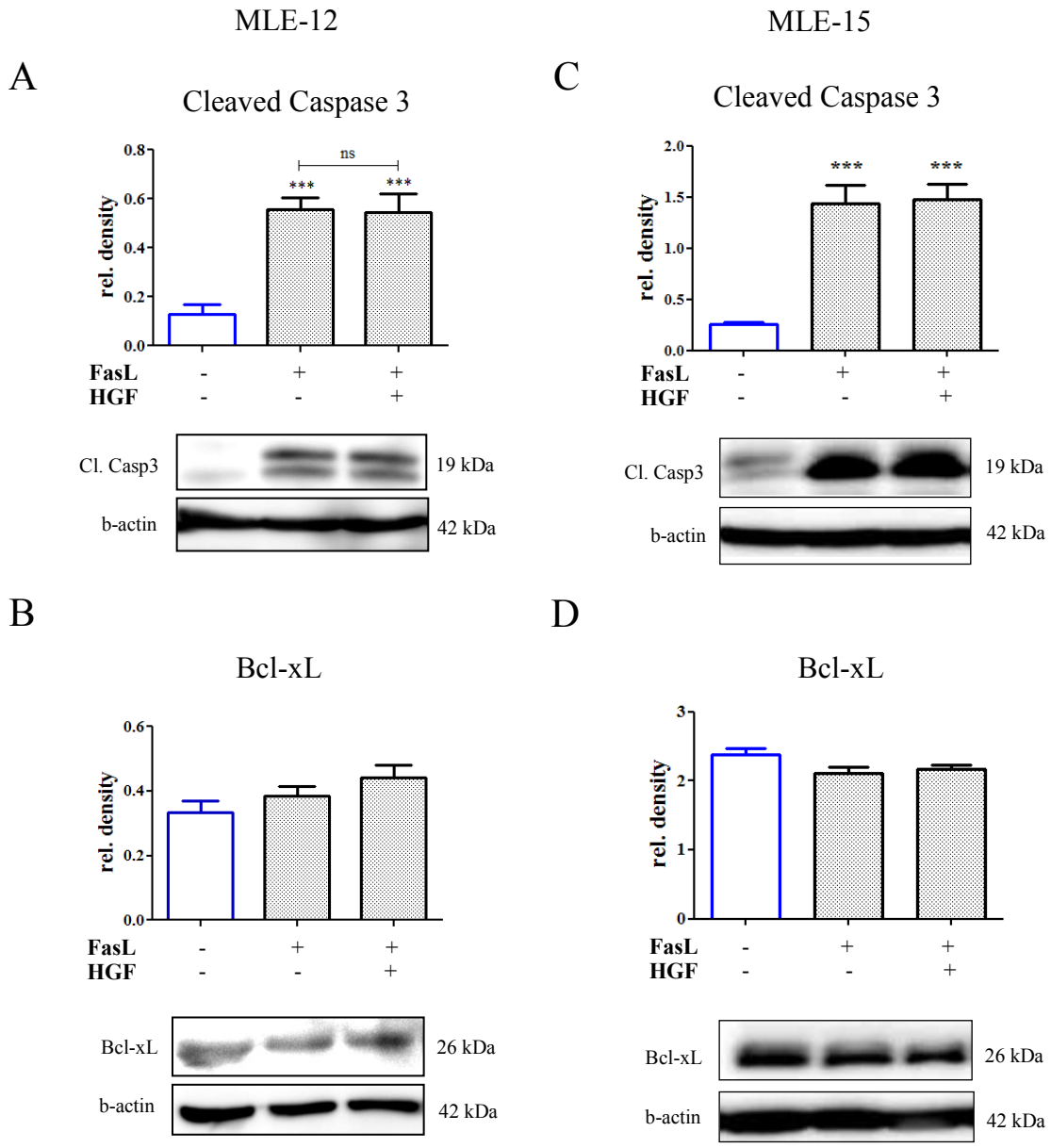

Figure 4.14: Lack of HGF protective effect on cells treated with FasL.
Confluent MLE-12 (**A**, **B**) and MLE-15 (**C**, **D**) cells were treated with FasL and HGF simultaneously for 24 h under no serum conditions. (**A**, **C**) Western blot analysis shows activation of Caspase 3 after FasL and/or HGF treatment. (**B**, **D**) Bcl-xL expression under different treatment conditions. The pictures are representative of at least three independent experiments. *** p=<0,001 *versus* control.

4.5 siRNA knock-down of Bcl-xL

Since HGF stimulation increased the expression of Bcl-xL under stress conditions, we used the siRNA targeted against Bcl-xL to further investigate its role in HGF-mediated epithelial cell protection.

4.5.1 Analysis of siRNA-mediated knock-down of Bcl-xL

Endogenous Bcl-xL expression was knocked-down with siGENOME SMARTpool siRNA oligonucleotides specific for murine Bcl-xL mRNA. Scrambled siRNA sequence was used as a specificity control. MLE-12 cells were transfected with DharmaFECT1 according to manufacturer's instructions. In the initial experiments two concentrations (25 nM and 100 nM) of targeted/scrambled siRNA were used. For characterization of the level of gene knock-down real time analysis was performed 24h and 48h after transfection. We observed significant decrease of Bcl-xL expression already at 24h time point. The negative control siRNA caused no change in Bcl-xL mRNA expression (Figure 4.15 A). For validation of the knock-down system on the protein level, we used 25 nM siRNA and performed Western blot analysis after 24h, 48h and 72h. (Figure 4.15 B). We demonstrated that Bcl-xL protein is markedly reduced already after 24h and its expression remains at low level up to 72h post-transfection.

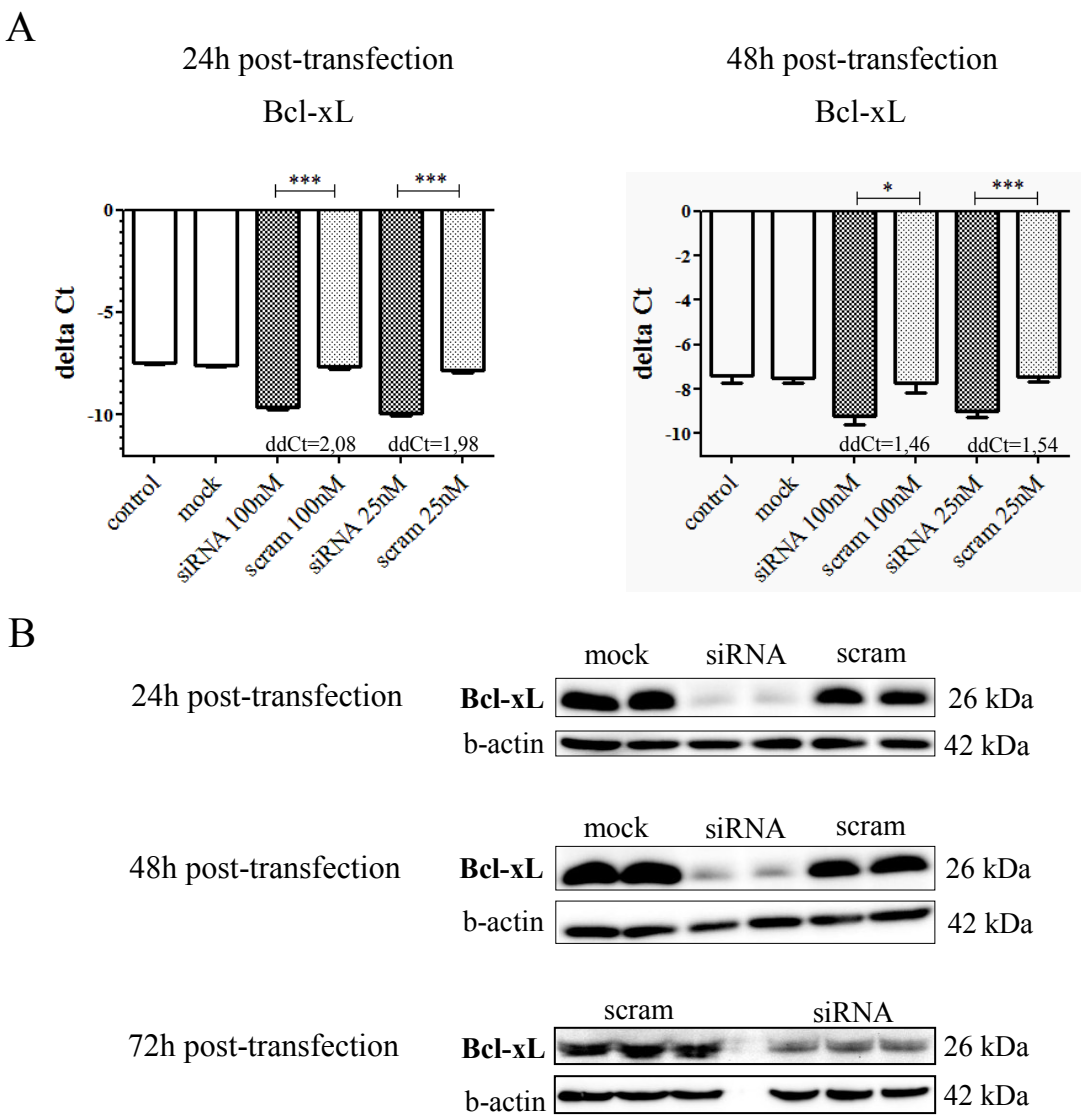

Figure 4.15: siRNA-mediated knock-down of endogenous Bcl-xL expression.
Epithelial MLE-12 cells were transfected with 25 nM siRNA targeted against Bcl-xL and scrambled siRNA as negative control. (**A**) Real time analysis shows reduced Bcl-xL gene expression after 24 h and 48 h after transfection, N=2. * p=<0,05, ** p=<0,01 *** p=<0,001. (**B**) Western blot demonstrates Bcl-xL protein level at 24 . 48 h and 72 h post-transfection. The pictures are representative of at least two independent experiments.

4.5.2 Effect of Bcl-xL knock-down on HGF-mediated survival of cells treated with hydrogen peroxide

To determine the role of Bcl-xL in HGF-mediated pro-survival effect on epithelial cells upon oxidative stress conditions, we treated the transfected cells with 650 µM hydrogen peroxide in the presence or absence of 24h long incubation with 20 ng/ml HGF. We noticed, that significant loss of Bcl-xL protein, caused increased sensitivity to injury of MLE-12 cells, demonstrated as a marked increase of cell death after treatment with H_2O_2 in comparison to cells transfected with scrambled siRNA. The cells with knock-downed Bcl-xL showed higher cell death level also in control conditions, without any stress stimuli. Nonetheless, normal expression of Bcl-xL was not crucial for HGF-mediated cell protection. HGF was able to rescue MLE-12 cells from cell death induced by hydrogen peroxide treatment in both cases, targeted and scrambled siRNA transfected variant (Figure 4.16 A). To validate if this effect is not a consequence of HGF-induced Bcl-xL re-expression, we performed Western blot analysis and found out that Bcl-xL level in transfected cells remains at a low level independently from HGF treatment (Figure 4.16 B).

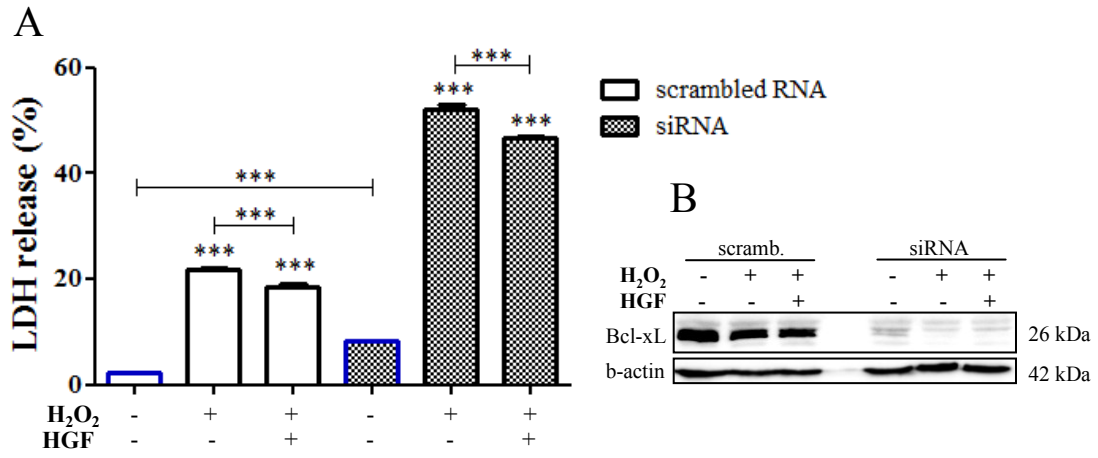

Figure 4.16: Role of Bcl-xL in HGF-mediated epithelial cell protection.
MLE-12 cells were transfected with anti-Bcl-xL siRNA or non-targeting siRNA, treated with 20 ng/ml HGF and then incubated with 650 µM hydrogen peroxide. (**A**) LDH cytotoxicity assay demonstrates cell death level under different cell culture conditions, N=5. *** p=<0,001 *versus* control if not indicated otherwise. (**B**) Western blot analysis shows Bcl-xL expression at the end of the experiment, N=3.

4.5.3 Effect of Bcl-xL knock-down on HGF prosurvival activity after thapsigargin treatment

In order to examine the stress stimuli dependent specific reaction of epithelial cells to HGF, we conducted an analogous experiment, using the 2,5 nM thapsigargin as cell death inducer. In agreement with previous results, we found out that down-regulation of Bcl-xL affected cell survival even without stress stimuli, drastically increasing sensitivity of MLE-12 cells to ER-stress caused by thapsigargin treatment. However normal endogenous expression of Bcl-xL did not seem to be crucial for HGF-driven cell protection (Figure 4.17 A). HGF-driven pro-survival effect on MLE-12 cells was not dependent on up-regulation of Bcl-xL (Figure 4.17 B).

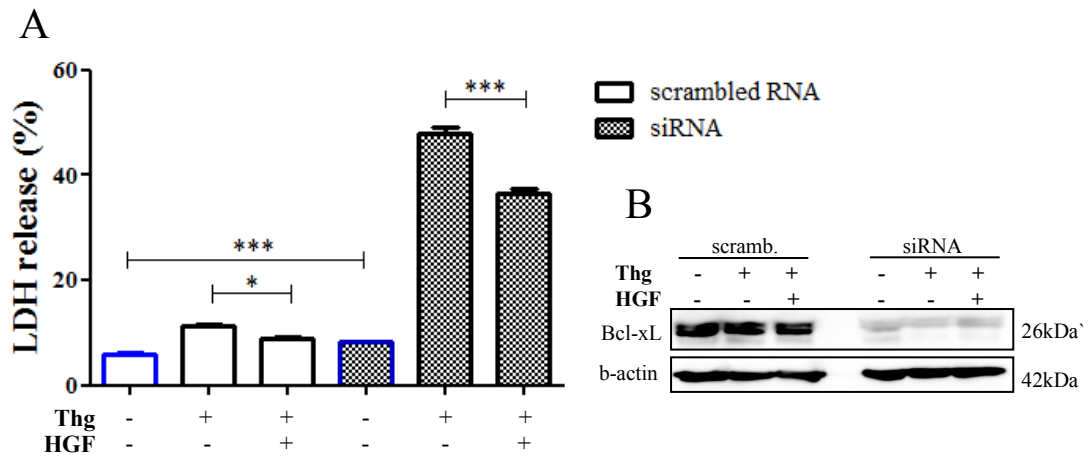

Figure 4.17: Role of Bcl-xL in HGF-mediated epithelial cell protection
MLE-12 cells were transfected with anti-Bcl-xL siRNA or non-targeting siRNA, treated with 20 ng/ml HGF and then incubated with 2,5 µM thapsigargin. (**A**) LDH cytotoxicity assay demonstrates cell death level under different cell culture conditions, N=3. ** p=<0,01, *** p=<0,001 (**B**) Western blot analysis shows Bcl-xL expression at the end of the experiment, N=2.

In conclusion, our *in vitro* data show that HGF has anti-apoptotic properties on alveolar epithelial cells under oxidative and ER stress conditions, which directly correlates with increased expression of Bcl-xL. Knock-down of Bcl-xL makes epithelial cells more sensitive to injury caused by hydrogen peroxide and thapsigargin. Bcl-xL is, however, not crucial for HGF cytoprotective activity. These data suggest that Bcl-xL is an important part of HGF/c-Met-mediated pro-survival response, but additional alternative molecular mechanisms are as well involved in the process.

5 Discussion

5.1 Epithelial apoptosis in IPF

5.1.1 What is the role of epithelial apoptosis in IPF?

IPF is a chronic interstitial pulmonary disease of unknown etiology. It is characterized by the formation of scar tissue within the lung, and results in a progressive decline in exercise capacity, restriction on lung function and ultimately a fatal outcome (King *et al*., 2011). The pathogenesis of IPF has been the subject of extensive investigation. According to a current hypothesis, repetitive injury to the alveolar epithelium is followed by impaired wound healing and this represents the early driving force for the development of the disease (Zoz *et al*., 2011). Persistent injury and excessive apoptosis of AEC seem to lead to an aberrant epithelial activation with synthesis of a variety of profibrotic factors (*e.g.* TGF-β, PDGF), fibroblast/myofibroblast expansion, deregulated remodeling and finally irreversible restructuring of the lung parenchyma (Selman *et al*., 2002).

Apoptosis has been clearly recognized in the alveolar epithelium in human tissue samples of patients with IPF. Such analysis of lung sections revealed DNA strand breaks as well as positive immunohistochemical signals for p53 and p21 in bronchial and alveolar epithelial cells in mild and moderate fibrotic lesions, with the most prominent staining in areas with so called “hyperplastic” type II cells (Kuwano *et al*., 1996). By employing electron microscopy it has been confirmed that dying pneumocytes are directly adjacent to fibroblastic foci, however, those cells displayed morphological characteristics of both apoptosis and necrosis (Uhal *et al*., 1998). Additionally, in a later study, it has been demonstrated that apoptotic type II cells can also be found in regions with still regular appearing architecture, suggesting they may be involved in early events during development of the disease (Barbas-Filho *et al*., 2001). In principle, alveolar epithelial type II cells play a key role in re-epithalization of intact alveoli. They have a high regenerative potential and are capable of abundant self-renewal and regeneration. AECII are also described to be progenitor cells with potential to differentiate into AECI following injury (Barkauskas *et al*., 2013, Fehrenbach, 2001). However, in IPF, these processes appear to be disturbed. Persistent damage, initial or secondary, with subsequent excessive cell death of epithelium is increasingly accepted as the key event in of IPF. In support of this theory, a targeted injury of AECIIs has

been shown to induce pulmonary fibrosis *in vivo*. In the study of Sisson *et al*., transgenic mice expressing the human diphtheria toxin receptor (DTR) under the SP-C promoter were exposed to diphtheria toxin (DT) to specifically aim the type II epithelial cells. Histological evaluation revealed that administration of DT resulted in diffuse collagen deposition with patchy areas of more confluent scarring associated with significant increase in hydroxyproline content in the lungs of DTR-expressing animals in comparison to controls (Sisson *et al*., 2010). Furthermore, the mutations in genes encoding surfactant protein C (*SFTPC*) and A (*SFTPA*) have been found in patients with familial forms of IPF (FPF). Clinical symptoms of FPF, treatment outcomes and histological findings (UIP) are indistinguishable from sporadic cases of IPF, except from the younger age at diagnosis. It has been suggested that up to 20% of cases might be familial (Kropski *et al*., 2013). Until now, several different mutations of *SFTPC* and *SFTPA* have been detected in FPF patients. The common feature of all identified mutations is that they involve amino acids in highly conserved regions and are believed to disrupt a C-terminal domain proven to be crucial for the proper folding and processing of the surfactant proteins (Thomas *et al*., 2002, van Moorsel *et al.*, 2010, Wang *at al*., 2009b). *In vitro* studies have demonstrated that the expression of mutant *SFTPC* in human or murine lung epithelial cell lines promoted up-regulation of multiple ER-stress species (*e.g*. Bip, Xbp-1) as well as activation of Caspase 3 (Mulugeta *et al.*, 2005, Thomas *et al*., 2002). Additionally, disruption of a C-terminal domain of *SFTPA2* in A549 cells led to accumulation of misfolded SP-A2 in ER and activation of the UPR (Wang *et al*., 2009b). Since *SFTPC* and *SFTPA* are expressed exclusively by AECII in the lungs, these findings support a model according to which mutations observed in FPF predispose to fibrosis *via* induction of type II cell death and provide evidence linking the type II cell injury to the development of lung fibrosis.

According to published studies, a number of factors contribute to the cytotoxic effect on lung epithelium, including DNA damage, oxidative and ER stress response pathways. Moreover, multiple different pathways are activated in fibrosis such as chemokines, pro-coagulant molecules and growth factors, that modify production of pro-apoptotic mediators (FasL, TNF-α, angiotensin peptides) (Günther *et al*., 2012, Hecker *et al*., 2011). In our study we focused on three major pro-apoptotic stimuli affecting type II cells in fibrotic lung: oxidative stress, ER stress and FasL. Knowing that HGF has anti-fibrotic properties in experimental models of lung fibrosis, we were interested if mediating survival of alveolar epithelial cells exposed to damaging factors may be one

of the mechanism *via* which HGF promotes resolution of fibrotic remodeling. At the centre of our focus was the role of Bcl-xL in this context.

5.1.2 Reactive oxygen species production in fibrotic lung

Oxidative stress has been described to contribute to the pathophysiology of interstitial lung diseases including IPF, fibrosing alveolitis associated with systemic sclerosis and sarcoidosis (Kliment *et al*., 2010). It is mainly referred to as the imbalance of oxidant production and antioxidant defenses, where oxidants dominate and lead to tissue damage. The main source of reactive oxygen species in IPF lung are alveolar macrophages, neutrophils and eosinophils. These cells release metabolic oxygen products including hydrogen peroxide and superoxide anions into the local microenvironment, and thereby affect adjacent cells (Kliment *et al*., 2010). Permanent oxidative stress may lead to increased susceptibility to injury and apoptosis of lung epithelium either directly or by activating redox-sensitive pathways, such as Ras/Raf/MAPK or PI3K/Akt that are implicated in TGF-β -induced EMT. Furthermore, ROS have been shown to directly activate latent TGF-β as well as induce TGF-β gene expression, thus positively affecting fibroblast proliferation and differentiation into myofibroblasts or enhanced production of ECM proteins (Liu *et al*., 2010). Changes in oxidant-antioxidant balance can be quantified in biological systems by measuring the levels of ROS themselves (*e.g*. hydrogen peroxide) or by measuring the end products of oxidation if cell components, such as carbonylated proteins or 8-isoprostane and exhaled ethane (as markers of lipid peroxidation). Elevated concentrations of all mentioned ROS or ROS by-products have been reported in IPF and have been found negatively correlated with pulmonary function (Bargagli *et al*., 2007, Kanoh *et al*., 2005, Montuschi *et al*., 1998, Psathakis *et al*., 2006). Furthermore, IPF fibroblasts have been shown to generate hydrogen peroxide in response to TGF-β1 and to induce cell death of co-cultured small airway epithelial cells (Waghray *et al*., 2005).

Concurrently to elevated levels of ROS, a marked reduction of anti-oxidant defense has been described in IPF. The antioxidant protection is mainly provided by intracellular glutathione (GSH), that functions *via* several enzymes catalyzing hydrogen peroxide and lipid peroxide reduction as well as retaining protein cysteine residues in their

reduced form (Liu and Pravia, 2010). In several studies deficiency of GSH in the lower respiratory tract of IPF patients has been described, including decreased levels of glutathione in BALFs, sputum and plasma of IPF subjects when compared to controls (Beeh *et al*., 2002, Cantin *et al*., 1989). Moreover, a marked reduction of major antioxidant enzyme of the extracellular matrix, superoxide dismutase was found in IPF lung tissues. Additionally, the immunohistological analysis of IPF/UIP sections revealed absence of EC-SOD in fibrotic areas and fibroblast foci (Kinnula *et al*., 2006).
In the current study, we used hydrogen peroxide to induce oxidative stress conditions *in vitro*. We observed that simultanous treatment of mouse epithelial cell lines MLE-12 and MLE-15 with HGF and H_2O_2 for 24h resulted in improved survival of the cells when compared to control conditions without HGF, and this appeared to be associated with increased expression of Bcl-xL (Figure 4.7). We confirmed this result using rat RLE-6TN cells, where we detected a reduced total cell death after HGF treatment. This effect was abolished by using a c-Met inhibitor (Figure 4.9). Similar results to ours were obtained in renal tubular epithelial cells. Treatment with HGF 48h prior to induction of apoptosis caused by oxidative stress resulted in improved cell survival. Both, Bad phosphorylation and increased Bcl-xL expression were the necessary events for that effect to occur. Simultaneous HGF and oxidative stress exposure failed to reduce apoptosis level in tubular cells, which was due to the fact that only phosphorylation of Bad took place, without up-regulation of Bcl-xL, which was assessed to be a critical event (Zhang et al., 2008). Knowing that Bcl-xL is involved in the HGF-driven cytoprotective actions against hydrogen peroxide on alveolar epithelial cells, we further investigated its impact on this process. In this purpose we transfected the cells with siRNA to specifically knock down Bcl-xL and subsequently exposed it to HGF and oxidative stress conditions. As expected, we observed that loss of Bcl-xL made cells more sensitized to injury, however HGF treatment remained profitable and resulted in improved cell survival despite the low level of Bcl-xL (Figure 4.16). Taken together, these findings indicate that up-regulation of Bcl-xL expression is one of the several mechanisms through which HGF/c-Met mediate prosurvival activity on the lung epithelium. However, Bcl-xL-based signaling seems not to be crucial for maintaining the HGF anti-apoptotic activity. A limitation in our study was that we used an siRNA-based approach, which did not result in a 100% knock-down of Bcl-xL. Hence, the dependency of HGF-elicited cytoprotective effect may have been much more evident if we would have been able to completely silence Bcl-xL in our experimental approach. In

line with this view, Takehara *et al.* observed that mice with a complete Bcl-xL gene knock-out in hepatic epithelial cells showed consistent apoptosis of these cells, and spontaneously developed liver fibrosis after 6 months (Takehara *et al*., 2004). Thus, prolonged and complete Bcl-xL deficiency, potentially resembling a more chronic process, may be necessary to assess its role *in vivo* in the context of fibrotic remodeling. This has been also indicated by the study performed by Staversky *et al*. They investigated the effect of disruption of Bcl-xL in AECII in context of lung development. Interestingly, they observed that short term knock-out of Bcl-xL in those cells did not affect pulmonary function or epithelial marker expression, but resulted in a shift of the lung toward a pro-apoptotic state and an increased sensitivity of the respiratory epithelium to oxygen-induced cytotoxicity (Staversky *et al*., 2010).

5.1.3 ER stress response in fibrosing lung

The endoplasmic reticulum contains chaperones that promote correct folding of a wide range of protein components in the cell. It has been well established that under normal conditions the activation of the ER stress response is a cytoprotective mechanism aiming to help misfolded proteins to re-fold and restore homeostasis. The process occurs through activation of several signaling pathways which affect synthesis of chaperones and proteasomal compounds responsible for degradation of irreparable products, lipid production or anti-ROS signaling. In principle, it is a fundamental process promoting cell survival. However, in case of overwhelming or prolonged stress conditions, a cell is driven into apoptosis *via* activation of CHOP and ATF-4 (Günther *et al*., 2012). Our group has previously shown that severe ER stress response triggers apoptosis of type II pneumocytes in IPF and may be implicated in development of the disease. By means of immunohistochemistry we were able to localize ER stress response elements, *e.g.* ATF-6, ATF-4 and CHOP, in AECII adjacent to fibroblast foci and in the regions of dense fibrosis of IPF patients, to a lower extent in still healthy appearing areas of IPF lung, but not in donor lungs. Moreover, the expression of ER stress markers co-localized with cleaved Caspase 3 and TUNEL signaling in those cells. We also detected enhanced ER stress response pathway activation in lung tissues obtained from IPF subjects (Günther *et al*., 2012, Korfei *et al*., 2008). Moreover, the

prominent role of ER stress in the pathogenesis of pulmonary fibrosis has been recently implied by Lawson *et al*. in an *in vivo* study in mice overexpressing the mutant form of protein surfactant C. In these animals, a constant ER stress response was associated with increased apoptosis of alveolar epithelial type II cells and enhanced fibrotic remodeling in the lungs after bleomycin application in comparison to control mice (Lawson *et al*., 2011).

Having determined that exaggerated activation of ER stress pathways may be one of the key mediators of AECII excessive apoptosis, we next focused on the impact of HGF on the survival of alveolar epithelial cells exposed to ER-stress inducing agent and the role of Bcl-xL in this context. To induce ER stress conditions we used thapsigargin, a potent inhibitor of ER and SR trafficking. As previously described for oxidative stress conditions, we observed similar effect and an interdependency between HGF and Bcl-xL on epithelial cells exposed to thapsigargin: HGF treatment improved survival of MLE-12 and RLE-6TN cells, which occurred through up-regulation of Bcl-xL level. This effect was abrogated in cells incubated with c-Met inhibitor (Figure 4.11 and 4.12). Knock-down, although incomplete, of Bcl-xL made MLE-12 cells markedly more sensitive to injury caused by thapsigargin, and again did not entirely suppress HGF cytoprotective activity (Figure 4.16).

Collectively, these data indicate that up-regulation of Bcl-xL expression is one of the potential mediators involved in HGF-promoted epithelial protection against oxidative as well as ER stress. The impairment of HGF production and activation observed in IPF may be one of the reasons for deregulation of Bcl-xL levels in lung epithelium potentially leading to insufficient Bcl-xL synthesis, thus favouring the development and progression of lung fibrosis.

5.1.4 Activation of death receptor pathway in IPF

The extrinsic pathway of apoptosis is activated by extracellular signals that result in the binding of specific ligands to the transmembrane receptors belonging to the tumor necrosis factor (TNF) receptor superfamily. Death receptor ligation triggers recruitment of the precursor form of Caspase 8 to a death-inducing complex through the adaptor protein FADD, which leads to subsequent Caspase 3 activation and execution of

apoptotic cell death (Kuwano *et al*., 2005). Best described mediators of that pathway are TNF-α and Fas ligand (FasL). Fas receptor (FasR)/FasL system is considered to be the most efficient start point of the extrinsic pathway, and therefore to play a crucial role in the regulation of cell survival (Kopiński, *et al*., 2011). It has been reported that IPF lungs exhibit increased FasL expression, especially in bronchial and alveolar epithelial cells when compared to healthy subjects. The enhanced levels of soluble FasL and FasL encoding mRNA has been also found in BALFs as well as in serum of IPF subjects (Kopiński *et al*., 2011, Kuwano *et al*., 1999, Kuwano *et al*., 2002). Although the exact role of FasR/FasL signaling in the development of ILDs has not been extensively explored, it has been accepted to be a significant factor contributing to the pathogenesis of IPF (Kuwano *et al*., 2002). In the present study, we showed that FasL-mediated induction of apoptosis in MLE-12 and MLE-15 cells did not influence the Bcl-xL expression. Interestingly, even upon strong activation of Caspase 3, the cellular level of Bcl-xL remained unaltered (Figure 4.13). In line with these results, we did not observe any effect of HGF treatment on cellular survival (Figure 4.14). Our findings can be related to the study performed by Hagimoto *et al.*. They observed that TGF-β-induced activation of Caspase 3 cleavage in bronchiolar epithelial cells also did not result in the down-regulation of Bcl-xL expression. Moreover, they demonstrated that TGF-β1 acts as an enhancer of Fas-mediated apoptosis in small airway epithelial cells (Hagimoto *et al*., 2002). Thus potentially, HGF which has an indirect effect on AECII survival, may still function through alternative mechanisms independent from Bcl-xL, for example by counteracting TGF-β signaling.

5.2 Epithelial protection, anti-apoptotic pathways in IPF

5.2.1 Impairment of the HGF system in IPF

HGF is a cytokine with pleiotropic functions during wound healing and repair. It's synthesized as a inactive precursor and proteolytically activated by several proteases specifically at sites of injury. Elevated levels of circulating HGF are observed as a response to a variety of insults. HGF can act as an endocrine, a paracrine or an autocrine factor for cells expressing the c-Met receptor, *e.g.* alveolar epithelial cells (Mason, 2002). However, the exact role of HGF during development and progression of IPF is

yet not fully understood. A few studies have reported that a total amount of HGF is increased in serum as well as in BALF obtained from IPF patients as compared to control subjects (Crestani *et al*., 2002, Hojo *et al*., 1997, Yamanouchi *et al*., 1998). Within this study we performed Western blot analysis of HGF expression in lung tissue homogenates and BALFs. We observed, that in lung tissue of IPF subjects, the level of activated HGF is decreased when compared to donors. There was no statistically significant difference with regard to the expression of the non-cleaved form of HGF (Figure 4.5 A). Analysis of BALFs showed no significant changes in active HGF levels observed in BALFs from IPFs in comparison to control subjects. The precursor form of HGF could not be detected, since it is normally stored in ECM or circulates in plasma. We speculate that, under pathological conditions in IPF, the up-regulation of pro-HGF does either not result in proper activation or insufficient downstream signaling in order to help the alveolar epithelium to better withstand endogenous as well as exogenous stress and to limit the magnitude of epithelial apoptosis and lung fibrosis. In this regard, application of exogenous HGF has been proven anti-fibrotic in several experimental models of various organ fibrosis including the lung (reviewed in Table 1). *In vivo* electroporation-mediated HGF gene expression in bleomycin treated mice resulted in reduction of pulmonary fibrosis, as assessed by Ashcroft's numerical score and hydroxyproline content of the lung. Moreover, HGF transfer to bleomycin-challenged lung markedly improved survival of animals and significantly reduced the apoptotic cell index compared with animals transfected with control vector (Umeda *et al*., 2004). Similarly, intratracheal application of recombinant HGF protein attenuated collagen deposition and led to reduction of fibrotic changes in the lung induced by bleomycin. In addition, exogenous administration of HGF has been shown to have other beneficial effects, such as induced pulmonary epithelial cell proliferation, stimulation of the fibrinolytic capacity of the lung and cell migration (Dohi *et al.,* 2000). These findings show that HGF is an essential factor for organ repair and protection. On the other hand, it is important to consider that long term use of HGF may stimulate extensive proliferation, survival or motility that in turn may lead to cancer. HGF/c-Met signaling has been shown to contribute to oncogenesis and tumor progression as well as promote cellular invasiveness strongly linked to tumor metastasis (Mizuno and Nakamura, 2013). Thus, before application, a proper time and dose of HGF have to be established.

In line with observations in animal models of pulmonary fibrosis, fibroblasts from IPF lungs have been shown to reveal a low capacity to activate pro-HGF due to decreased

production of HGF activator as well as increased synthesis of the HGFA inhibitors HAI-1 and HAI-2 (Marchand-Adam *et al*., 2006). In accordance with such observation, we detected a decreased expression of activated HGF in IPF versus donors lungs, in absence of a significant difference in non-cleaved HGF (Figure 4.5 A). Another factor limiting HGF-mediated signaling in IPF may be a posttranslational modification of the serine residue present in the juxtamembrane domain of c-Met receptor. c-Met is a tyrosine kinase, thus signal transduction occurs *via* phosphorylation of tyrosine residues localized in the receptor's catalytic domain and multifunctional docking site. Phosphorylation of serine at position 985 of c-Met has been described to be of critical importance for limiting cellular responsiveness to HGF depending on the extracellular environment. Ser 985 phosphorylation is regulated by reverse activities of protein kinase C and protein phosphatase 2A (PP2A) and results in an inhibition of biological responses to HGF (Gandino *et al*., 1994, Hashigasako *et al*., 2004). Once organ tissue undergoes injury, Ser 985 site becomes dephosphorylated *via* recruitment of PP2A, which leads to regenerative action triggered by HGF. Since tissue damage leads to an increase of plasma HGF, hereby to systemic exposure to the growth factor, this mechanisms allows intact organs to escape c-Met activation *via* Ser 985 phosphorylation (Nakamura and Mizuno, 2010). The status of c-Met Ser 985 phosphorylation in IPF lungs has not been investigated up to date. However, it has been reported that fibroblasts from IPF patients have a lower capacity to induce PP2A activity *in vitro*, as well as to exhibit a reduced PP2A expression in comparison to control fibroblasts (Xia *et al*., 2012). Studies performed on scleroderma fibroblasts suggest that decreased PP2A levels in fibrotic environment may be a result of constitutively activated TGF-β signaling (Samuel *et al*., 2010). TGF-β is a major profibrotic molecule involved in initiation and progression of pulmonary fibrosis. It has been described to be greatly increased in IPF lungs, especially in alveolar epithelium, which constitutes the main site of its synthesis (Kapanci *et al*., 1995). TGF-β can induce the recruitment of inflammatory cells, the proliferation and myofibroblastic differentiation of fibroblasts as well as epithelial-to-mesenchymal transition (Leppäranta *et al*., 2012). It has been shown that TGF-β1 suppresses HGF secretion and conversion into its biologically active form in IPF fibroblasts (Marchand-Adam *et al*., 2006, Matsumoto *et al*., 1992). Hence, excessive activation of TGF-β could contribute to a further impairment of the HGF system.

5.2.2 Role of Bcl-2 family in IPF

Bcl-2 family comprises proteins involved in mitochondrial control of apoptosis. Functionally, they can be classified into three groups: anti-apoptotic (*e.g* Bcl-2, Bcl-xL), pro-apoptotic (*e.g*. Bax, Bak) and regulatory BH-3-only proteins that posses the ability to bind to anti- as well as pro-apoptotic members of the family (*e.g*. Bid) and thus function as initial sensors that integrate and transmit apoptotic signal to other Bcl-2 family proteins (Adams and Cory, 1998, Chipuk *et al*., 2010). According to existing evidence, several proteins belonging to the Bcl-2 family have been implicated to play a role in the pathogenesis of various organ fibrosis, including lung, kidney, liver and heart (Budinger *et al*., 2006, Nakamura *et al*., 2000, Takehara *et al*., 2004, Zhang *et al*., 2001, Zhang *et al*., 2008). The group of Budinger *et al*. reported that the pro-apoptotic Bcl-2 family member Bid is required for the development of pulmonary fibrosis induced by bleomycin instillation in mice. Alveolar epithelial cells isolated from Bid-null mice were resistant to TGF- β1-induced cell death, whereas TGF-β1 levels in BALFs as well as TGF-β1-inuced fibroblast proliferation were not affected by loss of Bid. These findings suggest that Bid may play a crucial role in epithelial protection during development of pulmonary fibrosis (Budinger *et al*., 2006).

In line with this view, IPF lungs exhibit altered expression of Bcl-2 family members, as shown by Plataki *et al*. They have observed that hyperplastic AECII of IPF patients show increased expression of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2 in comparison to type II cells of control subjects (Plataki *et al*., 2005). Since it has not been investigated before, now we analyzed the expression pattern and total amount of Bcl-xL in IPF lungs. The immunohistochemical analysis of lung sections demonstrated presence of Bcl-xL in bronchial and alveolar epithelial cells in both IPF and control subjects (Figure 4.2). Interestingly, we noticed the most prominent expression in hyperplastic type II cells found in the regions of dense fibrosis. We could also observe strong signal in bronchial epithelial cells of fibrotic lung, whereas healthy donor sections revealed only weak staining, especially present in AECII. These findings were confirmed by lung homogenate analysis, which showed significant increase of total amount of Bcl-xL in IPF tissues *versus* control (Figure 4.1 A). Bcl-xL was below the detection level in the BALFs obtained either from healthy or IPF subjects (Figure 4.1 B).

Taking into consideration that we found elevated level of pro-survival Bcl-xL especially in the hyperplastic type II cells that have been shown previously to undergo DNA fragmentation and express pro-apoptotic markers including those that belong to Bcl-2 family, we hypothesize that up-regulation of Bcl-xL in the fibrotic lung may be a part of a compensatory mechanism, activated in attempt to rescue injured pneumocytes (Plataki et al., 2005, Uhal et al., 1998). A similar finding was obtained by Kitamura *et al.* in the context of Alzheimer's disease (AD), a neurodegenerative disorder associated with widespread neuronal death. Examination of temporal cortex showed elevated level of the pro-apoptotic Bcl-2 family members Bad and Bak coinciding with Bcl-xL up-regulation in AD patients in comparison to controls (Kitamura *et al*., 1998). Moreover, the exposure of cultured rat neurons to subtoxic dose of β-amyloid peptides, inducer of oxidative stress and cell death mediator, resulted in an increased level of Bcl-xL with no significant induction of apoptosis, whereas stable overexpression of Bcl-xL was still able to promote neuronal protection against cytotoxic concentrations of β-amyloids and apoptosis (Luetjens *et al*., 2001). These data suggest, that increased Bcl-xL expression may be an important event in response to stress stimuli such as oxidative stress. However the fact that ongoing and permanent apoptosis of neuronal cells, in case of AD, or alveolar epithelial cells in IPF, possibly leads to a constant progression of pathological condition, verifies that the system is insufficient or impaired.

Furthermore, we studied the expression pattern of Bcl-xL in IPF sections presenting normal lung structure with adjacent areas of fibrotic remodeling (Figure 4.3). Spatial heterogeneity is a characteristic feature of UIP/IPF. Since the disease constantly progresses with time, we believe that the regions with still unaltered parenchymal architecture potentially represent an early stage of fibrosis. In agreement with previous results, hyperplastic AECII revealed strong immunohistological staining for Bcl-xL, whereas type II cells present in the still regular appearing regions of IPF lung sections appeared to have much weaker Bcl-xL expression, resembling that observed for donor lung. This indicates that Bcl-xL may be a part of mechanism involved in suppressing the progression of already existing pro-fibrotic changes rather than in the initial development of this pathological condition.

5.3 Conclusions and future directions

Idiopathic pulmonary fibrosis is a chronic disease of unknown etiology, characterized by the formation of scar tissue within the lung (King *et al*., 2011). In agreement with a current concept, occurrence of a sequential injury and excessive apoptosis of alveolar epithelial cells followed by impaired wound healing process represents the leading cause for the development of IPF (Selman *et al*., 2002, Zoz *et al*., 2011). Apoptosis has been clearly recognized in the alveolar epithelium in human tissue samples with IPF, especially in the areas with adjacent to fibroblast foci with so called "hyperplastic" type II cells (Kuwano *et al*., 1996). In familial cases of IPF, observed mutations in the genes encoding surfactant protein C and A, have been suggested to predispose to pulmonary fibrosis *via* induction of enhanced ER stress and UPR response that leads to reduction of AECII viability (Kropski *et al*., 2013). For this reason the present study focused on alveolar epithelial cells. Since HGF has been proven to posses anti-fibrotic properties in experimental models of pulmonary fibrosis and, at the same time, to be impaired in human IPF, we investigated if HGF-promoted resolution of fibrotic changes may be due to its protective activity on type II cells driven into apoptosis by using three major stimuli present in fibrotic lung: oxidative stress, ER stress and FasL-triggered apoptosis. At the centre of our focus was the role of anti-apoptotic protein Bcl-xL in the HGF/c-Met-mediated cytoprotection. Our *in vitro* studies on mouse and rat lung epithelial cell lines revealed that up-regulation of Bcl-xL expression is one of the mechanisms involved in HGF/c-Met-mediated cytoprotective activity on epithelial cells in conditions of oxidative and ER stress. It would be beneficial to investigate the role of HGF in this regard on the primary alveolar epithelial cells isolated from bleomycin treated mice, or at best, from human fibrotic lung. Since it is hypothesized that acquired or genetic dysfunction of AECII may be the underlying cause for increased sensitivity of epithelium to injury and may lead to aberrant wound healing, employing primary type II cells from fibrotic lung would be of advantage, as they may respond differently than cells from healthy organ.

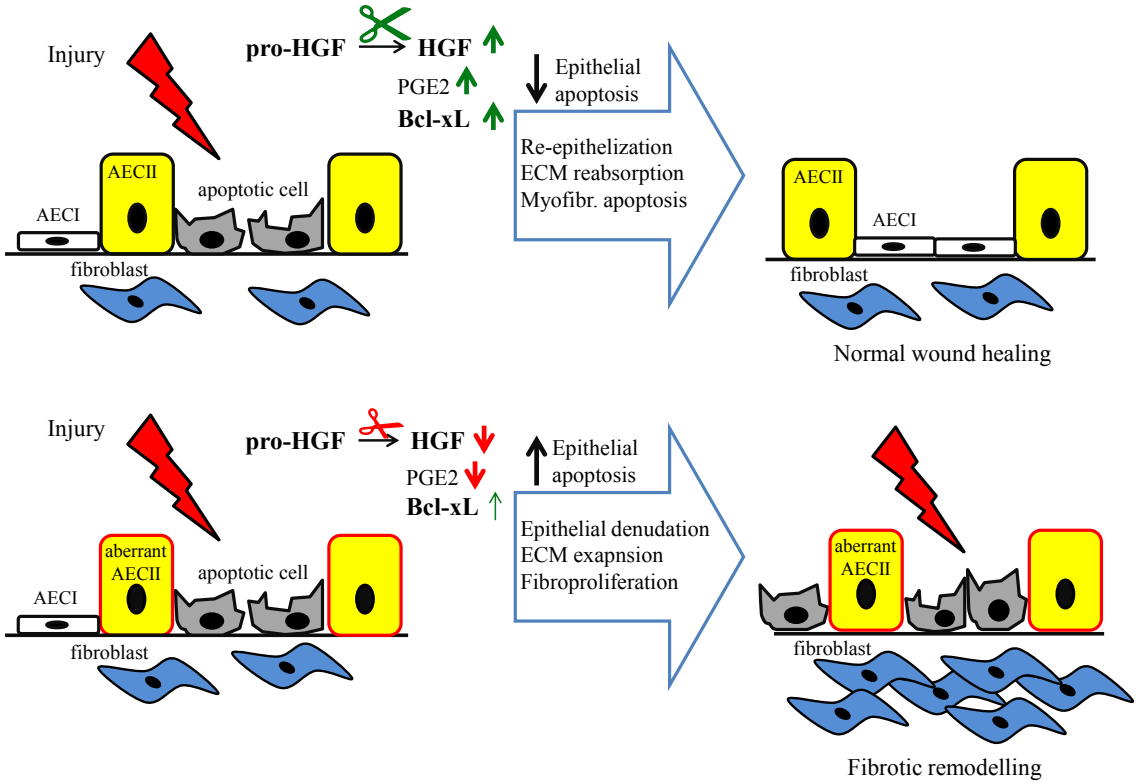

Fig 5.1: Schematic representation for proposed role of Bcl-xL and HGF in idiopathic pulmonary fibrosis.

Furthermore, we have found that activation of extrinsic apoptotic pathway *via* FasL treatment did not alter Bcl-xL expression and no anti-apoptotic effect of HGF could be observed in this case, suggesting that HGF is able to promote anti-apoptotic effect on epithelial cells only in response to an activation of the intrinsic pathway. It is possible that, HGF would be capable of mediating pro-survival signaling also when extrinsic pathway is triggered *in vivo*, however only in a indirect manner, for example through counteracting TGF-β signaling. Thus, *in vivo* studies using bleomycin model could provide additional information about a role of Bcl-xL and its dependency on HGF system in development and progression of pulmonary fibrosis.

Interestingly, we observed that IPF lungs exhibit a significant increase of total amount of Bcl-xL in comparison to donor lung, with the most prominent expression in hyperplastic type II cells found in the regions of dense fibrosis. Taking into account that an increase of Bcl-xL synthesis mediated by HGF treatment had a beneficial effect on injured epithelial cells as well as the observation of Plataki *et al.* group that hyperplastic type II cells show a decreased expression of another pro-apoptotic Bcl-2 family member, we speculate that Bcl-xL may be a part of compensatory mechanism, activated

in attempt to rescue injured pneumocytes (Plataki *et al*., 2005). However the fact that HGF production and activation is impaired in IPF may be a potential explanation for insufficient epithelial protection favoring the progression of lung fibrosis (Figure 5.1).

6 Appendix

6.1 List of primary antibodies

Antibody against	Source	Dilution	Application	Company
Bcl-xL	Rabbit	1:4000	WB	Abcam
Bcl-xL	Rabbit	1:50	IHC	Abcam
Beta-Actin	Rabbit	1:10000	WB	Abcam
Beta-Actin	Mouse	1:10000	WB	Abcam
cc-10	Rat	1:75	IHC	R&D Systems
Cleaved Caspase 3	Rabbit	1:1000	WB	Cell Signaling
c-Met	Rabbit	1:100	IHC	Abcam
c-Met	Rabbit	1:1000	WB	Abcam
c-Met Phoshpo	Rabbit	1:1000	WB	Cell Signaling
Cytokeratin 5	Rabbit	1:200	IHC	Abcam
HGF	Rabbit	1:400	WB	Santa Cruz
Pro-SPC	Rabbit	1:750	IHC	Millipore

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9 Declaration

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_____ _____

Ort, Datum Unterschrift

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