Therapeutic Effects of recombinant human Keratinocyte Growth Factor (rHuKGF) in an Elastase-Induced Emphysema Model in the Mouse

Inaugural-Dissertation zur Erlangung des Doktorgrades der Humanbiologie

(Dr. rer. physiol.)

dem Fachbereich Medizin der Philipps-Universität Marburg

vorgelegt von Vandana Muyal, geborene Bhatgain

aus Tehri, Indien

Marburg , 2010

Aus der Klinik für Innere Medizin, Schwerpunkt Pneumologie der Philipps-Universität Marburg

Direktor: Prof. Dr. Claus Vogelmeier

des Fachbereichs Medizin der Philipps-Universität Marburg

in Zusammenarbeit mit dem Universitätsklinikum Gießen und Marburg

Therapeutic Effects of recombinant human Keratinocyte Growth Factor (rHuKGF) in an Elastase-Induced Emphysema Model in the Mouse.

Inaugural-Dissertation zur Erlangung des Doktorgrades der Humanbiologie

(Dr. rer. physiol.)

dem Fachbereich Medizin der Philipps-Universität Marburg

vorgelegt von Vandana Muyal, geborene Bhatgain

aus Tehri, Indien

Marburg , 2010

Angenommen im Fachbereich Medizin der Philipps-Universität Marburg am:

Gedruckt mit der Genehmigung des Fachbereichs.

Dekan: Prof. Dr. M. Rothmund

Referent: Prof. Dr. H. Fehrenbach

Tag der Disputation: 25-10-10

1. Korreferent: Prof. Dr. H. P. Elsässer

TABLE OF CONTENTS

1 Introduction

1.1 Pulmonary Emphysema

Chronic obstructive pulmonary disease (COPD) is defined by slowly progressive development of airflow limitation (1). COPD is a major and increasing global health problem. It is predicted by the World Health Organization (WHO) to become the third most common cause of death and the fifth most common cause of disability in the world by 2020 (2). Indeed, in the United States, COPD is already the fourth most common cause of death and the only common cause of death that has increased over the last 30 years (3). Cigarette smoking is by far the most common cause of COPD but only a fraction of the smoking population develops this complication. Exposure to air pollution particles, occupational exposure to dust and fumes, and, in the developing world, exposure to biomass fuel used for cooking are also believed to be etiologic agents of COPD (4). In addition, genetic susceptibility factors must also contribute to the development of the disease (5). The two most common conditions of COPD are chronic bronchitis and pulmonary emphysema. Pulmonary emphysema is referred to as a pulmonary inflammatory disease in which destruction of the lung's gas-exchange structures, i.e. the alveoli or distal airspaces, lead to inadequate oxygenation, disability and frequently death (6). Such damage to the alveoli results in permanent "holes" in the tissues of the lower lungs and is thought to be irreversible. Emphysematous lung destruction reduces maximal expiratory airflow by decreasing the elastic recoil force that drives air out of the lungs. The present treatment of COPD consists of daily administration of bronchodilaters and or/steroids and oxygen supplimentation, often continuously for 24 hour for hypoxemic patients, however there are no drugs specifically developed so far that are able to cure emphysema.

1.1.1 Historical background

The physiologic and clinical concepts of pulmonary emphysema were developed in the 19th century; however the disease had been recognized as a pathologic entity long before following some of the earliest finding to describe emphysema that include for example, Bonet’s description of voluminous lungs in 1679 (7), Ruysch’s

(8) and Morgagni's description of 19 cases in which the lungs were turgid, particularly from air in 1769 (9). Matthew Baillie first provided the clear illustration and description of emphysema as enlarged air spaces in 1799 and 1807, and pointed out its essentially destructive characters (10-11). A French physician, Laënnec described emphysema as marked variations in the size of the air "vesicles", which might be smaller than a millet seed or as large as a cherry stone. He suggested that "vesicles" of the latter size were produced by the coalescence of adjacent air spaces following rupture of the alveolar walls (12-13).

Later in year 1892, Osler generated a report in the Textbook of Medicine about idiopathic emphysema. According to him, substantive or idiopathic emphysema is a well marked clinical affection, characterized by enlargement of the lungs due to distention of the air "cells" and atrophy of their walls, and clinically by imperfect aeration of the blood and more or less marked dyspnea (14).

In 1956, McClean suggested that emphysema is a chronic destructive lung disease, characterized by enlargement of existing airspaces in the lung parenchyma (15). In 1960s, both the World Health Organisation (WHO, 1961) and the American Thoracic Society (ATS, 1962) defined emphysema as a condition of the lung characterized by permanent enlargement of airspaces distal to the terminal, non-respiratory bronchiole accompanied by destruction of respiratory tissue (16-17). Subsequently, a workshop convented by the National Heart, Lung, and Blood Institute (NHLBI) defined tissue destruction in emphysema as non-uniformity in the pattern of respiratory enlargement so that the orderly appearance of the acinus and its components is disturbed and may be lost (6). The acinus is the functional unit of the lung which consists of a respiratory bronchiole and its alveolar ducts as well as alveolar sacs, and is involved in gas exchange.

1.1.2 Pathological definition and classification

Pulmonary emphysema is anatomically defined as the "abnormal permanent enlargement of the airspaces distal to the terminal bronchioles, accompanied by destruction of their walls" (18). Both anatomical aspects can be assessed using quantitative histopathology, which revealed airspace enlargement (19, 20) and loss

of distal lung tissue, as reflected by a significant loss of total alveolar surface area and total capillary length (21).

On the basis of acinus destruction, mainly two forms of emphysema with distinct morphologic and functional entities have been described so far, i.e. centrilobular (or centriacinar) emphysema (CLE) and panlobular (or panacinar) emphysema (PLE) (22, Fig.1). CLE is more common in patients who are cigarette smokers with the main site of destruction and inflammation being the respiratory bronchiole. In this form of emphysema, lesions occur more frequently in the upper than in the lower lobes of the lung. PLE is a type of emphysema which is not usually linked with cigarette smoking (23), and is generally associated with deficiency of A1AT (24) which prevents the function of neutrophil elastase by binding to it to produce an inactive complex and hence protects the respiratory regions. In this form of emphysema, the destruction of the alveolar walls occurs in a fairly uniform manner, i.e. it involves all the components of the acinus uniformly, and as a result involves the entire lobule. Lower lobes are more frequently affected in this form of emphysema than are the upper lobes. Additionally, Saetta and co-workers suggested that smokers with CLE have more abnormal and narrower small airways, and airflow limitation is correlated with the small airway abnormalities and not with the loss of elastic recoil. By contrast, patients with PLE have much less severe airway abnormalities, and airflow limitation seems to be due to the loss of elastic recoil (25). Studies have shown that patients suffering from the PLE form have higher lung compliances and PLE exhibits little relation to peripheral airway inflammation while patients with CLE have a higher degree of hyperreactivity and airway inflammation which reflects the distinctive mechanical properties of the two types of emphysema (22, 26).

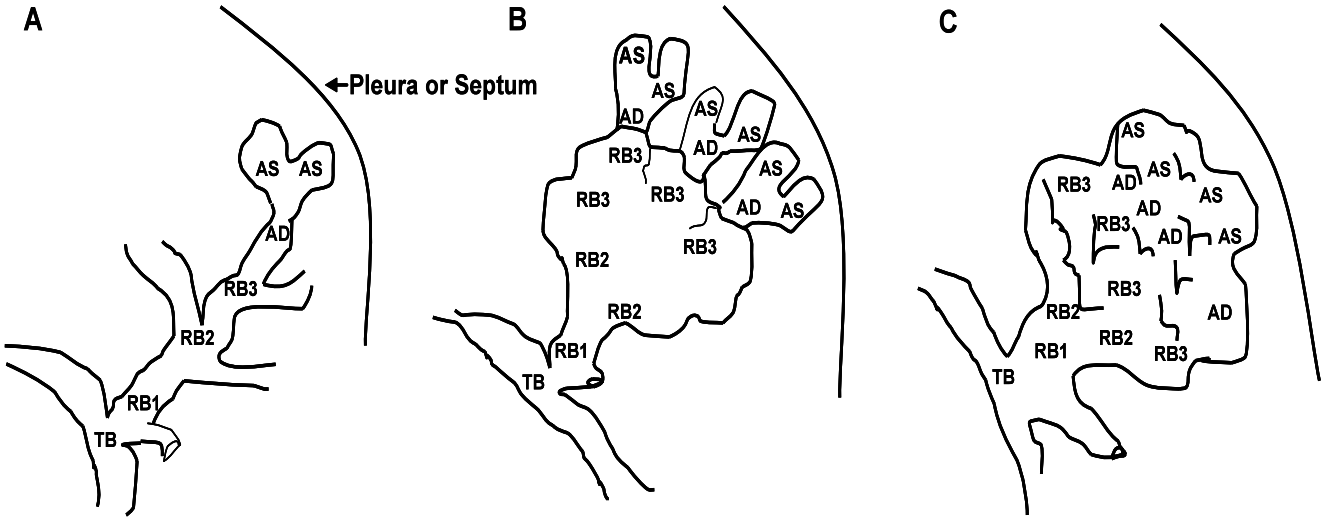


Figure 1 - *Schematic representation of centrilobular emphysema (CLE) and panlobular emphysema (PLE) in contrast to healthy lung acinar architecture (A). In CLE (B), respiratory bronchioles are predominantly involved, whereas in PLE (C), destructive enlargement of all air spaces distal to the terminal bronchiole is seen and the acinus is uniformly affected. AD-Alveolar duct; AS-alveolar sac; RB_{1-3}-respiratory bronchiole; TB-terminal bronchiole [Picture is modified after Turato et al (27)].*

1.1.3 Pathogenesis of emphysema

Multiple pathogenetic mechanisms contribute to the development of emphysema. No single mechanism can account for the complex pathology leading to destructive processes in emphysema. Over the past 50 years, our understanding of the pathogenesis of emphysema has advanced significantly, and new concepts continue to emerge. The critically important pathophysiological processes that interact in the periphery of the lung in emphysema are discussed below:

1.1.3.1 Inflammation and emphysema

It has been shown that chronic exposure to cigarette smoke leads to chronic inflammatory responses, which potentially cause alveolar destruction with an increase of inflammatory cells such as neutrophilic granulocytes (28-29), macrophages (30-33), CD8+T lymphocytes (34) and dendritic cells (35-36). These cells are capable of releasing inflammatory mediators and proteinases, which are

believed to play a role in the progressive lung destruction in emphysema. (For the schematic representation of pathogenesis of emphysema, see Fig.2)

Role of neutrophilic granulocytes

The neutrophilic granulocyte (or neutrophil) was the first inflammatory cell implicated in the pathogenesis of emphysema (37-38). Neutrophils release a multitude of mediators and tissue degrading enzymes including neutrophil elastases, proteinase-3, and oxidants causing chronic inflammation and tissue destruction (39-40). Increased numbers of activated neutrophils are found in sputum and BAL fluid of patients with COPD (37) while some reports have failed to find any correlations between neutrophil numbers in tissue sections and the severity of lung destruction (38) (Fig.2).

Role of alveolar macrophages

The second major cell type involved in the pathophysiology of COPD is the alveolar macrophage, which was noticed to be markedly increased in numbers (5- to 10-fold) in airways, lung parenchyma, BAL fluid, and sputum in patients with COPD. A mophometric study conducted by Retamales and co-workers revealed a 25-fold increase of the numbers of macrophages in the tissue and alveolar space of emphysema patients in comparison to smokers with normal lung function (30). Furthermore, alveolar macrophages release increased levels of inflammatory mediators including cytokines as e.g., interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α) and IL-6; and chemokines as e.g., IL-8, growth-related oncogene-α (GRO-α), macrophage inflammatory protein (MIP-1α), interferon-gamma inducible protein (IP-10), monokine induced by interferon-gamma (Mig) as well as the anti-inflammatory cytokines IL-10 and leukotriene (LTB4) (41-46). IL-8, GRO-α, MIP-1α and LTB4 are potent chemoattractants of neutrophils whereas IP-10 and Mig have the capacity to attract CD8+T lymphocytes (Fig.2).

Role of lymphocytes and dendritic cells

Increased numbers of lymphocytes such as $CD4^+$ and $CD8^+$ T-cells are also thought to contribute to the pathogenetic mechanisms underlying COPD (47-48). Although increased numbers of $CD8^+$ T-cells than $CD4^+$ T-cells are found in the airways of COPD patients (47), the mechanism by which $CD8^+$ T-cells and, to a lesser extent,

The airways contain a rich network of dendritic cells, which play a crucial role to activate a variety of other inflammatory and immune cells including macrophages, neutrophils, T and B lymphocytes (49). An increase in the number of dendritic cells has been evaluated in rat lungs exposed to cigarette smoke (50), and in the airways and alveolar walls of smokers (35-36). It therefore likely that the dendritic cells may play an important role in the progression of emphysema in response to cigarette smoke and other inhaled noxious agents (Fig.2).

1.1.3.2 Proteases/Anti-proteases and emphysema

Proteases are a large group of enzymes that conduct proteolysis, that is, begin protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. The central concept of protease/anti-protease imbalance hypothesis consists of switching on proteases due to inactivation of anti-proteases, which leads to the destruction of connective tissue components, particularly elastin, with ensuing development of emphysema. This concept derives from early clinical observations that subjects deficient in alpha1-antitrypsin (A1AT), a major circulating inhibitor of neutrophil elastase (51), develop severe emphysema. Takubo et al (52) and Cavarra et al (53) reported that pallid mice, which are naturally deficient in A1AT, developed emphysema earlier than strains with normal A1AT levels.

Several serine proteases such as neutrophil elastase (NE), proteinase-3 and cathepsin-G had been found to be involved in the progression of emphysema in experimental animals (54). A study performed by Shapiro and co-workers (55) showed that mice lacking NE were 59% protected against emphysema which supports a role of NE in the pathogenesis of emphysema.

Similarly, matrix metalloproteinases (MMPs) and cysteine proteases have also been observed in the lung in COPD (56-58). MMPs are a large family of zinc-dependent proteinases that regulate the turnover of extracellular matrix components (ECM) (59). Previous reports have demonstrated increased expression of MMP-1 (collagenase) and MMP-9 (gelatinase B) in BAL concentrations and in macrophages of emphysema patients (60-62). Some studies have shown the involvement of MMP-12 in the sputum, BAL, bronchial biopsies and peripheral lung tissue of patients with

Several lysosomal cysteine proteases (cathepsins) as e.g., cathepsins B, S, K, and L, which are expressed in alveolar macrophages, comprise potent elastolytic activity and hence might be involved in the pathogenesis of emphysema (66-67) (Fig.2).

1.1.3.3 Oxidants/Anti-oxidants and emphysema

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediate. Oxidative stress is thought to be implicated in the pathophysiology of emphysema. In the lung, oxidants that come from cigarette smoke may cause oxidative damage to lung matrix components such as elastin and collagen and may lead to impairment of elastin synthesis and repair, resulting in emphysema (68-69).

Cigarette smoke is a complex mixture of >4,700 chemical compounds and is characterized by high concentrations of free radicals and other oxidants in both the gas and tar phases (70). The gas phase of cigarette smoke contains approximately 10^{15} radicals per puff including reactive oxygen species (ROS), epoxides, peroxides, and nitrogen dioxide. The tar phase of cigarette contains more stable radicals, such as the semiquinone, which can react with oxygen to produce superoxide anion (O2-), the hydroxyl radical, and hydrogen peroxide (70).

Activated inflammatory and structural cells including neutrophils, eosinophils, macrophages and epithelial cells in the airways of emphysema patients further enhance the production of ROS in excess of their antioxidant defence mechanism to create oxidative stress (71). This oxidative stress may further participate in several processes including inactivation of A1AT by oxidizing methionine residue at its active site, leading to an acceleration of the breakdown of elastin in lung parenchyma (72-73). Apart from A1AT, another major inhibitor of neutrophil elastase is secretary leukoprotease, which can also be inactivated by oxidants (74-75).

Oxidative stress serve to activate the transcription factor such as NFkB and transcription factor activator protein-1 (AP-1) leading to increased release of many inflammatory mediators including cytokines IL-8, TNF-α, and nitric oxide (NO) thus

1.1.3.4 Alveolar cell apoptosis and emphysema

Initially an American pathologist, Averil Liebow, suggested that the loss of endothelial cells which are major structural cells of the alveolar wall, may lead to the development of emphysema (78). In support of this, Aoshiba and Nagai further discussed the concept of alveolar cell destruction due to apoptosis leading to emphysema (79). A year later the first documentation of lung cell apoptosis revealed that COPD lungs exhibited an enhanced number of TUNEL-positive cells, predominantly involving endothelial cells when compared with normal or non-COPD smoker's lungs (58). Aoshiba and Nagai in their further study demonstrated that administration of active caspase-3 (an apoptosis mediator (80) induces interpulmonary apoptosis as well as enhanced elastolytic activity in BAL leading to emphysema (81). Kasahara and co-workers first described the relationship between VEGF, endothelial cell apoptosis, and emphysema. These authors reported that blocking of the VEGF receptor due to the presence of a VEGF receptor kinase inhibitor in rat model, induced alveolar septal cell apoptosis and led to enlargement of the airspaces (82). A similar outcome was noticed by Tsao et al, who reported that mice over-expressing placental growth factor (PIGF) also developed VEGF-deficiency, thereby causing alveolar septal apoptosis, and emphysema (83).

Furthermore, evidence of the concept that apoptosis could initiate airspace enlagement was also provided by signal transducer and activator of transcription-3 (STAT-3)-deficient mice that were shown to develop emphysema with evidence of apoptosis and caspase-3 expression when infected by adenovirus (84). STAT-3 plays a critical role in the regulation of various biological processes, including cell survival, apoptosis, inflammation, and proliferation.

Additionally, increased number of CD8+T lymphocytes in the lungs of COPD patients could cause apoptosis of alveolar epithelial cells through the release of perforins, granzyme-B and TNF-α (85-86) (Fig.2).

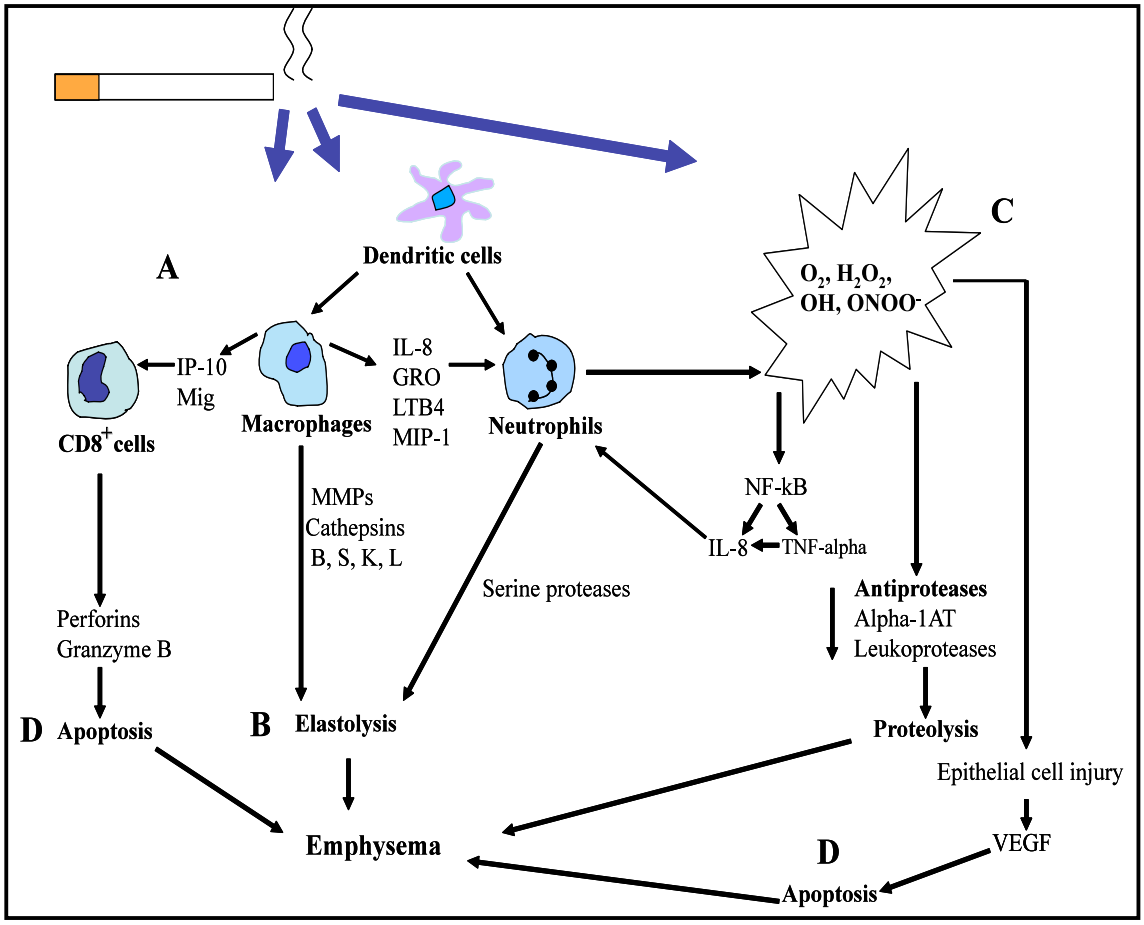


Figure 2 - *Schematic representation of pathogenesis of emphysema. A) Inflammation process, B) protease release in excess of their inhibitors, C) oxidatitive stress by cigarette smoke and inflammatory cells, and D) apoptosis process. GRO-α = Growth-related oncogene-α; IL-8 = interleukin-8; IP-10 = interferon-gama inducible protein; LTB4 = leukotriene; Mig = monokine induced by interferon-gama; MIP-1α = macrophage inflammatory protein; NFκB= nuclear factor kappa B; TNF-α = tumor nacrosis factor-α.*

1.1.4 Animal Models of Emphysema

Animal models are especially important for slowly progressing diseases such as emphysema and have a major impact on the investigation of the key mechanisms in the pathophysiology of emphysema (87). They would also facilitate the exciting prospect of treatment of disease for which there is currently no effective therapy.

1.1.4.1 Cigarette smoke-induced emphysema model

Cigarette smoking is a major environmental factor that predisposes patients to pulmonary emphysema. A variety of mammalian species have been exposed to cigarette smoke over the years to develop emphysema in dogs, rabbits, guinea pigs, and rodents (88-89). Out of these animals, Wright and colleagues reported that perhaps guinea pigs are the most susceptible species to cigarette smoke. In their study, they demonstrated that exposure of guinea pigs to tobacco smoke resulted in emphysema-like airspace enlargement within a few months (90). Other than guinea pig, several strains of mice have been described to develop emphysema after 6 months or longer exposure to cigarette smoke (91, 52-53). A clear cut comparison between mice (strain B6C3F1) and rat (strain F344) in order to develop emphysema in response to tobacco smoke exposure was conducted by March TH and colleagues (92). In their study, they revealed that B6C3F1 mice are more susceptible than F344-rats using morphometry and histopathology parameters to assess and compare emphysema. Guerassimov and co-workers compared five different strains of mice that were exposed to smoke for 6 months (93). They reported that only the AKR/J strain had significant emphysema, whereas A/J, SJL, and C57BL6/J strains appeared to be mildly susceptible to smoke and NZWLac/J stain was resistant. This study clearly points out towards the importance of genetic factors in determining individual susceptibility to cigarette smoke induced emphysema (93). The one important disadvantage of these models is that production of either emphysema or small airway remodeling takes months of smoke exposure (usually in the order of 6 months) and thus, is an expensive and time consuming proposition. However, the most important limitation of smoke models, no matter what species is selected and no matter how long animals are exposed, is that the disease they produce is mild, probably equivalent to human Global Initiative on Chronic Obstructive Lung Disease (GOLD) stage 1 or 2 disease (94-95).

1.1.4.2 Elastase-induced emphysema model

The elastase instillation based animal model is one of the fastest and easiest models available for emphysema. First, in 1965, Gross and colleagues reported that the exogenous application of a plant protease, papain, via intratracheal instillation destructs the elastin network in the lung and thereby leads to emphysematous like

elastase-based emphysema animal models in the laboratory because of the following reasons: a) it is relatively simple to perform, b) highly reproducible and c) models could be created by a single treatment with an inexpensive reagent in a short period of time with less labour as compared to cigarette smoke exposure. A variety of proteases (leukocytic proteases, porcine pancreatic elastase, cysteine proteinases), applied via a single intrapulmonary challenge, cause an efficient enzymatic induction of emphysema (88, 97-100). One of the key findings in this regard was that only enzymes that could degrade intact elastin, an elastic fibre that together with collagen determines the mechanical properties of connective tissue, produced emphysema. The administration of porcine pancreatic elastase (PPE; 1 to 4 mg/kg), for example, has produced the most consistent and impressive airspace enlargement alonge with the loss of gas exchange area in rodents, guinea pigs, dogs, and primates followed by inflammatory cells accumulation within the lungs (99, 101). PPE is a compact gobular protein of a single polypeptide chain of 240 amino acids cross-linked by four disulfides bridges and breaks down elastin. PPE also offers the advantages of being cheap and easy to obtain. Elastase-induced emphysema remains a useful model of pulmonary emphysema since it replicates many aspects of the disease and facilitates the assessment of the efficacy of therapeutic agents, particularly those with the capacity to repair lung damage, a critical need in the field.

1.2 Keratinocyte growth factor (KGF)

Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7 (FGF-7), was first purified from the human embryonic lung fibroblast cell line by Rubin et al (102). KGF consists of a single polypeptide chain of 26-28 kDa and has heparin-binding capability (103-104). KGF is a potent mitogen for different types of epithelial cells and assists in repair of the skin, cornea and gastrointestinal lining by stimulating cells to divide and grow (105-107). In vitro and in vivo studies revealed that various types of mesenchymal cells produce KGF including fibroblasts (102), smooth muscle cells (108), micro vascular endothelial cells (109), and γδT cells obtained from skin and intestine(110).

KGF is a member of the fibroblast growth factor (FGF) family of proteins. FGFs family

to 23 (111) and are involved in various biological processes, for instance, angiogenesis, wound healing, tumorigenesis and developmental and morphogenetic processes (112-115). Members of the FGF family bind to a variety of FGF receptors, which belong to tyrosine kinase receptor family. Currently, at least four FGF receptors (FGF receptor 1-4) (116-118) have been discovered and are composed of an extracellular ligand-binding domain that contains three immunoglobulin-like domains (D1-D3), a transmembrane domain, and a cytoplasmic domain which bears the tyrosine kinase activity (119-120). Unlike other members of FGF family that can bind to several FGFRs, KGF binds only to a splice variant of FGFR2 termed FGFR2-IIIb or KGFR (121), which is specifically expressed in epithelial cells and has intrinsic tyrosine kinase activity (122-123). KGFR differs from FGFR2 in the carboxyl-terminal half of D3 that is encoded by the alternatively spliced exon IIIb whereas in FGFR2 this region is encoded by exon IIIc. While FGFR2 binds FGF-1 and FGF-2 with high affinity and does not interact with the KGF, KGFR binds FGF-1 and FGF-7 with high affinity and FGF-2 with a 20-fold lower affinity (124-125).

1.2.1 Role of KGF in alveolar repair and maintenance

The restoration of healthy alveolar epithelium requires the proliferation and migration of alveolar epithelial type 2 cells (AE2) and their differentiation into alveolar epithelial type 1 cells (AE1). This mechanism is crucial to the effective repair of the alveolus after injury. Moreover, the proliferation and migration of lung fibroblasts which are believed to be cells crucial for the maintenance of interstitial tissue within lung occur early after lung injury and are necessary for ongoing lung healing. These mesenchymal cells have been shown to control proliferation and differentiation of alveolar epithelial cells through the secretion of soluble mediators such as keratinocyte growth factor (KGF).

Recombinat human keratinocyte growth factor (rHuKGF), also known as palifermin, is a modified version of naturally occurring KGF produced in Escherichia coli by recombinant DNA technology. Exogenous rHuKGF consists of a polypeptide chain of 16.3 kDa and differs from endogenous human KGF in that the first 23 N-terminal amino acids have been deleted to improve protein stability. Administration of exogenous rHuKGF has been demonstrated to stimulate hyperplasia of alveolar

the normal repair process after acute alveolar epithelial injury, as assessed by in vitro and in vivo studies (126-128). This role of exogenous rHuKGF of enhancing the spreading and motility of alveolar epithelial cells suggests that improved alveolar repair may underlie some of the protective effects of rHuKGF in lung injury models.

The first study on the protective effect of exogenous KGF in a rodent model with acute lung injury (ALI) was demonstrated by Panos and co-worker (129). They reported that intratracheal administration of rHuKGF stimulated AE2 cells proliferation in vivo and reduced hyperoxia-induced lung injury in rats. Subsequently, exogenous KGF has been extensively explored to protect lung against experimental lung injury including bleomycin (130-131), Pseudomonas aeruginosa pneumonia (132), hydrochloric acid (133-134), oleic acid (135), and radiation and bleomycin induced lung injury (136). Additionally, exogenous rHuKGF may have many other beneficial effects on acute lung injury that include scavenging of reactive oxygen species and increased DNA repair (137-138). In vitro and in vivo studies have shown that exogenous KGF increases alveolar epithelial fluid transport in both the uninjured and the injured lung by up-regulating the expression of sodium pumps, primarily the Na+-K+-ATPase alpha 1-subunit (139-140), and stimulates the production of lamellar bodies and expression of surfactant proteins (126, 141).

Moreover, KGF has been implicated as an important factor to stimulate the lung epithelial wound closure involving lung epithelial cells spreading, migration, and proliferation (142). Furthermore, the pattern of expression of KGF and its receptor suggests an important role for KGF in mediating mesenchymal–epithelial interactions and has been implicated to play a critical role in early lung branching morphogenesis (143). Many organs, including lung, are composed of two primary tissue layers, namely, epithelium and mesenchyme. In embryonic organs such as lung which are formed by a process of progressive branching of the epithelium, interactions of these two tissues have been demonstrated to play a determining role in the formation of the organ’s characteristic morphology (144). KGF has been demonstrated to be important for early postnatal alveolarization in rats as treatment of lungs with neutralizing antibodies to KGF lead to inhibition of alveoli-forming secondary crests, resulting in a significantly reduced alveolar number (145). KGF enhanced

increase in alveolar epithelial cell proliferation (146). Compensatory lung growth is achieved in part by formation of new alveoli and comprises all components of the alveolar septal wall, i.e. epithelium, endothelium, and interstitial tissue.

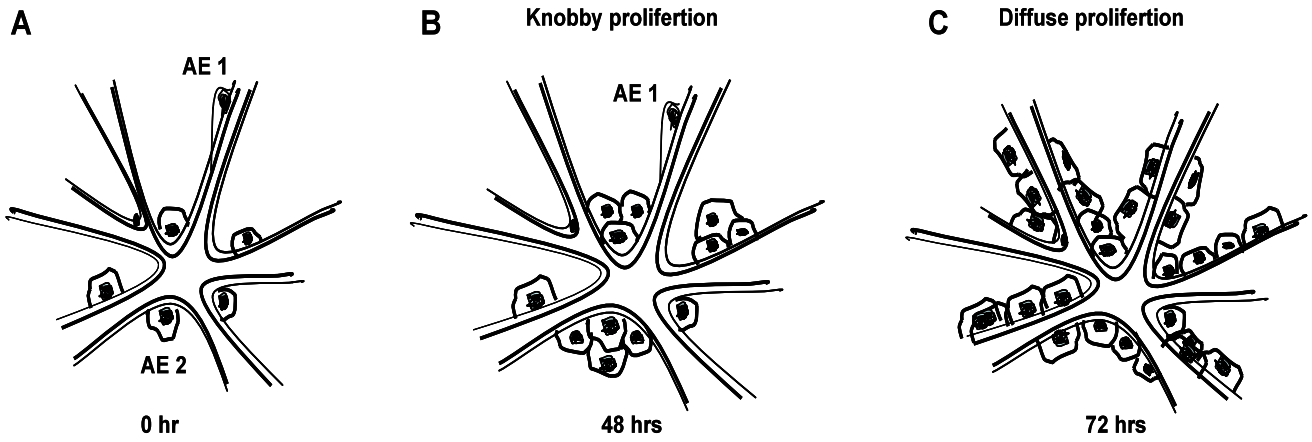


Figure 3 *- Schematic diagram shows the progression of alveolar epithelial type 2 cells (AE2) hyperplasia in the lung after a single intratracheal injection of rHuKGF. [Picture is modified after Ulich et al. (126)]*

1.3 Other growth factors implicated in alveolar repair and maintenance

Growth factors are diffusible proteins that act within a short distance of where they are produced to mediate tissue interactions. Growth factors are the key controller of repair and regulate a variety of cellular functions in lung that are critical for reestablishment of normal lung architecture.

Transforming growth factor β-1 (TGFβ-1) is a member of a family that regulates cell proliferation, differentiation, transformation as well as extra cellular matrix composition and remodling. TGFβ-1 is produced by various cell types including AE2 cells (147). It is particularly critical for induction of elastin gene expression (148) and formation of the elastin scaffold of alveoli (149), which was suggested to be the driving force for alveolarization (150). Disruption of TGFβ-1 signaling at various levels resulted in the development of emphysema-like phenotypes in mice (151-152). TGFß-signaling is initiated by binding of TGFß ligands to the two serine/threonine

2, or TβRII), which then complexes with a type 1 TGFβ receptor (TGFβ-R-1 or activin-like kinase [ALK5] or Acvrl1 or ALK-1 [activin receptor like kinase 1]) (153). The type 1 receptor transmits signals within the cell by means of specific intracellular signals mediators, called smad proteins and finally, nuclear translocation and regulation of gene transcription occurs.

TGFβ-2 transcript, one of the three isoform of TGFβ family, has been found exclusively in the endodermal bronchiolar epithelium and is expressed at high levels during normal murine lung morphogenesis (154). It has been shown that mice with targeted disruption of the TGFβ-2 gene died around birth due to respiratory failure (155).

Vascular endothelial growth factor (VEGF), a heparin binding protein, is highly specific mitogen for vascular endothelial cells. VEGF is integral for endothelial cell migration, proliferation, and survival (156). As the vast pulmonary capillary bed in the lung is crucial in allowing efficient gas exchange to occur between the alveoli and circulating blood supply, this angiogenic growth factor is essential for the development and maintenance of the pulmonary vasculature. Furthermore, during alveolarization process, VEGF contributes to the extensive vascular growth of lung which involves the formation of new blood vessels from endothelial cells within the mesenchyme (157).

VEGF exerts its biological effect on capillary endothelium cells through 2 specific tyrosine-kinase transmembrane receptors, VEGFR1 or fms-related tyrosine kinase-1 (Flt-1) and VEGFR2 or kinase domain receptor (KDR) or fetal liver kinase-1 (Flk-1) (158). VEGFR2 is thought to be the main signaling receptor (159), and VEGFR1 has been speculated to function as a decoy receptor (160). Both of these receptors were initially thought to be largely confined to the capillary endothelial cells, but studies in animal and developing human lung confirm expression in lung tissue on activated macrophages and respiratory epithelial cells (161). Furthermore, VEGF signaling play a pivotal role during airway growth as the lung progressively acquires a rich blood supply through the growth of endothelial cells in the pulmonary mesenchyme which is crucial for the gas exchange function of the human lung. Injury and loss of this tissue

Hepatocyte growth factor (HGF) was originally purified and cloned as a potent mitogen for mature hepatocytes (162). HGF is a multifunctional heterodimeric protein that is synthesized by a variety of lung cell types, including fibroblasts, alveolar macrophages, smooth muscle cells and epithelial cells (163). HGF has potent mitogenic and morphogenic activities on a wide variety of epithelial cells, including alveolar and bronchial epithelial cells (164-165). HGF has capacity to promote survival of endothelial cells and favors angiogenesis (166).

Platelet-derived growth factor (PDGF) was originally isolated from blood platelets as a growth factor for smooth muscle cells, fibroblasts and glial cells. The critical role of platelet-derived growth factor-alpha (PDGF-A) in lung structure maintenance is supported by the findings that a proportion of mouse PDGF-A null mutants (PDGF-A-/-) survive birth and develop generalized lung emphysema, involving the complete loss of alveolar smooth muscle cells (SMC) (167). Such cells are embedded in elastin fibres and normally build up the sphincter-like structures (alveolar ring muscles) around the openings of alveoli into alveolar ducts (168). The absence of alveolar SMC in PDGF-A-/- mice coincided with the loss of septal elastin deposits and failure of alveolar septal formation.

2 Aim of the study

Pulmonary emphysema is one of the most important causes of morbidity and mortality in our modern society. Alveolar destruction is the main morphological aspect of pulmonary emphysema that traditionally had been viewed as irreversible and irreparable. To date, there is no curative therapy available that can restore functional lung parenchyma that has been lost in an emphysematous lung. Recombinant human keratinocyte growth factor (rHuKGF) is known to enhance the regenerative capacity of epithelial tissues.

Hence, it was hypothesized that supplementation of rHuKGF facilitates the induction of a regenerative response in distal lung parenchyma in elastase induced emphysematous lungs of mice. The current study was initiated by treating emphysematous lungs with 10 mg/kg body weight of rHuKGF at three occasions. Substantiation regarding beneficial regenerative response in distal lung parenchyma was validated by performing non invasive lung function test and quantitative morphometry. Furthermore, on molecular level, candidate genes that are associated with biological processes implicated in the repair of alveolar gas exchange area, i.e. alveolar epithelium, capillary endothelium and interstitial tissue were also evaluated by means of quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR), immunohistochemistry and Western blotting.

In order to assess if the beneficial effects of rHuKGF on the interstitial tissue compartment are linked to alveolar epithelial type 2 (AE2) cell-derived transforming growth factor β-1 (TGFβ-1), the effect of rHuKGF on mRNA expression levels in and release of active TGFβ-1 protein from in-vitro model of primary mouse AE2 cells, murine AE2-like cell line LA-4 and AE1-like cell line E10 as well as LA-4/mouse lung fibroblast (MLF) co-cultures was studied. The effect of rHuKGF on MLF cells was examined in order to evaluate whether rHuKGF directly interact with fibroblast cells to affect mRNA expression of TGFβ-1 and its target genes. The importance of TGFβ-signaling as a potential mediator of the AE2-linked effects of rHuKGF was investigated in LA-4/MLF co-cultures in the presence or absence of the neutralizing

3 Materials

3.1 Animals

Male C57BL/J mice (Harlan Winkelmann, Hannover, Germany).

3.2 Antibodies

Table 1 -

Antibodies	***Supplier***
Anti-TGF-β neutralizing antibody	R&D System GmbH Wiesbaden-Nordenstadt, Germany
Biotinylated anti-Mouse-IgG	Vector Laboratories, CA, USA
Biotinylated anti-Rabbit-IgG	Vector Laboratories, CA, USA
Donkey anti-rat IgG conjugated to fluoresceine Isothiocyanate (FITC)	Dianova, Hamburg, Germany
Goat anti-rabbit conjugated to Texas red	Dianova, Hamburg, Germany
Goat horseradish peroxidase-linked secondary antibody	Pierce, Rockford, U.S.A.
Monoclonal mouse anti-PCNA antibody	DakoCytomation, Denmark
Monoclonal rat anti-Mouse Ki-67 antibody	Dako Deutschland GmbH, Hamburg, Germany
Mouse β-actin antibody	Abcam, Cambridge, UK
Polyclonal rabbit anti-TGF-β1 antibody	Biozol, Eching, Germany
Polyclonal rabbit anti-TGF-β2 antibody	Santa Cruz, Burlingham, CA, USA
Polyclonal rabbit anti-VEGFR2 antibody	Abcam, Cambridge, UK

3.3 Biochemicals and chemicals

Table 2 -

Biochemicals and chemicals	***Supplier***
1% SDS	Biorad Laboratories, Hercules, USA

4-15% Tris-HCL Criterion™ pre-cast gel	Bio-Rad, Hercules, USA
3, 3`-diaminobenzidintetrahydrochloride-(DAB)	Sigma, Steinheim, Germany
5x Tris-Borate-EDTA	Sigma-Aldrich, Steinheim, Germany
6x Loading Dye Solution	MBI Fermentas, Germany
Agar-Agar	Merck, Darmstadt, Germany
Agarose NEEO Ultra-Qualitat	Roth, Karlsruhe, Germany
Absolute ethanol	Roth, Karlsruhe, Germany
Alcoholic Eosin	Sigma, Steinheim, Germany
Beta-Marcaptoethanol (98%)	Sigma, Steinheim, Germany
CL- X Posure™ film	Pierce, Rockford, USA
DMEM nutrient medium	Life Technology, Karlsruhe, Germany
Ethidiumbromide	Roth, Karlsruhe, Germany
F12K nutrient medium	Gibco, Carlsbad, CA, USA
Glacial acetic acid	Merck, Darmstadt, Germany
Glycine	MP Biomedicals, CA, USA
KH_2PO_4	Merck, Darmstadt, Germany
Mayer's Hematoxylin	Merck, Darmstadt, Germany
Methanol	Roth, Karlsruhe, Germany
Milk powder	Merck, Darmstadt, Germany
NaCl	Roth, Karlsruhe, Germany
$NaHPO_4$	Sigma, Steinheim, Germany
Novex® Tris-Glycine SDS sample	Invitrogen, Karlsruhe, Germany
Paraffin	Vogel, Giessen, Germany
Paraformaldehyde (PFA)	Roth, Karlsruhe, Germany
Phosphate buffered saline (PBS)	PAA Laboratories, Colbe, Germany
Porcine pancreatic elastase (PPE)	Roche, Basel, Switzerland
PVDF transfer membranes	Life Science Product, Boston, USA
rHuKGF	Amgen, USA
rHu TGFβ-1	R&D system, Minneapolis, MN, USA
Streptomycin	PAA Laboratories, Pasching, Austria
Tris	Roth, Karlsruhe, Germany
Tri-Na-citrate-dihydrate	Roth, Karlsruhe, Germany
Xylene	Merck, Darmstadt, Germany

3.4 Buffers and Solutions

Table 3 -

Buffer	***Composition***
10 mM Citrate buffer	Tri-Na-citrate-dihydrate – 29.41 g Distilled water – 1000 ml The pH was adjusted with HCl to 6
0.05 M Tris-HCl buffer	Tris – 60.57 g Distilled water – 1500 ml The pH was adjusted with HCl to 7.4
SDS buffer	Tris--Glycine buffer (10 x) – 2000 ml 1% SDS – 20 ml
TBS buffer	NaCl – 53 g Tris – 12 g Distilled water – 1000 ml The pH was adjusted with HCl to 7.4
Transfer buffer	Tris glycine buffer (10 x) – 640 ml Methanol – 1600 ml Distilled water – 5760 ml
2% Agar Agar	Agar-Agar – 2 g Tap water – 100 ml
2% Milk solution	Milk powder – 2 g PBS – 100 ml
6% PFA solution	Paraformaldehyde – 6 g PBS- 100 ml
Alcoholic acetic acid solution	Alcoholic Eosin – 200 ml Glacial acetic acid – 3 drops
DAB Solution	3, 3`-diaminobenzidintetrahydro-chloride – 0.2 g

	Tris-HCl buffer – 200 ml

3.5 Cell Lines

Table 4 -

Cell lines	***Supplier***
Mouse lung epithelial cell (LA4)	American Type Culture Collection, Rockville, MD,USA
Mouse embryonic lung fibroblast cell (MLF)	American Type Culture Collection, Rockville, MD,USA

3.6 Enzymes

Table 5 -

Enzymes	***Supplier***
Recombinant RNasin Inhibitor	Promega, Madison, USA
Taq DNA Polymerase	Invitrogen, Karlsruhe, Germany

3.7 Equipment

Table 6 -

Equipment	***Supplier***
A/D converter	DT301 PCI, Marlboro, MA
Amplifier	Gould Universal Amplifier, Dietzenbach, Germany
Biofuge Centrifuge	Heraues , Wehrheim, Germany
Criterion electrophoresis chamber for western blotting	Biorad, Hercules, USA
Electrophoresis chamber for agarose gel	Gentechnology System, Heidelberg, Germany
Gel documentation system	Biorad, Hercules, USA

Heating-blocks	Roth, Karlsruhe, Germany
iCycler PCR machine	Biorad, Hercules, USA
Microplate autoreader	Tecan, Salzburg, Austria
Notocord hem 3, 5	Notocord, Paris, France
Olympus BX 51	Olympus GmbH, Germany
Pneumotechograp	PTM 378/1.2 Hugo Sachs Electronic March-Hugstetten, Germany
Polyester transwell membranes	Fisher Sci. Schwerte, Germany
Pressure transducer	8T-2, Gaeltec, Dunvegan, Scotland
RoboCycler® thermocycler	Stratagene, USA
Rotation microtome	Leica, Germany
Stereology Software Package	Olympus, Denmark
UV-spectophotometer	Pharmacia Biotech, Cambridge, UK
Vortex	IKA®, Wilmington, USA

3.8 Fragment Length Standards

Table 7-

Fragments length standards	***Supplier***
100 bp DNA Ladder	Promega, Madison, USA
Novex® Sharp Pre-stained Protein Standards	Invitrogen, Karlsruhe, Germany

3.9 Kits

Table 8 -

Kits	***Supplier***
Avidin/Biotin Blocking Kit	Vector Laboratories, USA
BCA™ protein assay Kit	Pierce, Rockford, USA
Chemiluminescence's detection Kit	SuperSignal® West Femto, Pierce, USA
Elisa Kit	R&D System Gmb Wiesbaden-ordenstadt, Germany

Rneasy Kit	Qiagen, Hilden, Germany
SYBR™ Green PCR mix Kit	Abgene® Thermo Fischer Scientific, UK
Total Protein Extraction Kit	Biochain Institute, Hayward, CA, USA
Vector M.O.M. (Mouse on mouse) Kit	Vector Laboratories, USA
Vecta Stain Elite ABC Kit	Vector Laboratories, USA

3.10 Oligodeoxynucleotides

Oligo (dt) 12-18 Primers were obtained from Invitrogen, Karlsruhe, Germany. Target genes primers were obtained from Biomer, Ulm, Germany.

Table 9 -

Target	***Left primer (5´-3´)***	***Right primer (5´-3´)***	***Size (bp)***	***Tm (°C)***
AQP-5	tcctggctgcaatcctctac	cagctcgatggtcttcttcc	143	60
Cyclin D1	gcgtaccctgacaccaatct	atctccttctgcacgcactt	110	60
Elastin	ctggtgttggtcttccaggt	gctttgactcctgtgccagt	112	60
FGF-7	ccatgaacaaggaagggaaa	tccgctgtgtgtccatttag	122	57
FOXA-2	agcaccattacgccttcaac	ccttgaggtccattttgtgg	111	60
GAPDH	aatggtgaaggtcggtgtgaac	gaagatggtgatgggcttcc	262	60
HGF	aggaacaggggctttacgtt	gctgcctcctttaccaatga	180	60
Nrf-2	aggacatggagcaagtttgg	tctgtcagtgtggcttctgg	121	60
PAI-1	gtagcacaggcactgcaaaa	atcacttgccccatgaagag	208	53
PCNA	ccacattggagatgctgttg	ccgcctcctcttctttatcc	128	60
PDGFA	gagataccccgggagttgat	cttgtctccaaggcatcctc	192	60
SP-1	tgggtacttcagggatccag	tccttctccacctgctgtct	143	60
SP-C	cagctccaggaacctactgc	agcttagaggtgggtgtgga	130	60
Smad 2	ggaacctgcattctggtgtt	gcagaacctctccgagtttg	130	60
TGFβ-1	gtccttgccctctacaacca	gttggacaactgctccacct	129	60

TGF-β-R1	attgctggtccagtctgctt	cctgatccagaccctgatgt	188	60
TGFβ-R-2	atctggaaaacgtggagtcg	tttcatgcctccactgatgg	187	60
VEGF	aatgatgaagccctggag	atgctgcaggaagctcatct	114	60
VEGFR1	ttaggggttctccataccc	tttctttggggtctttgtgc	148	60
VEGFR2	acagttcccagagtggttgg	gtcactgacagaggcgatga	129	60

3.11 Serum

Table 10 –

Serum	***Supplier***
Fetal bovine serum	Gibco-BRL, Eggenstein, Germany
Normal goat serum	Vector Laboratories, CA, USA

4 Methods

4.1 Experimental design: In-vivo experiments

4.1.1 Mouse model

Eleven weeks old C57BL/6 male mice of weight 28.3±1.9 g were included in the study. All animal experiments were approved by the regional government (Regierungspräsidium Giessen, Dezernat V 54, Giessen, Germany) in accordance with the guidelines that complied with national and international regulations. Mice were housed in humidity- and temperature-controlled rooms and were allowed food and water ad libitum. Experimental mice were distributed into A) treatment groups and B) therapy groups as per described below (Fig.4). Instillation of porcine pancreatic elastase (PPE), phosphate buffered saline (PBS), and rHuKGF was done by oropharyngeal aspiration method as per follow:

Oropharyngeal aspiration: To instill either PPE or rHuKGF (or PBS) into the mice lungs, mice were initially anesthetized by isofluran and immediately suspended by their upper incisors from a rubber band. The tongue was gently extended out and with the help of forceps, the nose was occluded to force the animal to swallow via the mouth. Thus, the liquid volume of either PPE or rHuKGF (or PBS) that was placed into the distal part of the oropharynx was aspirated into the lungs.

A) Treatment groups-

PPE group: To induce emphysema by elastase in the mice lungs (n=7), mice were initially anesthetized and 2.25mg/kg b.w of PPE, which was dissolved in PBS to make a final volume of 80 µl, was instilled by oropharyngeal aspiration method at day 0 & 10.

PBS group: As a control to PPE-instillation, 80 µl of PBS was instilled directly into the lungs of control mice (n=5), at day 0 & 10, by oropharyngeal aspiration as per described for the PPE group.

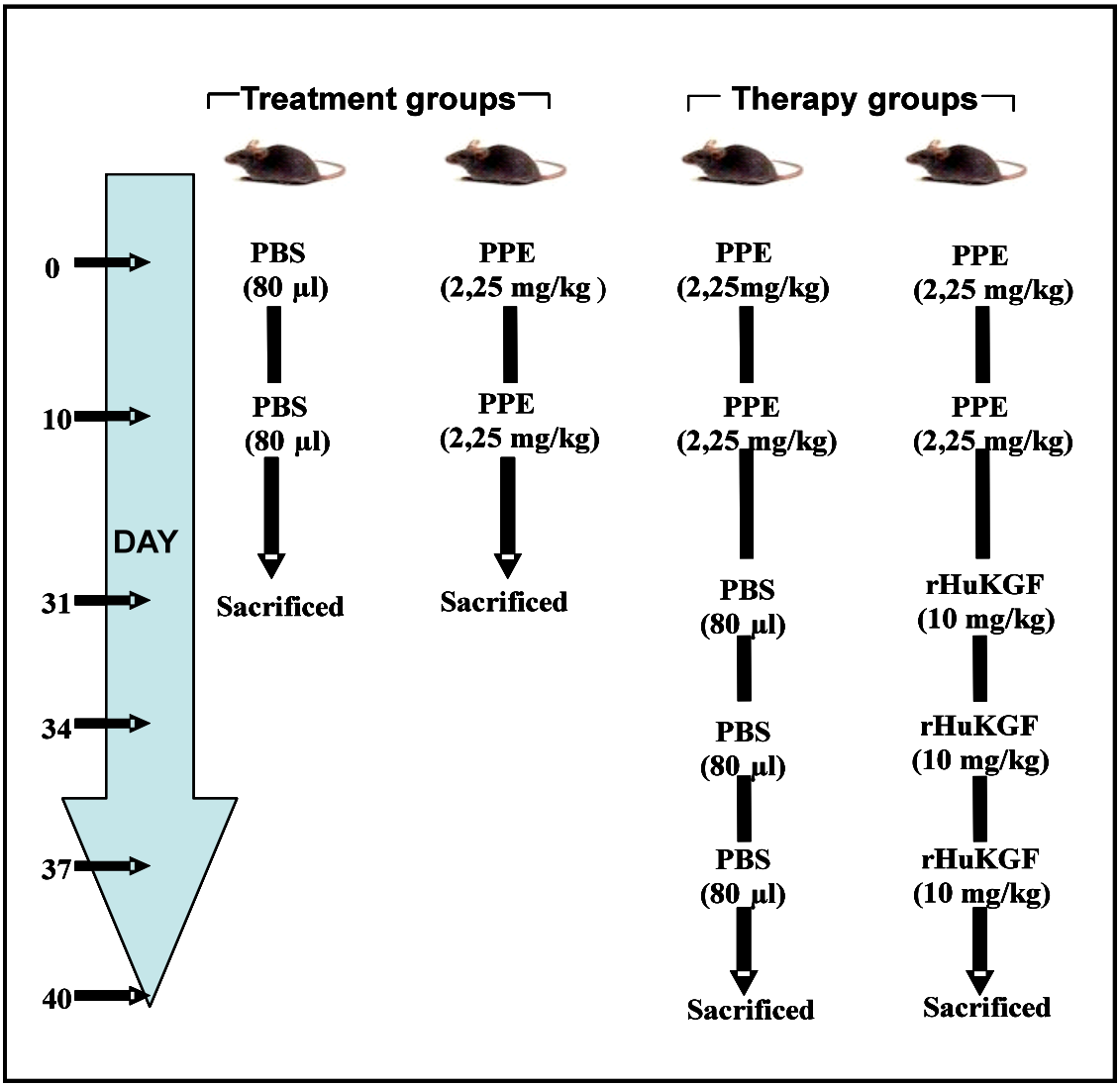
B) Therapy groups-

PPE+KGF group: Emphysema in mice (n=6) was induced as per described in PPE group. On day 31, 34, and 37 respectively, PPE treated lungs received 10 mg/kg b.w of rHuKGF dissolved in PBS to make a final volume of 80 µl per oropharangeal aspiration.

PPE+PBS group: Emphysema in mice (n=6) was induced as per described in PPE group. On day 31, 34 and 37 respectively, PPE treated lungs to be used as a treatment control received 80 µl of PBS alone per oropharyngeal aspiration.

.

Body weight of each mouse was measured on day 0, 10 and 31 or 40.



4.1.2 Lung function analysis by non invasive head-out body plethysmography

Lung function was assessed in each group by head-out body plethysmography (169). This method was used to analyse the breathing pattern of each animal by continuously registering changes in airflow during inspiration and expiration. Mice were positioned in the head-out body plethysmograph with the head protruding through a neck collar (9-mm ID, dental latex rubber) attached to an exposure chamber. Before starting the monitoring of respiratory function, mice were allowed to acclimatize for 15 min in the body plethesmograph. The airflow was measured with a pneumotachograph and a differential pressure transducer coupled to an amplifier. These devices were attached to the top port of each plethysmograph chamber. For each mouse, the amplified analog signal from the pressure transducer was digitized by an analog-to-digital converter at a sampling rate of 2,000/s and the mid-expiratory airflow (EF50, i.e. the expiratory airflow when 50% of the tidal volume (VT) is exhaled) was calculated by means of the software program in ml/s for each breath over a period of 15 minutes. Difference in EF50 values were calculated to the controls (set to 100%) (Fig.5).

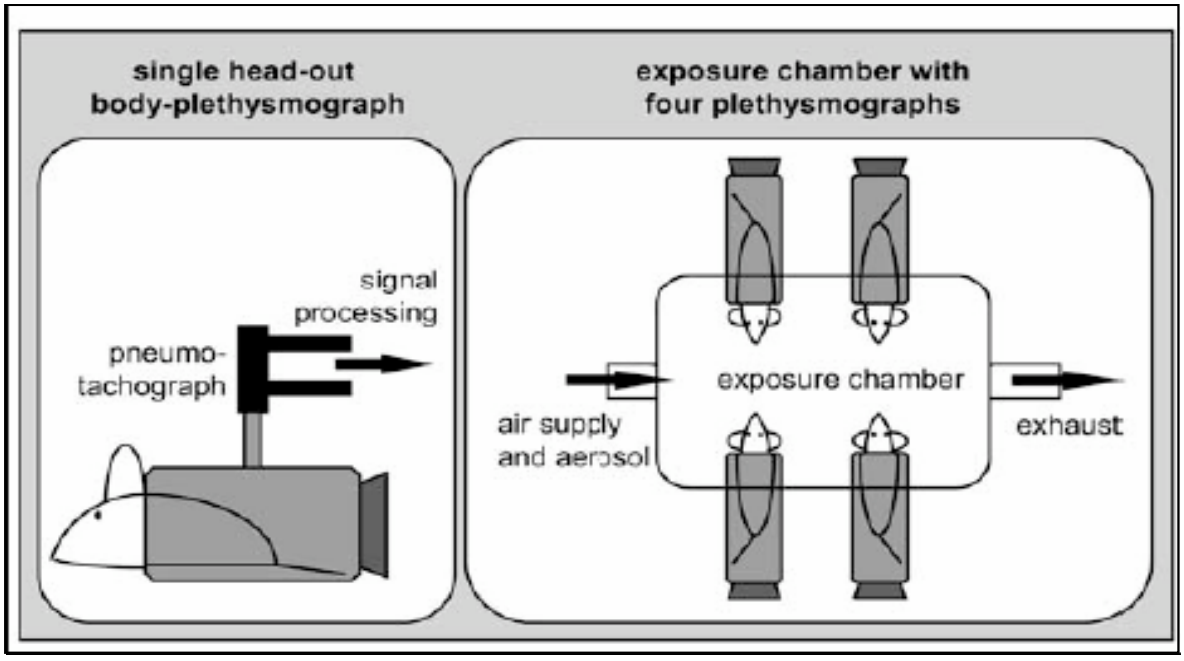


Figure 5 - *Schematic drawing of the head-out plethysmograph system used for the measurement of breathing patterns in mice.*

4.1.3 Lung fixation

Mice were euthanized by cervical dislocation on day 31 (PPE- and PBS- group) or on day 40 (PPE+KGF- and PPE+PBS- group). Through the tracheotomy, a cuffed endo-tracheal tube was inserted and securely tied to the trachea. The thoracic cavity was opened and the lungs were perfused free of blood with isotonic saline through the right ventricle of the heart. The left lung was removed for molecular biology studies whereas the right lung was inflated through the trachea with 6% phosphate-buffered paraformaldehyde at a pressure of 20 cm of fluid column for 20-25 minutes. The right main bronchus was ligated tightly and after submersion in the same fixative, lungs were stored in the refrigerator overnight. Lung volume was measured by fluid displacement (170). Subsequently, the lungs were embedded into 2% aqueous agarose and stored for 2 hour in the cold. According to the principle of systematic uniform random sampling (171), embedded lungs were cut into equidistant slices of 2-mm thickness.

4.1.4 Lung tissue preparation: Paraffin embedment

Paraffin embedding is a process in which fixed tissue (utilizing neutral buffered formalin or another fixative) is infiltrated by paraffin to harden the tissue and to stabilize it for long-term storage and thin sectioning. Agarose embedded lung tissue slices were placed into 10% neutral buffered formalin for 1 hour at a temperature of 40°C. Further, tissue slices were placed in 96% ethanol and incubated at 40°C for 20 min. This process was repeated two times. Thereafter, tissue slices were transferred to 100% ethanol and incubated for 20 min at 40°C. This step was repeated twice by transferring the tissue slices to fresh 100% ethanol. Further, tissue slices were transferred to xylene and incubated for 60 min at 40°C. This step was repeated one more time by transferring the tissue slices to fresh xylene. Afterwards, tissue slices were added to the melted paraffin at 60°C. Paraffin was changed three times until the tissue slices were infiltrated completely. Subsequently, infiltrated tissue slices were placed into a block of molten paraffin and allowed to cool and solidify before making tissue sections.

4.1.5 Lung tissue sectioning and histochemical staining

Paraffin blocks were placed into the microtome in a position that top and bottom edges were parallel with the blade in order to get even 2 µm thick cuts. With the help of small paintbrush, rolling and wrinkling of sections were prevented and sections were placed in clean slides. To evaporate the water and to fix the sections on glass, all slides were kept in heater at 37°C overnight. In order to proceed with histological staining, slides were first de-paraffinized by immersing in xylene for 15 minutes twice until the paraffin had been dissolved. Thereafter, slides were hydrated by passing them through graded alcoholic solutions, in 100% for 10 min, 96% for 5 min and 70% for 10 min.

Hematoxylin is the oxidized product of the logwood tree known as hematein and in order to use it as a stain it must be "ripened" or oxidized. Hematoxylin, being a basic dye, has an affinity for the basophilic structures containing nucleic acids, such as the ribosomes, chromatin-rich cell nucleus, and nucleic acids of the cell nucleus. Eosin is fluorescent red acidic dye with an affinity for cytoplasmic components of the cell. After hydrogenation, slides were rinsed off in tap water and were immersed in Mayer's Hematoxylin for 5 min and rinsed off in running water to get blue in colour. Slides were then stained with alcoholic eosin with glacial acetic acid solution for 5 min and rinsed off with running water to remove extra staining. In order to dehydration, stained slides were taken through a series of alcoholic solution with ascending order with 70% alcohol for 10 min, 96% alcohol for 5 min, 100% alcohol for 10 min and finally slides were kept in xylene for 10 min and afterwards tissue sections were covered with cover-slip to preserve them for long time.

4.1.6 Quantitative stereological analysis

Morphometric-based quantification was conducted on H&E-stained slides using a BX51 Olympus light microscope linked to a computer assisted stereology toolbox (C.A.S.T.). To assess air space enlargement, the mean chord length (MCL), the most commonly used indicator of emphysema (172), was quantified by superimposing a line grid on the images of lung sections at a magnification of 852.6x. Points on the lines of the grid hitting the air spaces and intercepts of the lines with alveolar septa

$$MCL = \Sigma P_{air} \times L_{(p)} / (I_{septa} / 2) \quad (\mu m)$$

where ΣP_{air} is the sum of the points of the grid overlaid air spaces, $L_{(p)}$ is the line length per point and I_{septa} is the sum of the intercepts of alveolar septa with the lines of the grid.

Morphometric assessment of alveolar surface area per unit volume of lung parenchyma (S_v), absolute volume of alveolar air spaces (V_{air}), absolute volume of alveolar septal tissue (V_{ast}) were determined by counting the number of points that fell on alveolar septal tissue, alveolar space, other structures such as airways and capillary lumen, and by counting the number of intercepts with alveolar septal surface at a magnification of 852.6x (Fig.7) according to the following formulas:

$$S_v = 2 \times I_{septa} / (\Sigma P_{par} \times 78.7/4) \quad (1/\mu m)$$

$$V_{air} = [(\Sigma P_{air}) \times 16] / (\Sigma P_{par} \times Vv_{(par, lung)} \times V_{RL}) \quad (mm^3)$$

$$V_{ast} = \Sigma P_{ast} / (\Sigma P_{par} \times Vv_{(par, lung)} \times V_{RL}) \quad (mm^3)$$

where ΣP_{par} is the sum of the points of hitting parenchyma, 78.8/4 is the line length per test point in μm, ΣP_{ast} the sum of points of hitting alveolar septal tissues, and Isepta the sum of the intercepts with alveolar septa.

$Vv_{(par, lung)}$ is the volume of parenchyma per volume of lung and was estimated by counting the points that fell on parenchyma relative to points on total lung using 425.4 x magnification (Fig.8) and calculated as:

$$Vv_{(par,lung)} = \Sigma P_{par} / \Sigma P_{lung\ tiss}$$

V_{RL} is the actual volume of the right lung obtained by the following formula.

$$V_{RL} = V_{flu\ disp} * \Sigma P_{lung} / \Sigma P_{all} \quad (mm^3)$$

where $V_{flu\ disp}$ is the volume measured by fluid displacement (175), ΣP_{lung} the test point hitting lung cross section and ΣP_{all} the test points hitting lung and adhering tissues.

For correction of global shrinkage of tissues due to processing, area shrinkage was measured and correction factors for both linear and volume shrinkage were derived using the following formulas:

$$\text{Section area} = \Sigma P_{lung} * A_{(p)} \quad (cm^2)$$

$$\text{Expected area} = V_{RL} / 0.2945 \quad (cm)$$

Area Shrinkage = (Section area – Expected area) / Expected area

$$\text{Volume Shrinkage} = (\text{Linear Shrinkage})^{3}$$

where $A_{(p)}$ is the area per test point and 0.2945 is the mean thickness of the lung slices. Therefore, MCL was corrected for shrinkage by dividing it by the group-mean linear shrinkage factor. Total volume of right lung (V_{RL}) and total alveolar surface area (S_a, in cm2) were corrected for tissue shrinkage as follows:

$$V_{RL\ (corrected)} = V_{RL} * (1- \text{volume shrinkage})$$

$$S_a = S_v * V_{RL\ (corrected)} \quad (cm^2)$$

In addition, arithmetic mean thickness of the alveolar septal wall (τ_{ast}) was calculated according to the formula:

$$\tau_{ast} = V_{ast} / S_a \quad (cm)$$

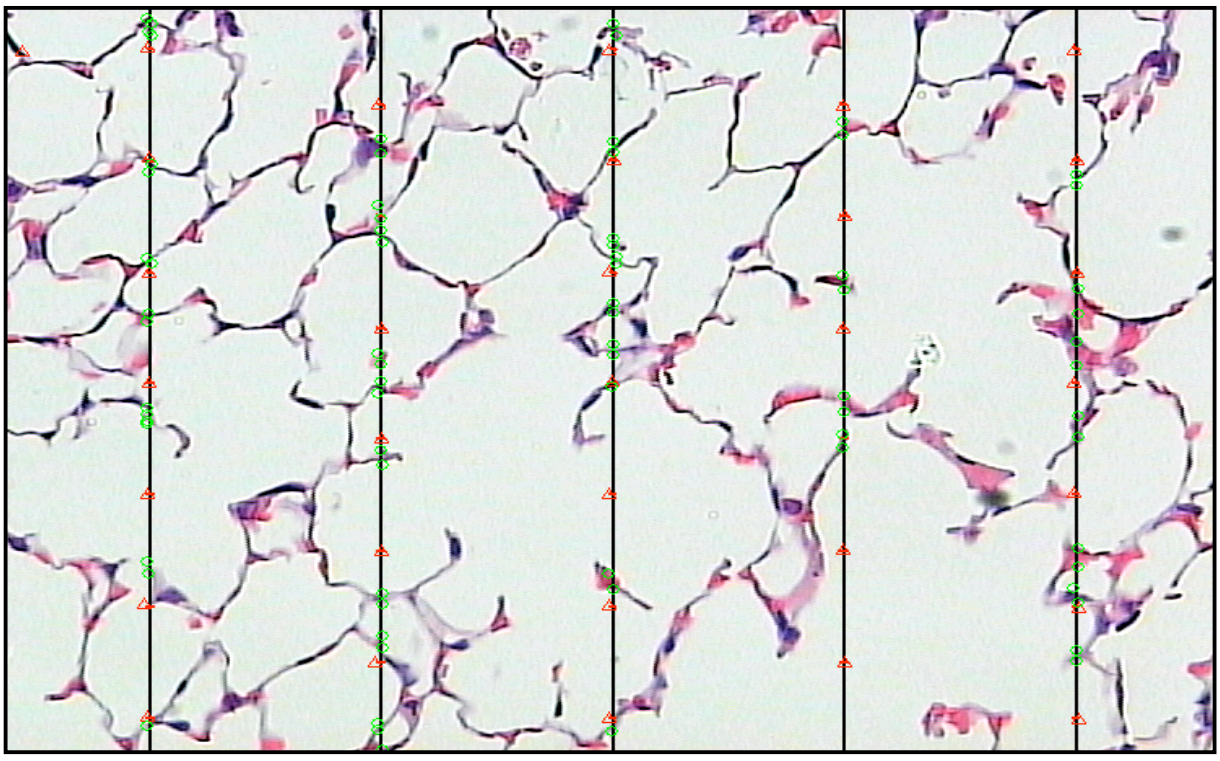


Figure 6 - *A lines grid generated by C.A.S.T. was superimposed on each section of lung tissue. Points of lines hitting air spaces [(ΣP_{air}), marked with red triangle] and total number of intecepts of the lines with alveolar septal surfaces [(I_{septa}), marked with green circle] were counted to calculate MCL. (This image is a screenshot*

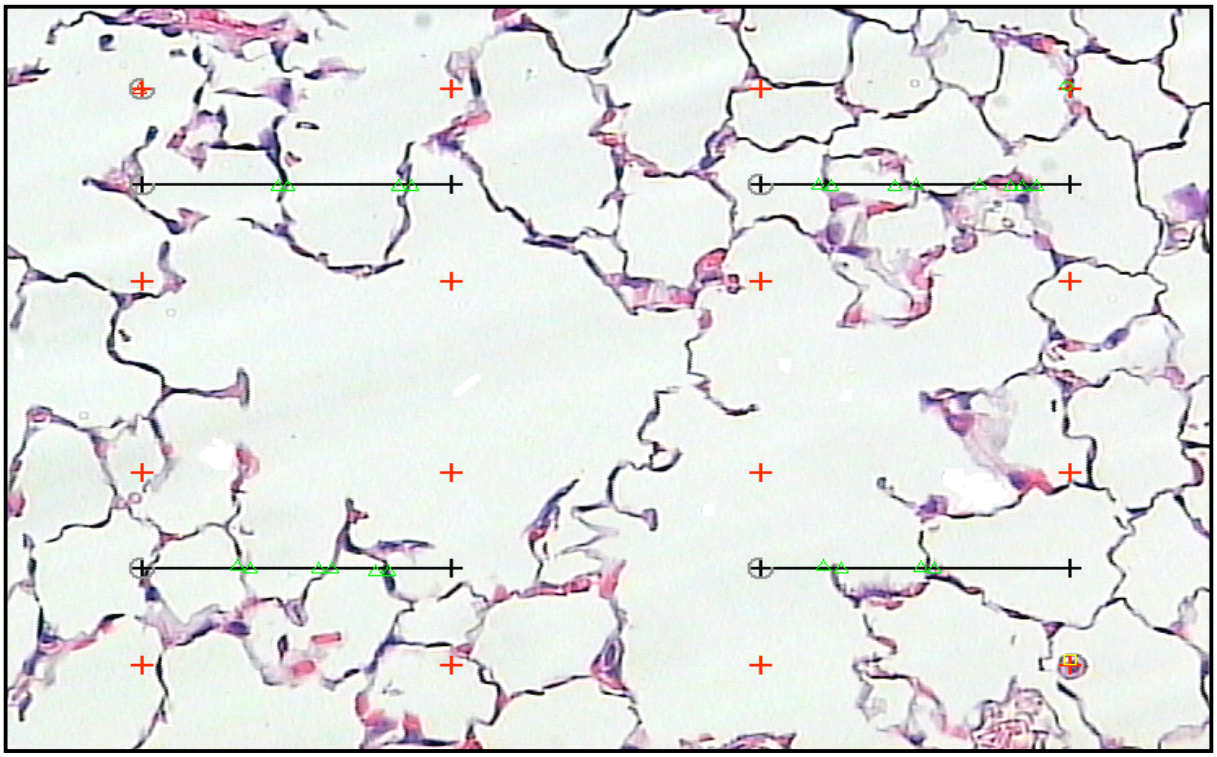


Figure 7 - *A grid with points and line segments generated by C.A.S.T. was superimposed on each section of lung tissue. Points (red crosses) overlying alveolar air space [(ΣP_{air}), marked with red triangle], alveolar septal tissue [(ΣP_{ast}) marked with green circle] and capillary lumens [(ΣP_{caplu}), marked with yellow square] were counted to estimate points on parenchyma (ΣP_{par}). Additionally, the total number of intercepts of the line segments with the alveolar septal surfaces [(I_{septa}), marked with green triangle] were counted. (This image is a screenshot captured on C.A.S.T. to demonstrate the counting procedure.)*

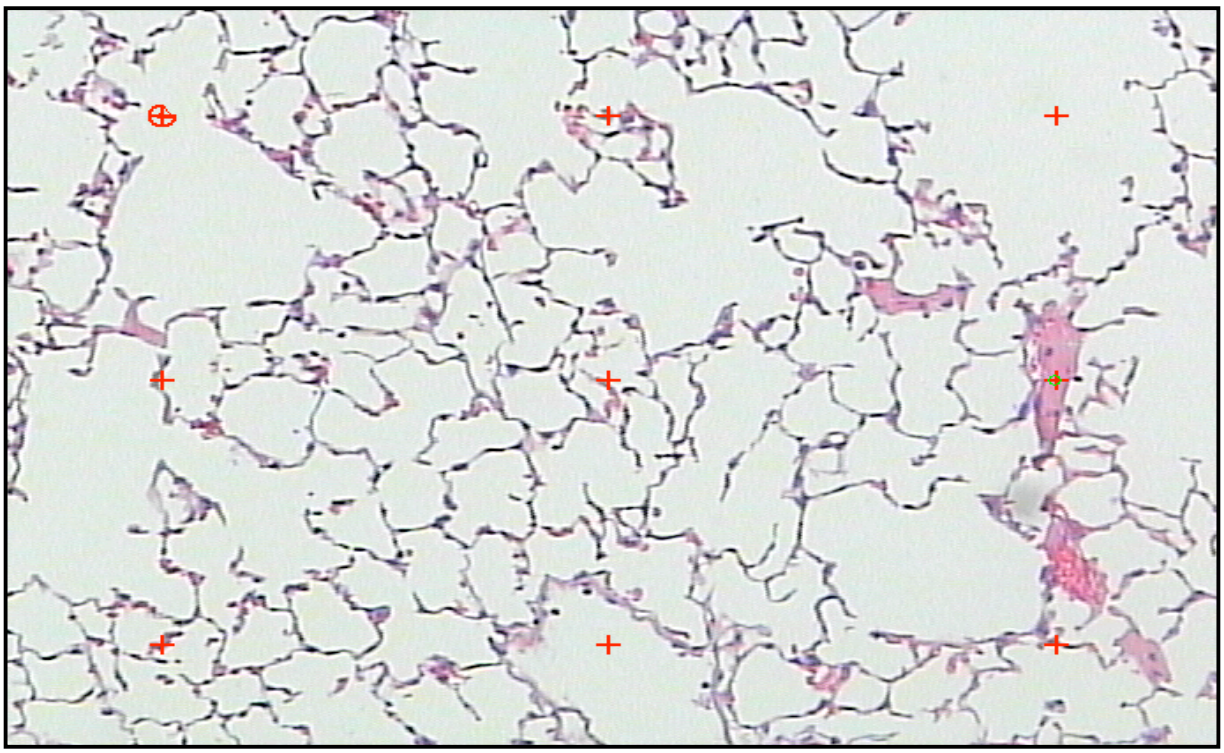


Figure 8 - *A grid with points generated by C.A.S.T. was superimposed on each section of lung tissue. Points (red crosses) falling on lung parenchyma i.e. alveolar air spaces, alveolar septa [(ΣP_{paren}), marked with red triangle] and non-parenchyma i.e. airways, blood vessels [($\Sigma P_{nonparen}$), marked with green circle] were counted. The sum of ΣP_{paren} and $\Sigma P_{nonparen}$ represented ΣP_{lung} and the sum of ΣP_{paren}, $\Sigma P_{nonparen}$ and $\Sigma P_{nonlung}$ represented points on all embedded tissues (ΣP_{all}) and were used to calculate $Vv_{(par,\ lung)}$ and V_{RL}. (This image is a screenshot captured on C.A.S.T. to demonstrate the counting procedure.)*

4.1.7 Immunohistochemistry

4.1.7.1 PCNA immunostaining

To study the PCNA protein expression in the tissues, immunohistochemistry with anti-PCNA antibody was accomplished using a mouse-on-mouse (M.O.M.) peroxide kit. Paraffin embedded tissue sections were deparaffinized by immersing in thrice changes of xylene for 15 min each and then in absolute alcohol. In order to inhibit endogenous peroxidase, sections were blocked with 3% H_2O_2 in methanol for 30 min. Thereafter, to recover antigenicity of tissue, antigen retrieval was performed by boiling sections in citrate buffer (at 450 W, 1mM, pH 6) for 5 min in a microwave oven for three times. Sections were then washed with PBS for 5 min and subsequently pre-treated with 100 µl avidin for 15 min, washed with PBS for 5 min followed by treatment with biotin solution for 15 min. Thereafter, sections were incubated with blocking reagent (M.O.M. Kit) for 1 hour to block non-specific binding of immunoglobulin and subjected to washing in 2% milk powder solution for 5 min and treated with 100µl M.O.M. diluent for 5 min. Afterwards, sections were incubated with mouse anti-PCNA primary anitibody (dilution 1:600 in PBS) for 1 hour at 37°C temperature. Once the primary antibody was bound to the antigen of interest, the biotinylated anti-mouse IgG secondary antibody was added (dilution 1:100 in PBS) for 10 min, forming a biotinylated antibody complex bound to the target antigen. Thereafter, sections were exposed to avidin-biotin-conjugated peroxidase for 30 min at room temperature and to diaminobenzidine tetrahydrochloride (DAB) for 10 min which resulted in brown staining. After rinsing in water, counterstaining was performed with Mayer's hematoxylin solution and sections were dehydrate through 70% alcohol for 10 min, 96% alcohol for 5 min and absolute alcohol for 10 min and kept in xylene for 15 min.

4.1.7.2 TGF-β1 immunostaining

TGFβ-1 immunostaining was done using avidin-biotin-complex (ABC) technique. Paraffin embedded sections were deparaffinised and rehydrated followed by blocking in 3% H_2O_2 in methanol as described above. After antigen retrieval and washing in TBS for 5 min, sections were blocked with 10% normal goat serum (dilution 1:10 in

1:100 in PBS) for 1 hour at 37°C and sequentially subjected to biotinylated anti-rabbit secondary antibody (dilution 1:100 in PBS) for 30 min. After washing sections with PBS for 5 min, signels were visualzed using avidin-biotin-conjugated peroxidase for 30 minutes and diaminobenzidine tetrahydrochloride (DAB) for 10 min. Thereafter, sections were rinsed in water, counterstained with Mayer's hematoxylin solution and dehydrated through 70% alcohol for 10 min, 96% alcohol for 5 min and absolute alcohol for 10 min and kept in xylene for 15 min.

.

4.1.7.3 TGF-β2 immunostaining

TGFβ-2 immunostaining was performed using avidin-biotin-complex (ABC) technique. Dewaxed paraffin sections were blocked with 0, 9% H2O2 in methanol as described above. After washing, the slides were incubated with normal goat serum (dilution 1:10 in PBS) for 20 min. The slides were then incubated with polyclonal rabbit anti-TGF-β2 primary antibody (dilution 1:50 in PBS) for 1 hour at 37°C, rinsed and subsequently incubated with biotinylated anti-rabbit secondary antibody (dilution 1:100 in PBS) for 30 min. After washing in PBS, signals were visualized using avidin-biotin peroxidase with diaminobenzidine tetrahydrochloride (DAB) for 10 min and counterstained with Mayer’s hematoxylin solution. Unless stated otherwise, all steps were performed at room temperature.

4.1.8 Double immunofluorescence microscopy

In order to perform double immunofluorescence staining, dewaxed and microwave pre-treated paraffin sections of lung were incubated with monoclonal rat anti-mouse Ki-67 (clone TEC-3; dilution 1:3 in PBS) which is a marker of proliferating cells (191), and polyclonal rabbit antipodocalyxin (kind gift from Dr. M. Farquhar, La Jolla, CA; dilution 1:1000 in PBS) which is a marker of endothelial cells (192), for 1 hour at room temperature followed by specific donkey anti-rat IgG conjugated to fluoresceine isothiocyanate (FITC, dilution 1:200 in PBS), and goat anti-rabbit IgG conjugated to Texas Red (dilution 1:100 in PBS) for 1 hour at room temperature.

4.2 In-vitro experiments

To have a better understanding if the effects of rHuKGF on interstitial tissue compartment observed in-vivo were mediated via alveolar epithelial type 2 (AE2) cells derived TGFβ-1, an in-vitro model of primary AE2 cells isolated from C57BL/6 mouse lungs, murine cell line LA-4 which exhibits an AE2-like phenotype (173) and E10 which exhibits an alveolar epithelial type 1 (AE1)-like phenotype (174), as well as of murine embryonic lung fibroblast cell line MLF and LA-4/MLF co-cultures were utilized as described below.

4.2.1 Isolation of primary lung alveolar epithelial type 2 (AE2) cells

Mouse primary AE2 cells (which were kindly provided by Prof. Bernd Müller, Philipps-University, Marburg) were isolated from C57BL/6 mice using a slightly modified protocol described previously (175). Briefly, mice were anaesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg b.w.) mixed with 100 IU heparin sodium. As soon as deep anaesthesia was achieved, thoracotomy was performed and the trachea was cannulated. An incision was made in the vena cava and the lungs were perfused free of blood via the pulmonary artery and removed from the thorax. Lungs were instilled with 650 U of PPE. Digestion with elastase was allowed to take place at 37°C for 20 min before the large airways were removed. In the presence of DNAse I (250 µg/ml), the lungs were minced with scissors and the elastase reaction was then stopped by addition of 5 ml fetal bovine serum per lung. The final cell suspension was filtered several times through gauze nylon and washed by gentle centrifugation. The cell pellet was resuspended in DMEM cell culture medium and transferred to mice immunoglobulin G-coated bacteriological Petri dishes to a density of $30x10^6$ cells. After incubation for 1 hour at 37°C in a 10% CO2-air incubator, alveolar macrophages were adherent to the plastic dishes whereas AE2 cells were unattached and could therefore, be removed, centrifuged, and used for subsequent cell culture experiments. The purity of the cell preparation was evaluated by means of Papanicolaou staining to be greater than 95%. Finally, the primary AE2 cells were cultured in 12-well plates at concentrations of $1x10^5$ cells/well using DMEM cell culture medium. After 24 hours, cells received fresh medium supplemented with 50 ng rHuKGF per ml or an equivalent amount of PBS as a

time RT-PCR analysis of gene expression and cell culture supernatants were collected for measurement of activated TGFβ-1 concentrations by standard ELISA procedures as described below.

4.2.2 Cell culture conditions

Cells of the mouse lung epithelial cell lines LA-4, E10 and MLF were cultured in F12K nutrient mixture supplemented with 10% fetal calf serum, 1% glutamine, 10.000 U/mL penicillin, and 10.000 µg/mL streptomycin. The murine lung epithelial cell line E10 was kindly provided by M. Williams (Pulmonary Center, Boston University School of Medicine, Boston, MA, USA). This cell line was generated by A. Malkinson (Denver, CA) and has an AE1 cell like phenotype e.g. expression of caveolin-1, aquaporin-5, T1α and other AE1 cell−related proteins (174).
Cells were seeded into 12-well plates at concentrations of $1x10^5$ cells/well. After 2 days in culture, cells had reached 80% confluency, and culture medium was changed. Cells received fresh medium supplemented with either 50 ng rHuKGF per ml, 10 ng recombinant human TGFβ-1 (rHuTGFβ-1) per ml or an equivalent amount of PBS as a control. After treatment for 6 hours and 24 hours cell culture supernatants were collected for measurement of activated TGFβ-1 concentrations by standard ELISA procedures as described below or cells were harvested using RLT buffer for real time RT-PCR analysis of gene expression.

4.2.3 Co-culture of epithelial (LA-4) and fibroblast (MLF) cells

LA-4 were seeded onto Polyester Transwell membranes (Costar, insert membranes with pore size 0.4 µm) at concentrations of $1x10^5$ cells/well and incubated for 8 hours. F12K nutrient cell culture medium, supplemented with 10% FCS, 1% glutamine, 10.000 U/mL penicillin, and 10.000 µg/mL streptomycin was changed, and 1x105 MLF cells were placed into the 12-well Costar Transwell plates. After incubation for 48 hours, cells received fresh medium supplemented with 50 ng rHuKGF per ml or an equivalent amount of PBS as a control. Co-cultures were incubated with rHuKGF or PBS in the presence or absence of anti-TGFβ neutralizing antibody (clone 1D11, 20 µg/ml) or control IgG1. Clone 1D11 neutralizes the activity of TGFβ-1, -2, and -3. Cell supernatants were collected for measurement of activated

4.3 RNA isolation, RNA quantity, and purity measurement

Thirty milligram frozen lung tissues were chopped off with sterile scalpel. Chopped off pieces were collected in 1.5ml sterile eppendorf tubes. Fine chopped tissue pieces were immersed in 800µl of RLT buffer with 8µl beta-mercaptoethanol. Tissue homogenate was prepared using a sterile needle and syringe until a fine tissue mixture was obtained. Immediately afterwards, total cellular RNA was isolated using Qiagen's RNeasy column technology method as per the instructions given for the Qiagen RNeasy Miniprep kit.

Total RNA quantification and purity was determined with an Ultraspec 2100-spectrophotometer. The quantity of the isolated RNA was calculated from the absorbance at 260 nm, and the purity was determined by calculating the ratio at optical density$_{260\ nm}$/optical density$_{280\ nm}$.

4.4 cDNA synthesis

First-strand cDNA was synthesized by introducing equal amounts of RNA (2.0µg) from each sample in a total reaction volume of 20 µl using an Omniscript RT kit by adding 1µl RNasin inhibitor, 2µl oligo dTs primers, 2µl 10X RT buffer, 2µl 5mM dNTP, 1µl reverse transcriptase. Total reaction volume of 20µl was adjusted with RNase free water. Reverse transcription reactions were performed for 1 hour at 37°C and 5 min at 93°C.

4.5 Quantitative real-time PCR

To determine the relative mRNA expression of target genes (see table 9), real-time PCR was performed in 96-well format iCycler Detection System from BioRad system. Each 20 µl reaction mixture contained 10 µl of SYBR Green PCR mix, 1 µl of each gene-specific forward and reverse primer (10 pmol/ µl), 1 µl cDNA, and 7 µl water. The thermal cycle conditions used for all reactions were as follows: 95°C, 15 min; 95°C, 50 sec; sequence-specific primer's annealing temperature, 40 sec; 72°C, 40 sec; 72°C, 5 min. Amplification curves were evaluated using the instrument's

data [Cycle threshold (Ct) values] were exported to spread sheet software (MS-Excel) for further analysis.

The relative mRNA expression of the target genes were normalized with the endogenous reference gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) using the formula 2 raised to the power of delta cycle threshold ($2^{\Delta Ct}$), where ΔCt = Ct, reference gene – Ct, target gene. Differences between groups were calculated with respect to controls (1%).

4.6 Protein extraction and quantification

Protein was extracted from 100 g lung tissue using total protein extraction kit from Biochain according to the manufacturer's protocol. The protein concentration of each sample from lung tissues was determined using a BCA™ protein assay kit according to their respective protocol.

To quantified β-actin, PCNA and VEGFR2 expressions, 25 µg of protein from each sample was mixed with Tris-Glycine SDS sample buffer and were fragmented by adding 5 µl of β-mercaptoethanol followed by heating for 5 min at 95°C. Proteins were subjected to electrophorosis on 4-15% Tris-HCL Criterion™ pre-cast gel at 60 V for 30 min and 120 V for 90 min and blotted to PVDF transfer membrane. Novex® sharp pre-stained protein standard was used as standard in SDS-PAGE. Non-specific sites on blots were blocked with 10% skim milk in PBS for 1 hour. Blocked blots were incubated with primary antibodies against β-actin, PCNA and VEGFR2 for overnight at 4°C. Blots were washed with PBS twice for 10 min and then incubated with horseradish peroxidase–linked secondary antibody (1:400) for 1 hour at room temperature. Blots were washed again with PBS twice for 10 min; specific bands were visualized by autoradiography using enhanced chemiluminescence according to the manufacturer's instructions. Blots were scanned and quantified using computer based densitometer and analysis software, which was carried out on negative images of the western blots. Expressions of PCNA and VEGFR2 were normalized with β-actin expression.

4.7 Enzyme Linked Immunosorbent Assay (ELISA)

Levels of active TGFβ-1 in cell culture supernatants were determined using Quantikine ELISA kit according to the manufacturer's instructions. Plates were read in a microplate autoreader at 405 nm. A standard curve was generated using the known amounts of purified recombinant TGFβ-1 provided with the kit.

4.8 Statistical analysis

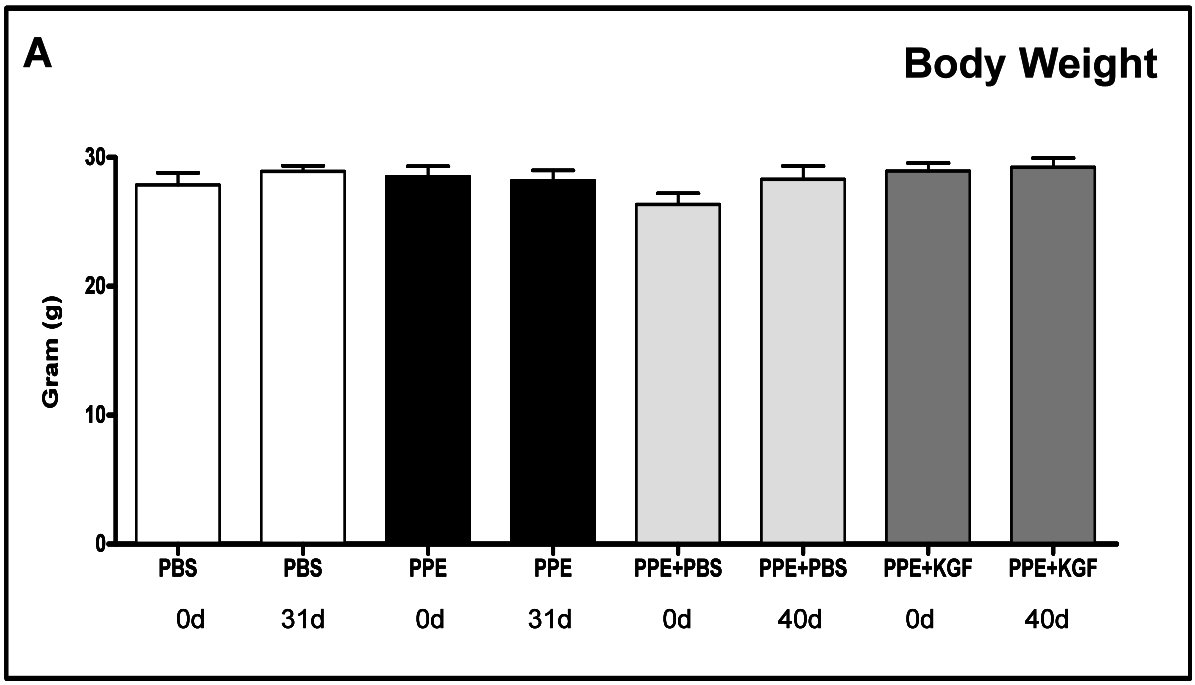
All data are presented as mean ± SD. Differences between PBS- versus PPE- group, and PPE+PBS versus PPE+KGF, were analyzed by t-test if normality and equal variance were given ($P>0.1$). Otherwise the nonparametric Mann-Whitney U-test was used. All analyses were performed by using GraphPad Prizm 4 software program (EI, Camino Rea, San Diego, CA, USA). Values of $P<0.05$ were considered to be significant.

5 Results

5.1 Effects of rHuKGF in in-vivo animal model

5.1.1 Body weight and lung volume

The body weights of mice from 4 different experimental groups were examined at day 0, 31 or 40, respectively. No significant differences were observed between day 0 and day 31 or 40, respectively (Fig.9A). The volume of the right lung (V_{RL}) was significantly increased at day 31 in elastase treated lungs compared with PBS treated lungs. However, no difference was observed between elastase treated lungs which had received rHuKGF as compared with elastase treated lungs which had received PBS (Fig.9B).



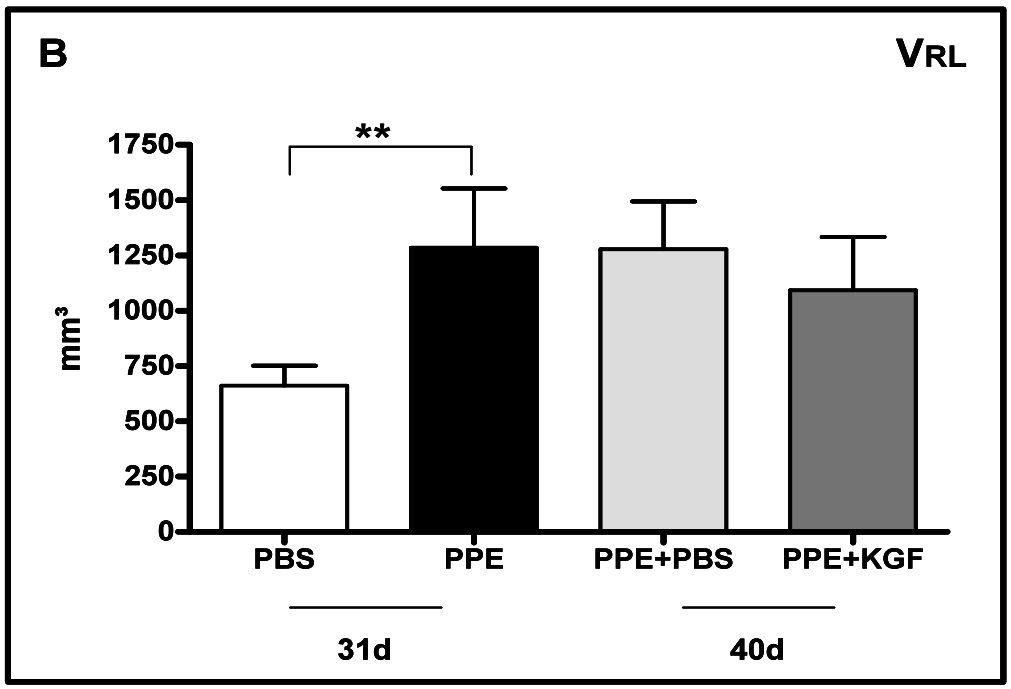


Figure 9 - *Body weight and volume of right lung (V_{RL}) measurements. (A) Body weight of each mouse was measured at two different time points i.e. day 0, 31 or 40. No significant difference was obtained on comparison of day 0 with 31 (or 40). (B) Mice lungs treated with elastase showed a significant increase in right lung volume compared to PBS treated mice. Elastase treated mice lungs receiving rHuKGF versus PBS did not show significant changes in lung volume. **P<0.05 versus the respective control group.*

5.1.2 Lung function analysis

To evaluate the influence of rHuKGF on airflow limitation, measurement of mid-expiratory airflow (EF50) were performed on day 31 for elastase treated lungs versus PBS, and on day 40 for elastase treated lungs which had received rHuKGF versus PBS. With respect to the lungs treated with PBS (set to 100%), lungs treated with elastase showed a significant decline of the EF50 values to 66%. Interestingly, rHuKGF significantly reversed the EF50 values to 98% in elastase treated lungs in comparison to elastase treated lungs which had received PBS (53%) (Fig.10).

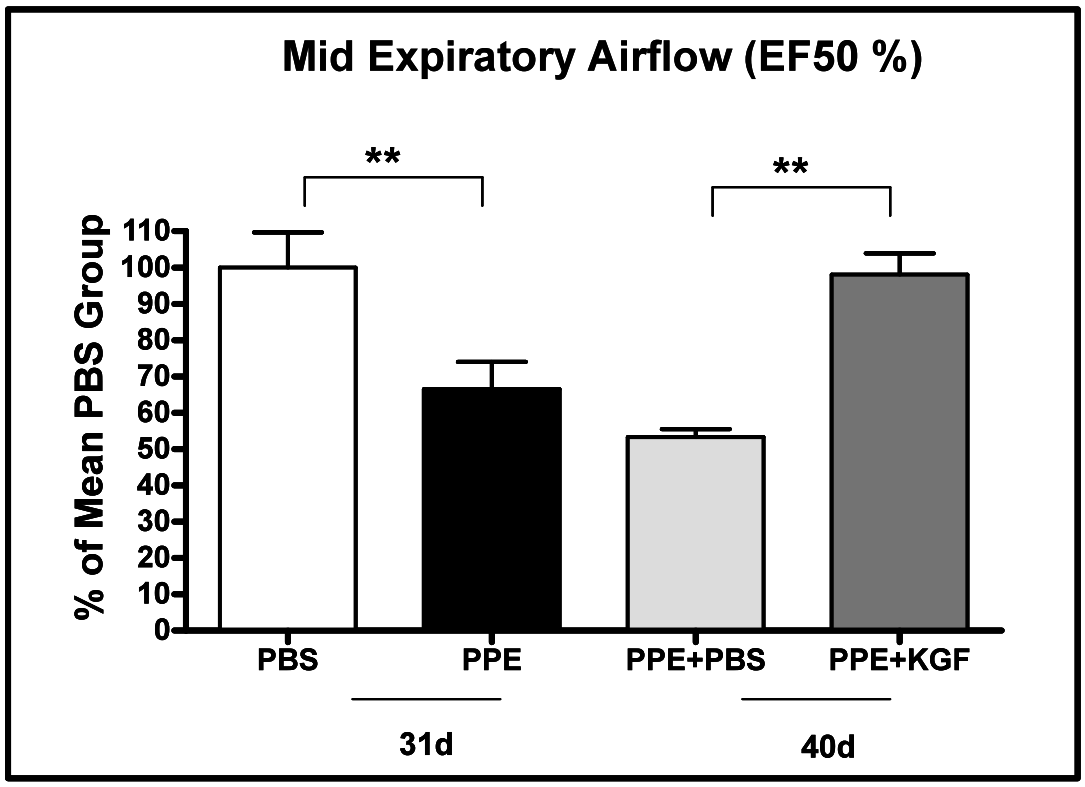


Figure 10 *- Lung function analysis. Mid expiratory airflow (EF50) values were reduced in lungs treated with elastase (66%) as compared to PBS treated lungs (100%). rHuKGF significantly reversed the impairment of respiratory function (98%) in elastase treated lungs in comparison with PBS received elastase treated lungs. **P<0.05 versus the respective control group.*

5.1.3 Quantitative stereological analysis

5.1.3.1 Air space enlargement

The destruction of alveolar wall leading to airspace enlargement and loss of alveolar surface area are classic characteristics of emphysema. To characterise airspace enlargement, mean chord length (MCL), absolute volume of air spaces (V_{air}) and alveolar surface area per unit volume (S_v) were quantified (Fig.11). The elastase treated lungs showed a significant increase in mean chord length (Fig.11A), absolute volume of air spaces (Fig.11B), and a significant reduction in alveolar surface area per unit volume (Fig.11C) reflecting marked airspace enlargement in comparison with the PBS treated lungs. Interestingly, supplementation of rHuKGF in elastase treated

significant increase in alveolar surface area per unit volume (Fig.11C) in comparison with elastase treated lungs which had received PBS. However no significant difference was noticed for absolute volume of air spaces between elastase treated lungs receiving rHuKGF versus PBS (Fig.11B).

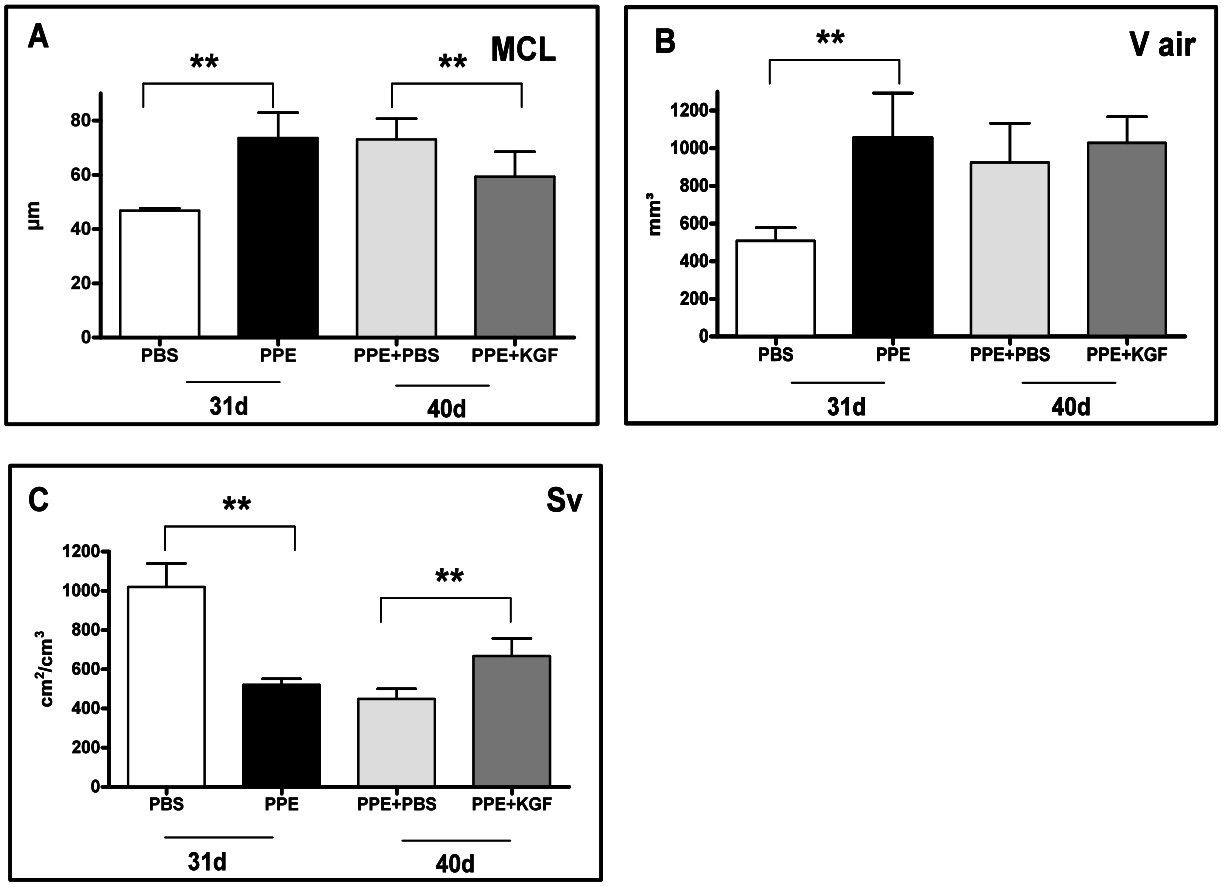


Figure 11 *- Quantitative Morphology. A) Mean chord length (MCL). B) Absolute volume of air spaces (V_{air}). C) Alveolar surface area per unit volume (S_v). MCL and V_{air} were found to be significantly increased whereas S_v was significantly reduced in elastase treated lungs in comparison with PBS treated lungs. Further, elastase treated lungs which had received rHuKGF showed a significant reduction in MCL and a significant increase in S_v compared with elastase treated lungs which had received PBS whereas no marked difference was observed in V_{air}. **P<0.05 versus the respective control group.*

5.1.3.2 Loss of alveolar septa

Loss of alveolar septa was depicted in elastase treated lungs by a significant reduction in alveolar surface area (S_a) when compared to PBS treated lungs (Fig.12B). No marked change was observed for absolute volume of alveolar septal tissue (V_{ast}) (Fig.12A). In addition, arithmetic mean thickness of the alveolar septal wall (τ_{ast}) was observed to be significantly increased in elastase treated lungs as compared to PBS treated lungs (Fig.12C). Elastase treated lungs receiving rHuKGF exhibited a significant increase in absolute volume of alveolar septal tissue (Fig.12A), alveolar surface area (Fig.12B) and arithmetic mean thickness of alveolar septal walll (Fig.12C) in comparison with elastase treated lungs receiving placebo therapy with PBS.

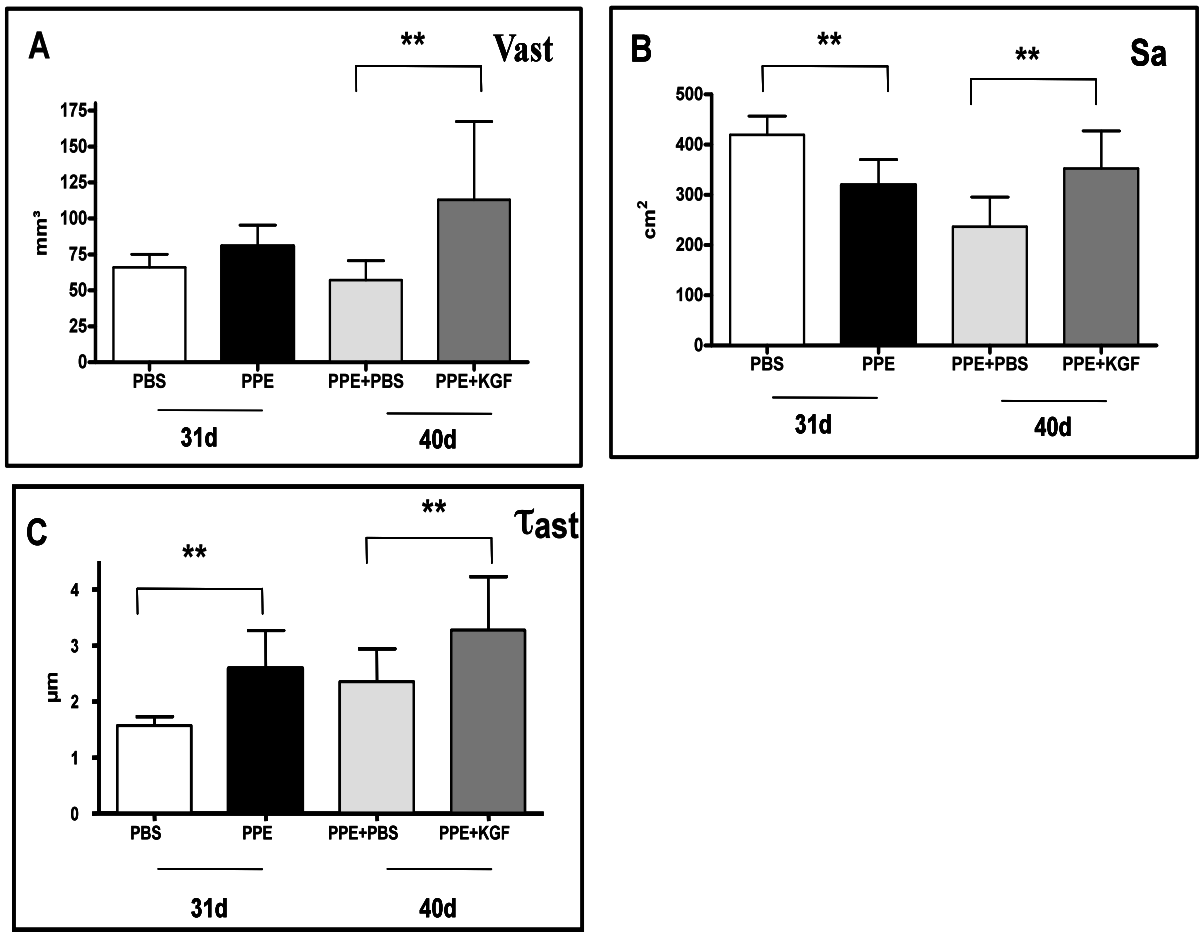


Figure 12 - *Quantitative Morphology. A) Absolute volume of septal tissue (V_{ast}). B) Alveolar surface area (S_a). C) Arithmetic mean thickness of alveolar septal wall (τ_{ast}).*

*lungs whereas S_a was significantly reduced and τ_{ast} was found to be significantly increased in elastase treated lungs in comparison with PBS treated lungs. Further, elastase treated lungs receiving rHuKGF showed a significant increase in V_{ast}, S_a and τ_{ast} compared with elastase treated lungs receiving PBS. **P<0.05 versus the respective control group.*

5.1.4 Lung compartments affected by rHuKGF

5.1.4.1 Alveolar epithelium

Proliferative cell nuclear antigen (PCNA), a marker for proliferating cells, is a major component of the DNA repair pathway (176-177). To evaluate the proliferative effects of rHuKGF on epithelium in elastase induced lung emphysema, immunohistochemical analysis for PCNA was performed. rHuKGF therapy resulted in a considerable increase in PCNA positive alveolar and airway epithelial cells in emphysematous lungs that had received rHuKGF (Fig.13D) while a very rare PCNA positive cell was detected in emphysematous lungs that had received PBS (Fig.13C). However, in emphysematous lungs, PCNA stained positive cells were very poorly observed (Fig.13B) when compared to PBS treated lungs (Fig.13A).

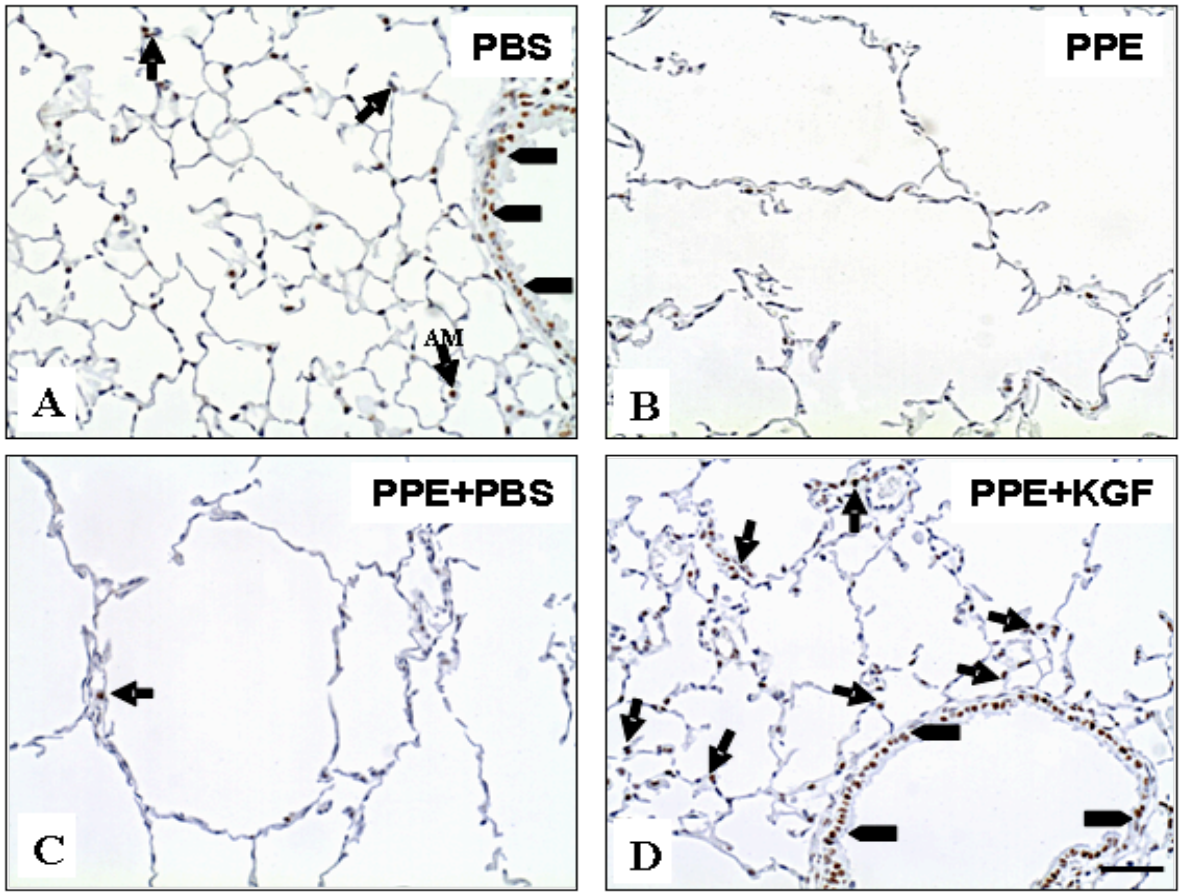


Figure 13 - *Immunohistochemical staining for PCNA in paraffin sections of lung tissue. rHuKGF aspiration in emphysematous lungs resulted in abundant PCNA positive alveolar (shown by arrows) and airway epithelial cells (shown by thick arrowheads) as well as alveolar macrophages (AM) (D) compared with emphysematous lungs receiving PBS (C). In contrast, PCNA positive cells were very poorly seen in emphysematous lungs (B) compared to PBS treated lungs (A). Scale bar = 40 µm.*

Furthermore, supplementation of rHuKGF in emphysematous lungs resulted in a significant induction of PCNA mRNA expression in comparison with emphysematous lungs receiving PBS (Fig.14A).

In addition, mRNA expression of Cyclin-D1, which is an important regulator of cell cycle progression and considered to be in association with PCNA (178), was significantly induced in emphysematous lungs receiving rHuKGF as compared to PBS treated emphysematous lungs (Fig.14B).

Likewise, mRNA expression of nuclear factor erythroid-related factor 2 (Nrf2), which is a basic leucine zipper transcription factor involved in wound healing and being necessary for the appropriate proliferation of alveolar epithelial cells (179), was significantly increased after therapy with rHuKGF in emphysematous lungs as compared with PBS treated emphysematous lungs (Fig.14C). No significant differences were observed between lungs treated with PBS or elastase (Fig.14A-B).

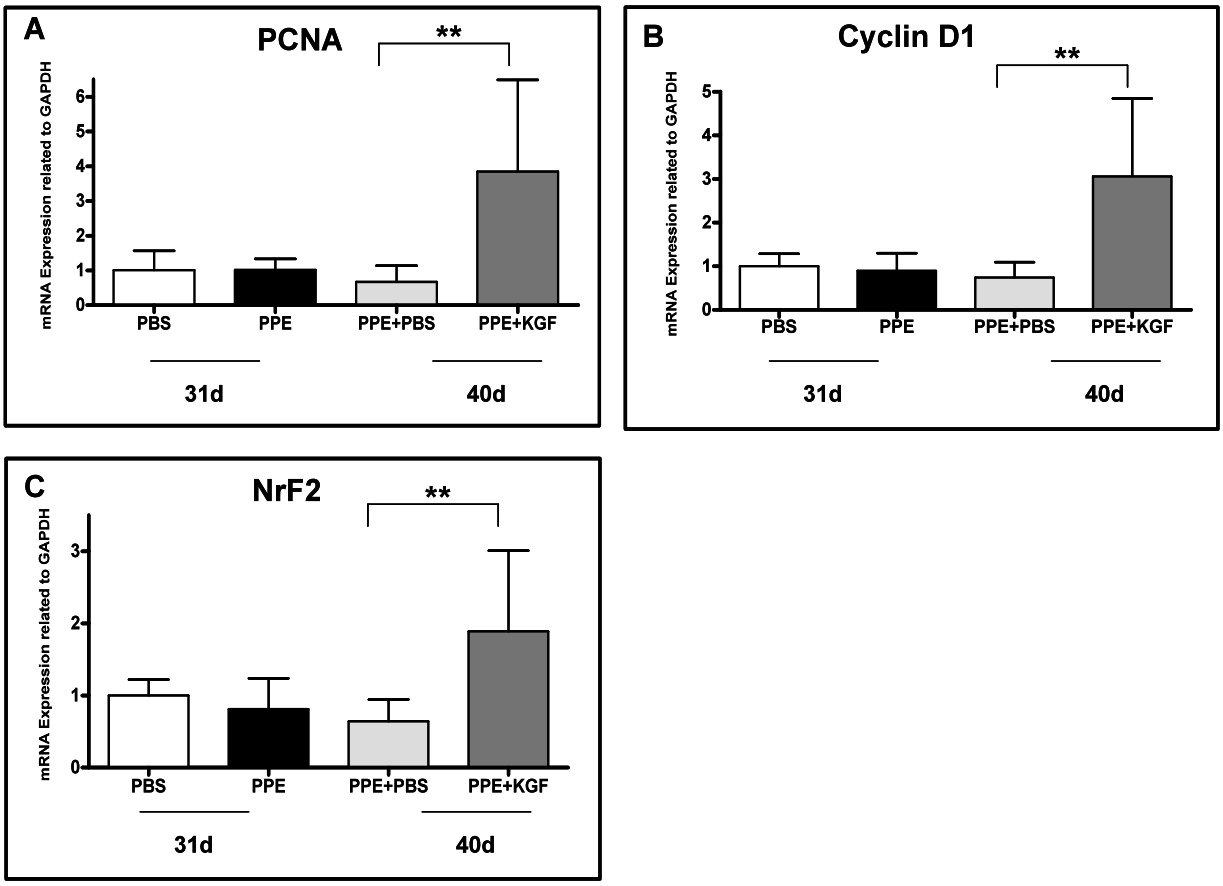


Figure 14 - *rHuKGF therapy was associated with increased expression of marker genes of epithelial cell proliferation in vivo. Quantitative real time RT-PCR demonstrated significantly increased mRNA levels of PCNA (A), Cyclin-D1 (B) and Nrf2 (C) in emphysematous lungs receiving rHuKGF in comparison with emphysematous lungs which had received PBS. No significant changes were seen in any of the genes between elastase versus PBS treated lungs. **P<0.05 versus the respective control group.*

Interestingly, in line with the RT-PCR data, Western blot analysis revealed that the

supplemented with rHuKGF as compared with emphysematous lungs receiving PBS. No significant difference in the protein levels was found between lungs treated with elastase compared with PBS treated lungs (Fig.15A-B).

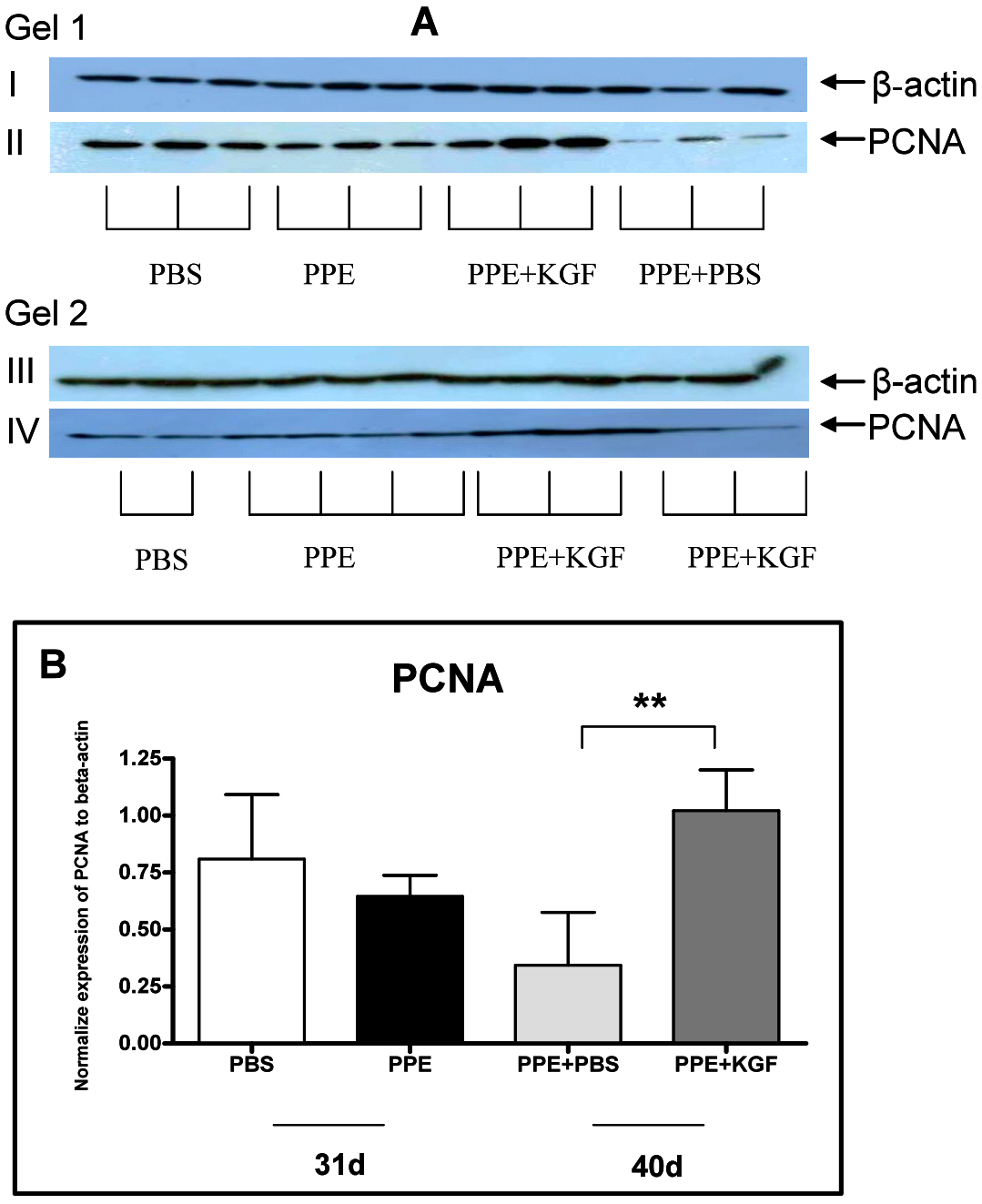


Figure 15 *- Western blot analysis. Protein expression of β-actin (A-I and A-III) and PCNA (A-II and A-IV) were detected by Western blotting. Densitometry based expression of PCNA normalised to β-actin revealed that PCNA protein expression was markedly higher in emphysematous lungs supplemented with rHuKGF as*

*compared with emphysematous lungs receiving PBS. No difference was observed in elastase versus PBS treated lungs (B). **P<0.05 versus the respective control group.*

Furthermore, the influence of rHuKGF on differentiation of alveolar epithelial (AE) cells was investigated at the mRNA level by looking at the AE type 2 cell (AE2) specific marker surfactant protein-C (SP-C) (180-181) and the AE type 1 cell (AE1) specific marker aquaporin (AQP)-5 (182) (Fig.16). The mRNA levels of SP-C (Fig.16A) and AQP-5 (Fig.16B) were significantly increased in emphysematous lungs supplemented with rHuKGF compared with emphysematous lungs receiving PBS. Moreover, mRNA levels of forkhead transcription factor (Foxa-2), which has been shown to regulate the differentiation of alveolar epithelial cells (183-184), was found to be significantly induced in emphysematous lungs supplemented with rHuKGF compared with emphysematous lungs receiving PBS (Fig.16C). In contrast, a marked reduction in AQP5 and Foxa-2 mRNA levels was observed in emphysematous lungs compared to PBS treated lungs (Fig.16B, C).

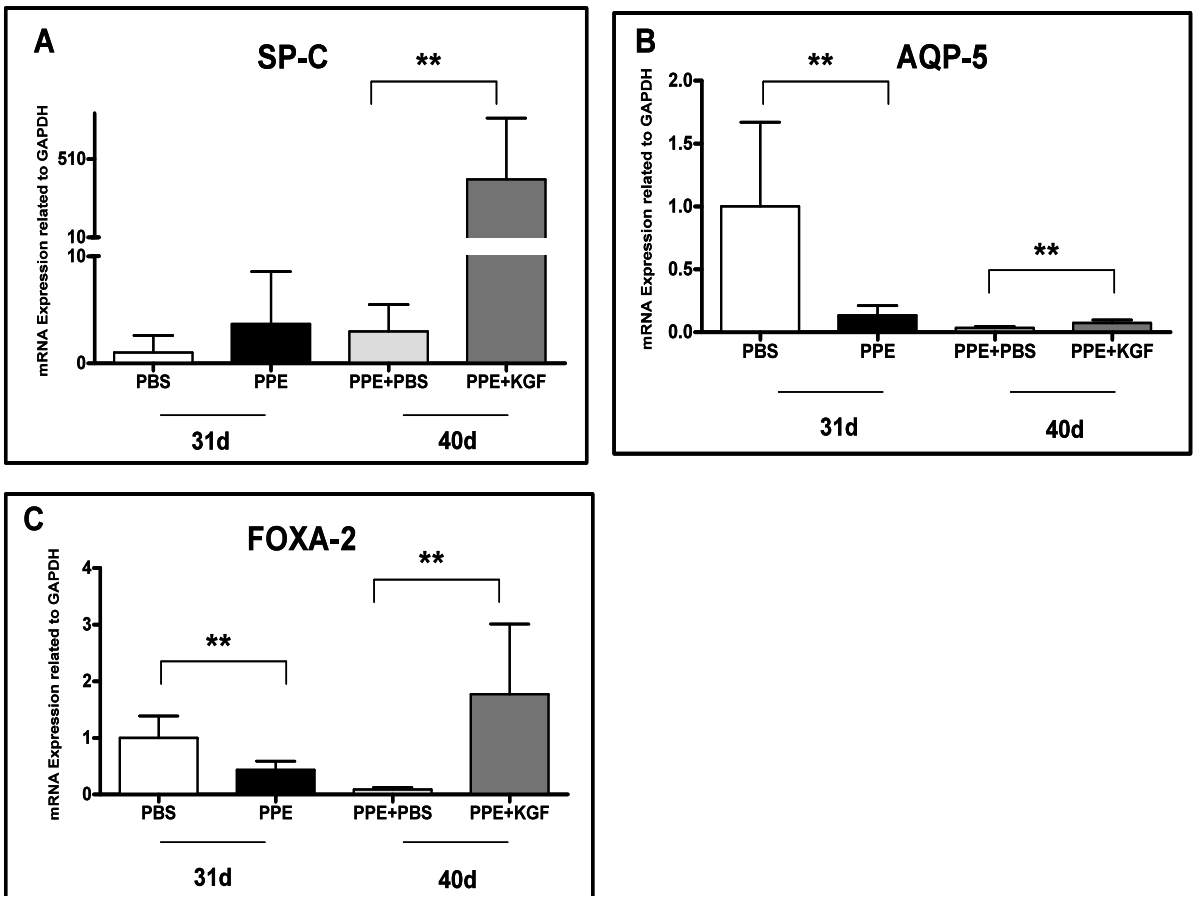


Figure 16 - *rHuKGF therapy resulted in increased expression of marker genes of alveolar epithelial cell differentiaion in vivo. Quantitative real time RT-PCR demonstrated significantly increased mRNA expression levels of SP-C (A), AQP-5 (B) and Foxa-2 (C) in emphysematous lungs receiving rHuKGF therapy compared to supplementation with PBS. On other hand, SP-C mRNA levels remained uneffective between PBS and emphysematous lungs whereas a significant reduction in mRNA levels of AQP-5 and Foxa-2 was observed in elastase treated in comparison with PBS treated lungs. **P<0.05 versus the respective control group.*

In addition, mRNA expression of HGF, which stimulates DNA synthesis of airway epithelial cells (165) and AE2 cells that contribute to regenerating the alveolar structure (164), was significantly increased in rHuKGF administered emphysematous lungs as compared with emphysematous lungs receiving PBS (Fig.17A).

Besides, fibroblast growth factor (FGF-7) mRNA level was markedly induced in emphysematous lungs receiving rHuKGF versus PBS (Fig.17B).

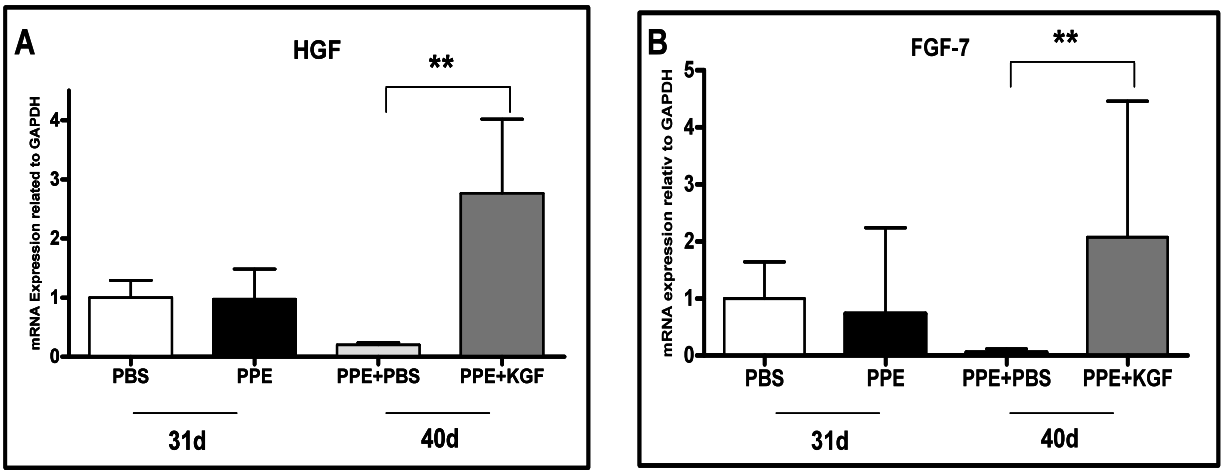


Figure 17 - *rHuKGF therapy resulted in increased expression of HGF and FGF-7. Quantitative real time RT-PCR revealed that aspiration of rHuKGF in emphysematous lungs resulted in a marked induction in mRNA levels of HGF (A) and FGF-7 (B) versus emphysematous lungs receiving PBS. No significant changes were seen in any of the genes between elastase versus PBS treated lungs. **P<0.05 versus the respective control group.*

5.1.4.2 Capillary endothelium

Capillary endothelial cells are one of the major cell types constituting the alveolar septal wall. To determine whether rHuKGF therapy in emphysematous lungs could effect on proliferation of capillary endothelial cells, double immunohistochemistry for marker for endothelial cells and Ki-67 (which is a cellular marker associated with cell proliferation and defines the proliferation status of capillary endothelial cells) was performed which revealed that rHuKGF resulted in proliferation of capillary endothelium cells (Fig.18A-C) while no Ki-67 stained cell was noted in PBS treated emphysematous lungs (Fig.18D-F).

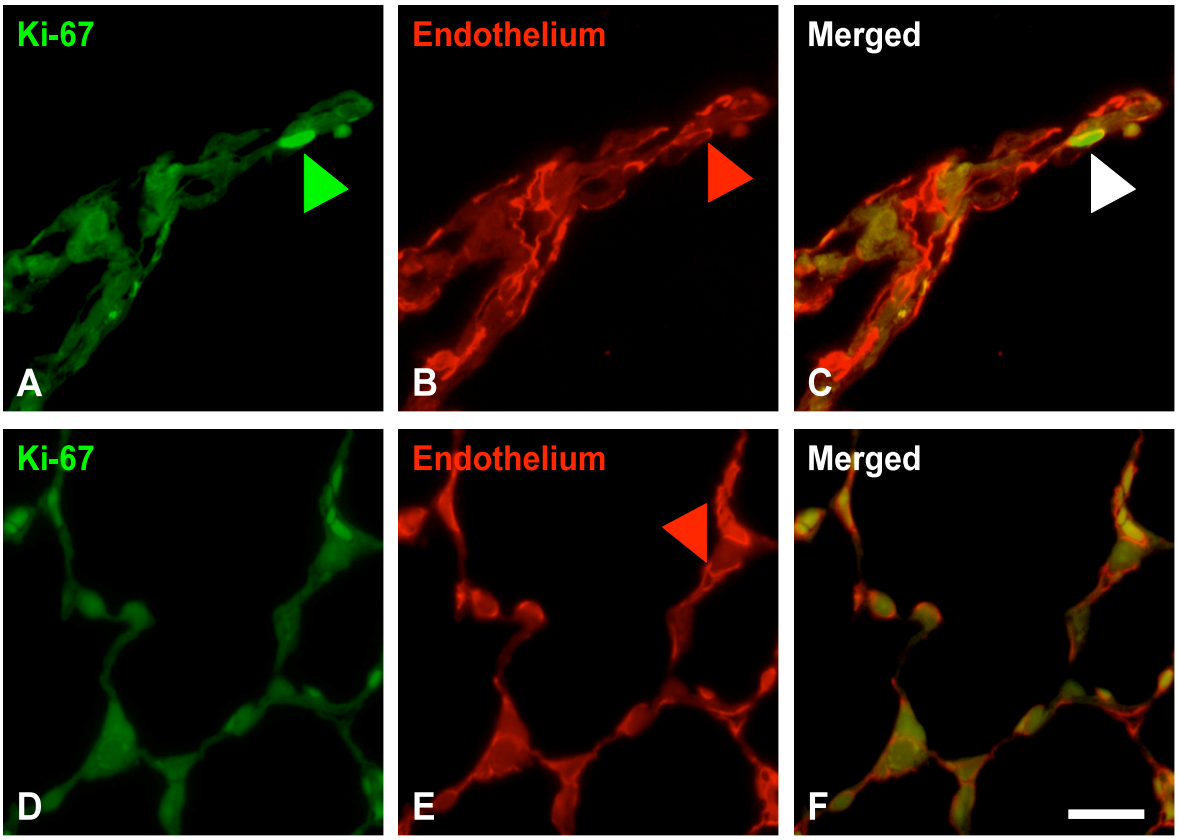


Figure 18 - *Double immunohistochemical staining for the proliferation marker Ki-67 (green fluorescence) and podocalyxin, a marker for endothelial cells (red fluorescence). rHuKGF administration in emphysematous lungs induced proliferation of capillary endothelium cells (A-C) whereas in PBS treated emphysematous lungs colocalization of Ki-67 and podocalyxin was not observed (D-F).*

In addition, therapeutic application of rHuKGF caused a significant increase in mRNA expression of vascular endothelial growth factor (VEGF), which is crucial for capillary endothlial cells maintenance (82), in emphysematous lungs in comparision with emphysematous lungs receiving PBS (Fig.19A).

The mRNA expression levels of VEGF specific receptors, VEGFR1 and VEGFR2 were significantly induced in emphysematous lungs which had received rHuKGF when compared to emphysematous lungs which had received PBS (Fig.19B-C).

On the other hand, transcript levels of VEGF and VEGFR2 were found to be significantly reduced in emphysematous lungs as compared to control lungs (Fig.19A, C).

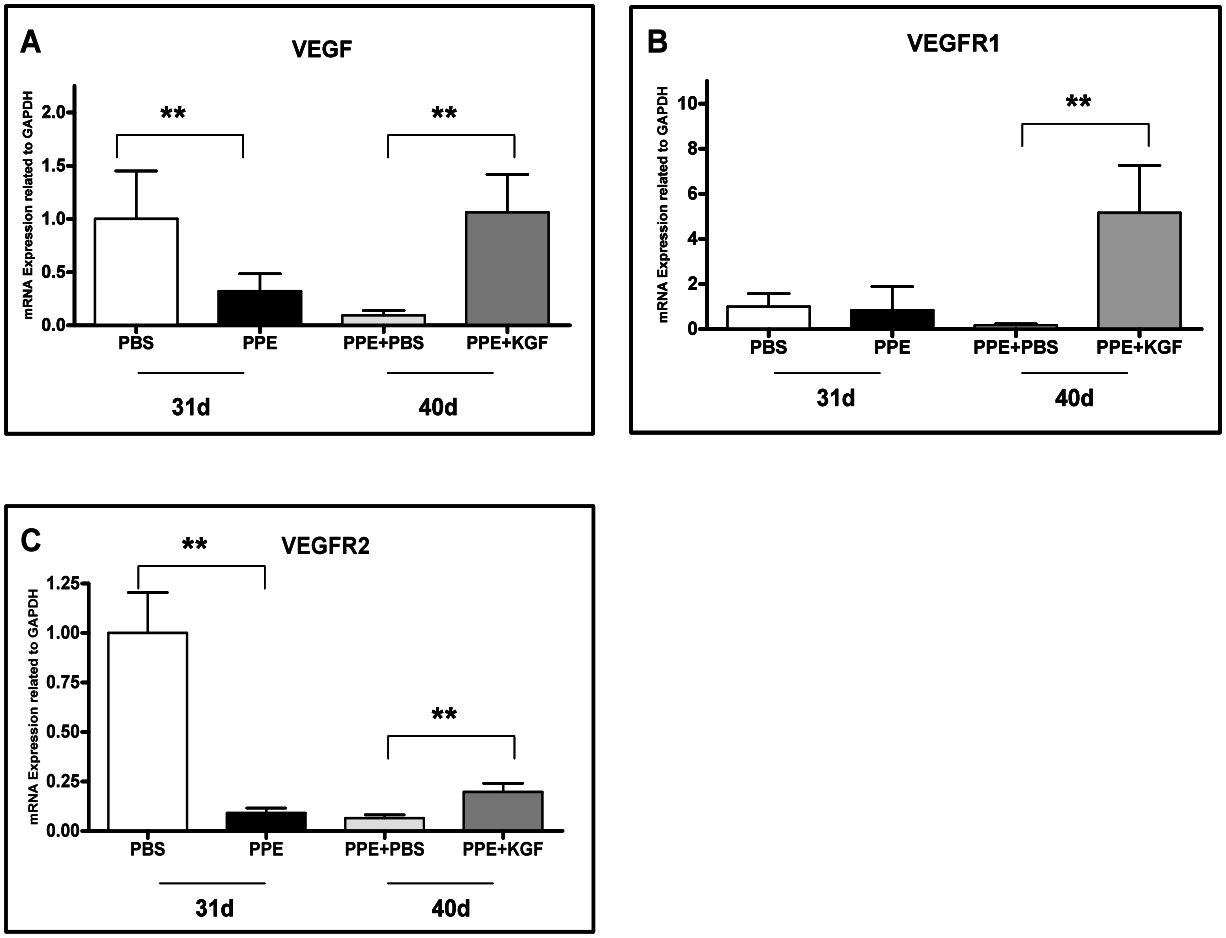


Figure 19 - *rHuKGF therapy was associated with increased expression of genes implicated in capillary endothelium maintenance in vivo. Quantitative real time RT-PCR revealed significantly increased mRNA levels of VEGF (A), VEGFR1 (B) and*

*saline treated emphysematous lungs. Elastase treatment alone resulted in reduction of VEGF and VEGFR2 mRNA expression as compared to controls (A and C). **P<0.05 versus the respective control group.*

To confirm and validate the RT-PCR analysis, Western blot analysis for VEGFR2 was performed. Likewise, a significantly increased expression of VEGFR2 was detected in emphysematous lungs receiving rHuKGF therapy as compared to emphysematous lungs receiving PBS (Fig.20).

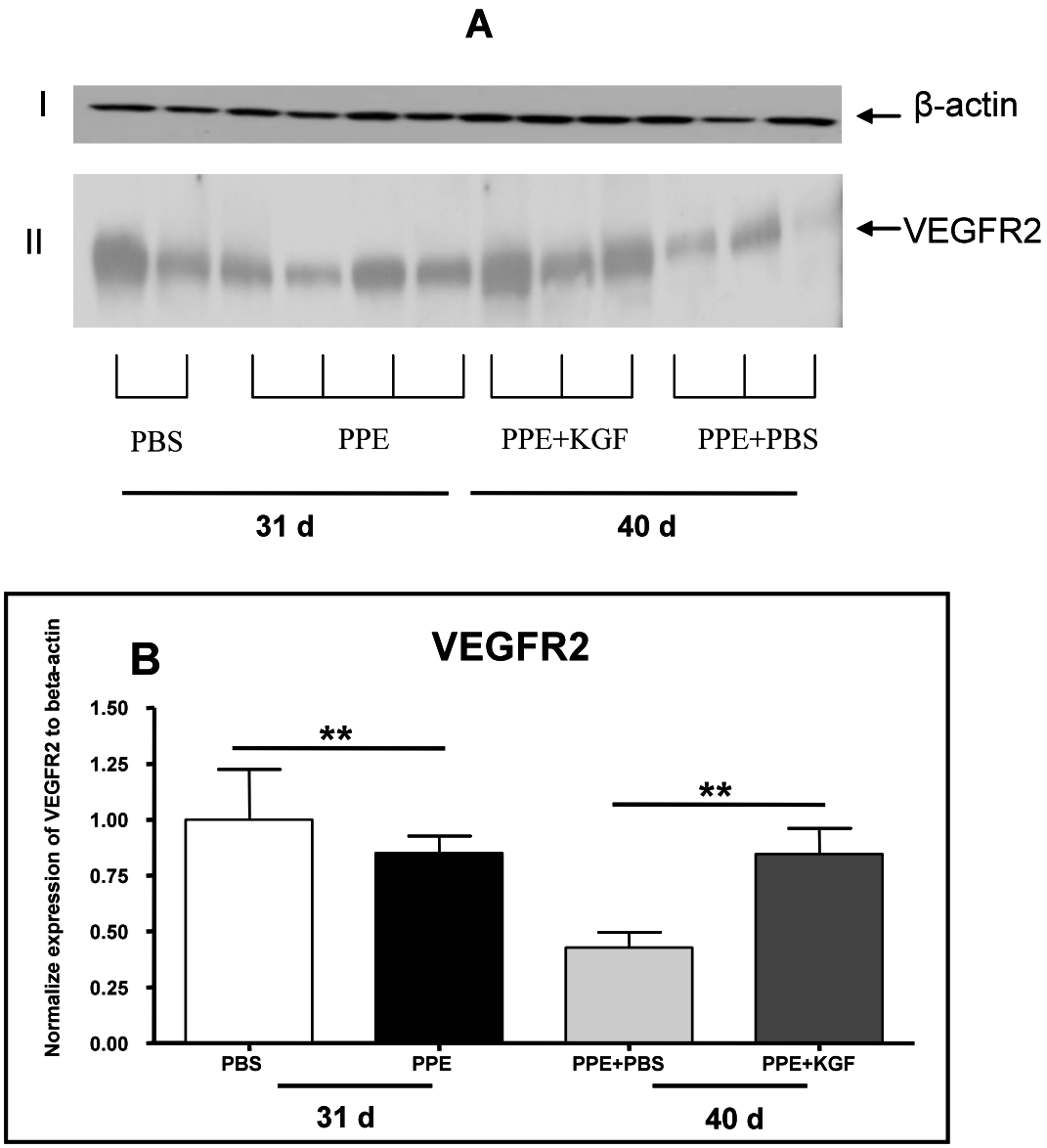


Figure 20 - *Western blot analysis. Protein expression of β-actin (A-I) and VEGFR2 (A-II) were detected by Western blotting. Densitometry based expression of VEGFR2 normalised to β-actin revealed that VEGFR2 protein expression was markedly induced in rHuKGF supplemented emphysematous lungs versus emphysematous lungs which had received PBS. A marked reduction was noticed in emphysematous lungs versus PBS treated lungs (B). **P<0.05 versus the respective control group.*

5.1.4.3 Interstitial tissue

To assess whether rHuKGF influences interstitial tissue maintenance in elastase induced emphysematous lungs, immunohistochemical detection of transforming growth factorβ-1 (TGFβ-1), a prototypic fibrogenic cytokine which stabilizes interstitial tissue formation (185), was performed. Immunohistochemistry revealed an enhancement of TGFβ-1 positive alveolar and airway epithelial cells in rHuKGF supplemented emphysematous lungs (Fig.21D) in comparison with emphysematous lungs receiving PBS (Fig.21C). Emphysematous lungs exhibited a marked reduction in TGFβ-1 positive epithelial cells (Fig.21B) when compared with PBS treated lungs (Fig.21A).

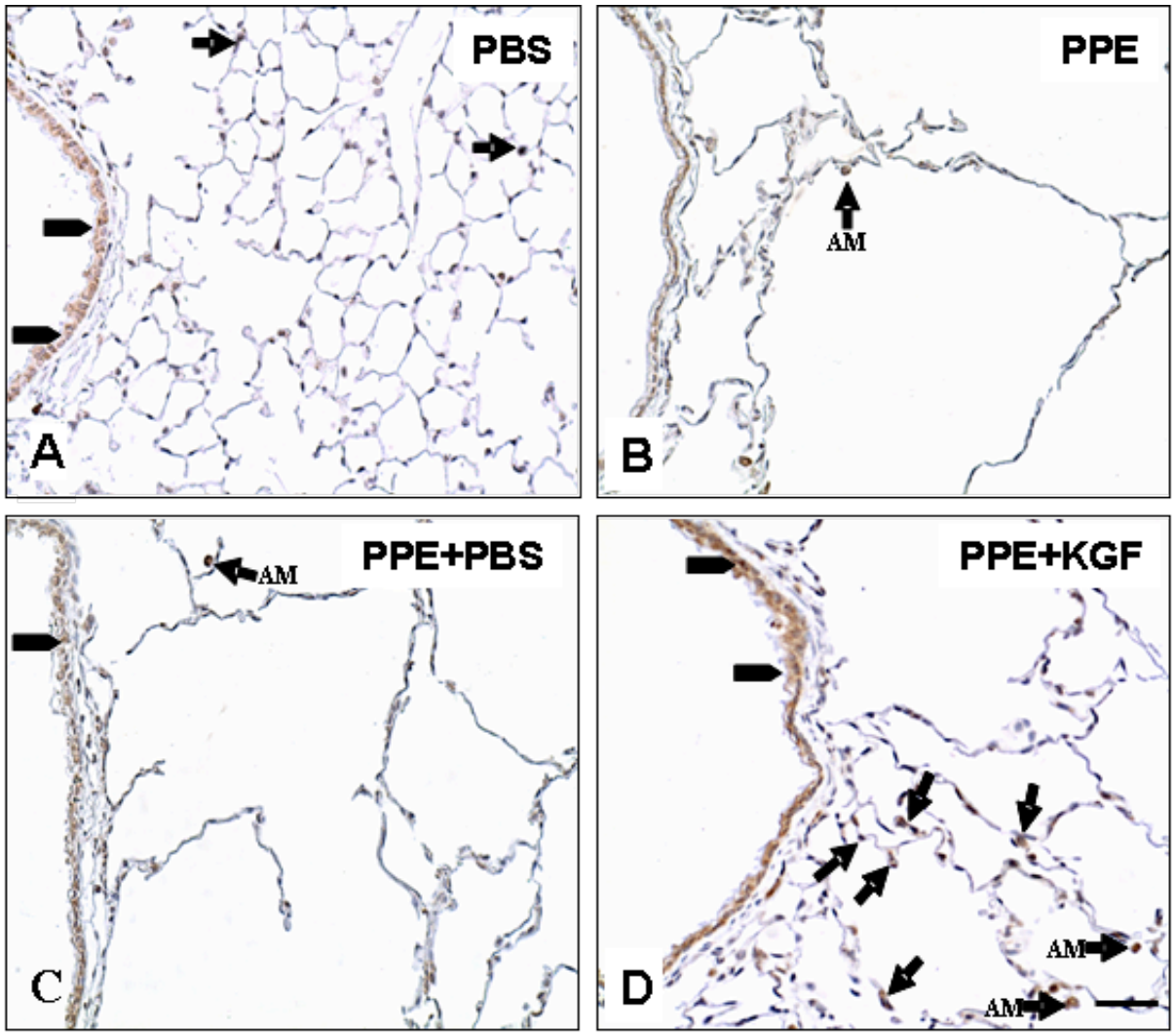


Figure 21 - *Immunohistochemical staining for TGFβ-1 in paraffin section of lung tissue. rHuKGF therapy resulted in a marked increase in TGFβ-1 positive alveolar (shown by arrows) and airways epithelial cells (shown by thick arrowheads) as well as alveolar macrophages in emphysematous lungs (D) in comparision with emphysematous lungs which had received PBS (C). Lungs treated with elastase showed only very few TGFβ-1 positive epithelial cells and alveolar macrophages (B) compared to PBS treated lungs (A). Scale bar = 40 µm.*

Moreover, TGFβ-1 is implicated to increase the expression of multiple matrix genes including elastin, involving TGFβ transmembrane receptor (type 1 and 2) and specific smads (153).

Elastin is a major constituent of alveolar interstitial tissue and its synthesis is an

rHuKGF on regulators of interstitial tissue formation, mRNA levels of TGFβ-1, TGFβ-R-1, TGFβ-R-2, Smad 2 and elastin were examined. The mRNA levels of TGFβ-1, TGFβ-R-1, TGFβ-R-2, Smad 2, and elastin were found to be significantly induced emphysematous lungs receiving rHuKGF compared with emphysematous lungs receiving PBS (Fig.22A-D, Fig.24B). In contrast, mRNA levels of TGFβ-1, Smad 2, and elastin were significantly reduced in elastase versus PBS treated lungs (Fig.22 A, C, D).

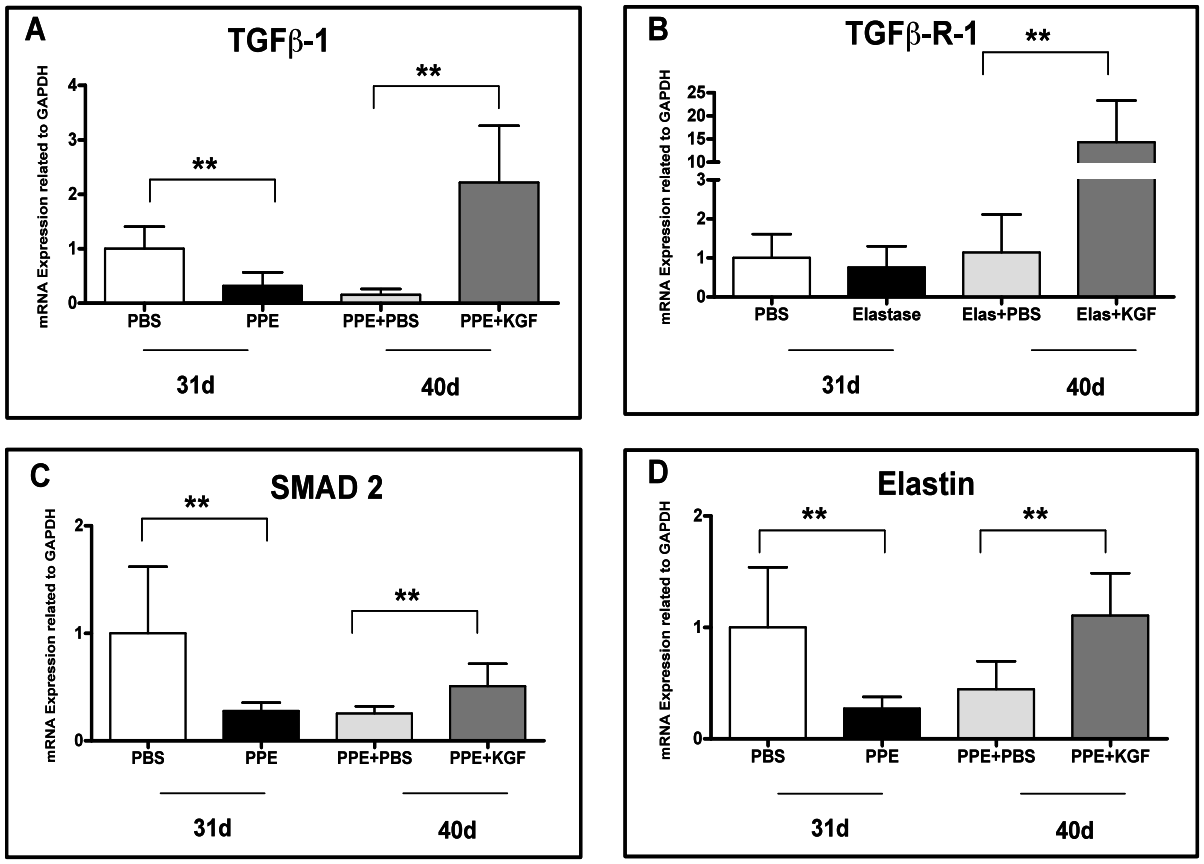


Figure 22 - *rHuKGF therapy resulted in increased expression of genes implicated in interstitial cell maintenance in vivo. Quantitative real time RT-PCR revealed significantly increased mRNA levels of TGFβ-1 (A), TGFβ-R-1 (B), Smad 2 (C), elastin (D) in emphysematous lungs receiving rHuKGF therapy as compared with emphysematous lungs which had received PBS. A marked reduction in relative mRNA levels of TGFβ-1, Smad 2, and elastin was observed in elastase versus PBS treated lungs. **P<0.05 versus the respective control group.*

In addition, aspiration of rHuKGF in emphysematous lungs resulted in significantly induced mRNA expression levels of plasminogen activator inhibitor (PAI)-1, which is regulated by TGFβ and plays a key role to decline the proteolysis of interstitial tissue constitutes (186-187), and of zinc finger transcription factor (SP-1) which regulates TGFβ-1 transcription (188) as compared to emphysematous lungs instilled with PBS (Fig.23A-B). Elastase treatment alone resulted in a significant reduction of SP-1 mRNA level compared to PBS treatement (Fig.23B).

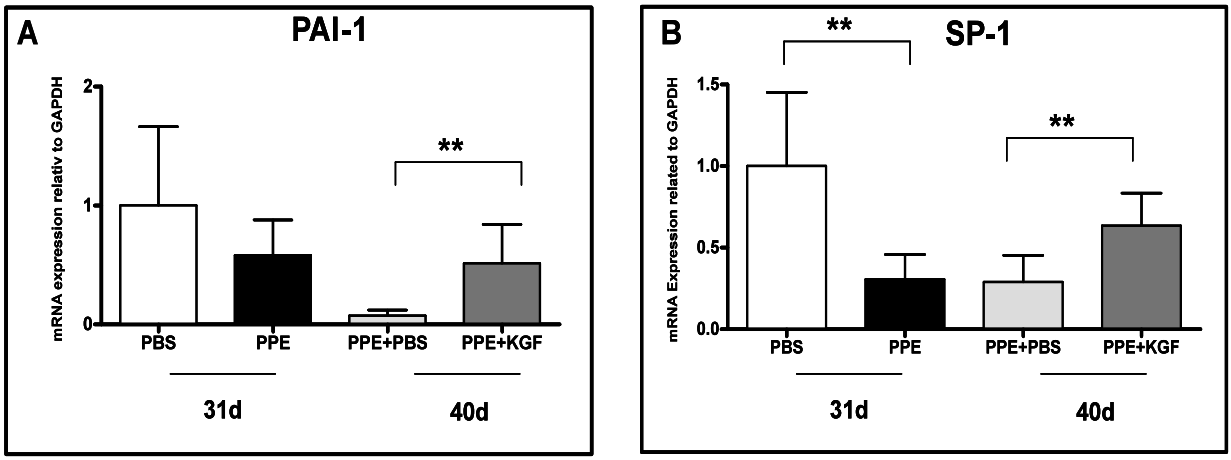


Figure 23 - *rHuKGF therapy resulted in increased expression of PAI-1 and SP-1 in vivo. Quantitative real time RT-PCR revealed that aspiration of rHuKGF in emphysematous lungs resulted in a marked induction in mRNA levels of PAI-1 (A) and SP-1 (B) versus emphysematous lung which had received PBS. A marked reduction in relative mRNA level of SP-1 was observed in emphysematous lungs versus control lungs (B). **P<0.05 versus the respective control group.*

In addition, administration of rHuKGF was associated with significantly increased mRNA expression of TGFβ-2 in emphysematous lungs in comparison with saline treated emphysematous lungs (Fig.24A). In contrast, emphysematous lungs showed a significant reduction in mRNA expression of TGFβ-2 as compared with control lungs (Fig.24A).

At the protein level, TGFβ-2 expression was assessed by indirect imunohistochemistry in rHuKGF treated emphysematous lungs as compared emphysematous lungs receiving PBS. TGFβ-2 positive cells were more frequently detected in rHuKGF administered emphysematous lungs where TGFβ-2 was localized to the tips of short

side-branches of alveolar septal walls (Fig.24D) while saline treated emphysematous lungs showed TGFβ-2 expression with lesser magnitude (Fig.24C).

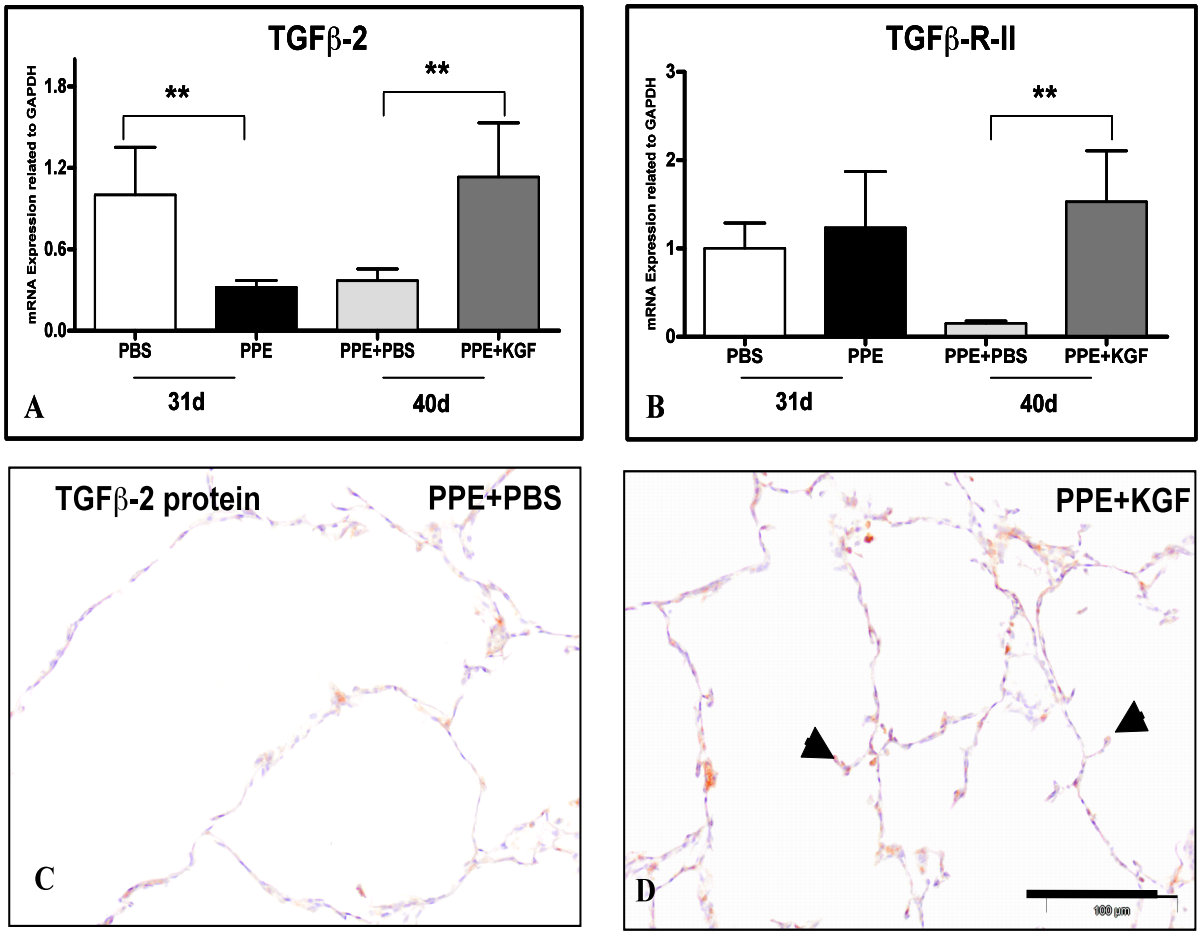


Figure 24 - *rHuKGF therapy was associated with increased expression of TGFβ-2 and TGFβ-R-II in vivo. Quantitative real time RT-PCR demonstrated a significant upregulation of relative mRNA levels of TGFβ-2 (A) and TGFβ-R-II (B) in emphysematous lungs receiving rHuKGF therapy when compared to saline treated emphysematous lung. In emphysematous lungs, a significant reduction in the expression of TGFβ-2 (A) was seen as compared to controls. Indirect immunohistochemistry revealed that expression of TGFβ-2 was localised in the tips of short side-branches of alveolar septal walls (shown by arrows) in emphysematous lungs receiving rHuKGF therapy as detected by immunohistochemistry (D). **P<0.05 versus the respective control group.*

Likewise, in emphysematous lungs receiving rHuKGF, PDGF-A mRNA expression

mice (167), was significantly increased as compared to emphysematous lungs receiving saline (Fig.25). Moreover, there was significantly reduced mRNA expression of PDGF-A in the lungs with emphysematous changes as compared to PBS treated lungs (Fig.25).

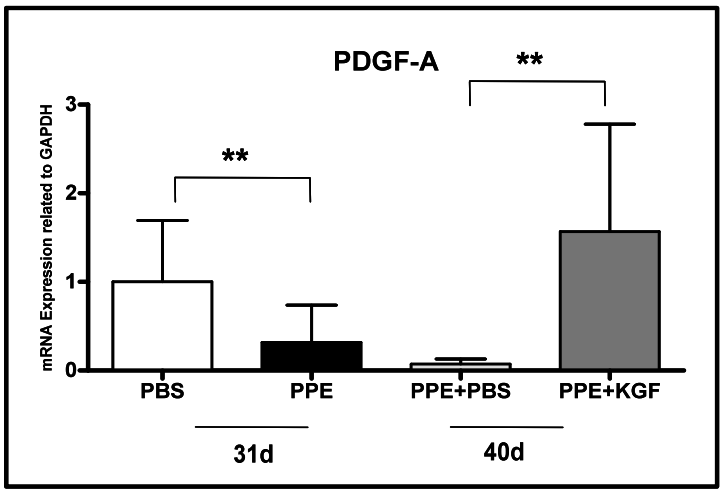


Figure 25 *- rHuKGF therapy resulted in increased expression of PDGF-A in vivo. Quantitative real time RT-PCR demonstrated significantly increased mRNA level of PDGF-A in rHuKGF administered emphysematous lungs when compared to saline treated emphysematous lungs. In emphysematous lungs, significant reduction in the expression of PDGF-A was seen as compared to controls. **P<0.05 versus the respective control group.*

5.2 Effects of rHuKGF in in-vitro cell culture:

5.2.1 TGFβ-1 mRNA expression in and release of active TGFβ-1 of alveolar epithelial type 2 (AE2) cells.

In order to investigate if the regenerative effects of rHuKGF on interstitial tissue compartment observed in vivo, were caused by AE2 cells derived TGFβ-1, mouse primary lung AE2 cells, murine AE2-like cells LA-4 and AE1-like cells E10 were incubated with 50 ng/ml of rHuKGF for 6 hours and 24 hours, respectively. Incubation with rHuKGF resulted in significantly increased transcript levels of TGFβ-1 in primary

cells failed to respond (Fig.26A-C). In addition, release of active TGFβ-1 into the supernatant was significantly increased above control levels after 24 hours of incubation with rHuKGF in primary AE2 cells, in LA-4 cells, and after 6 as well as 24 hours of incubation with rHuKGF in LA-4 cells co-cultured with mouse lung fibroblast (MLF) cells (Fig.27A-C).

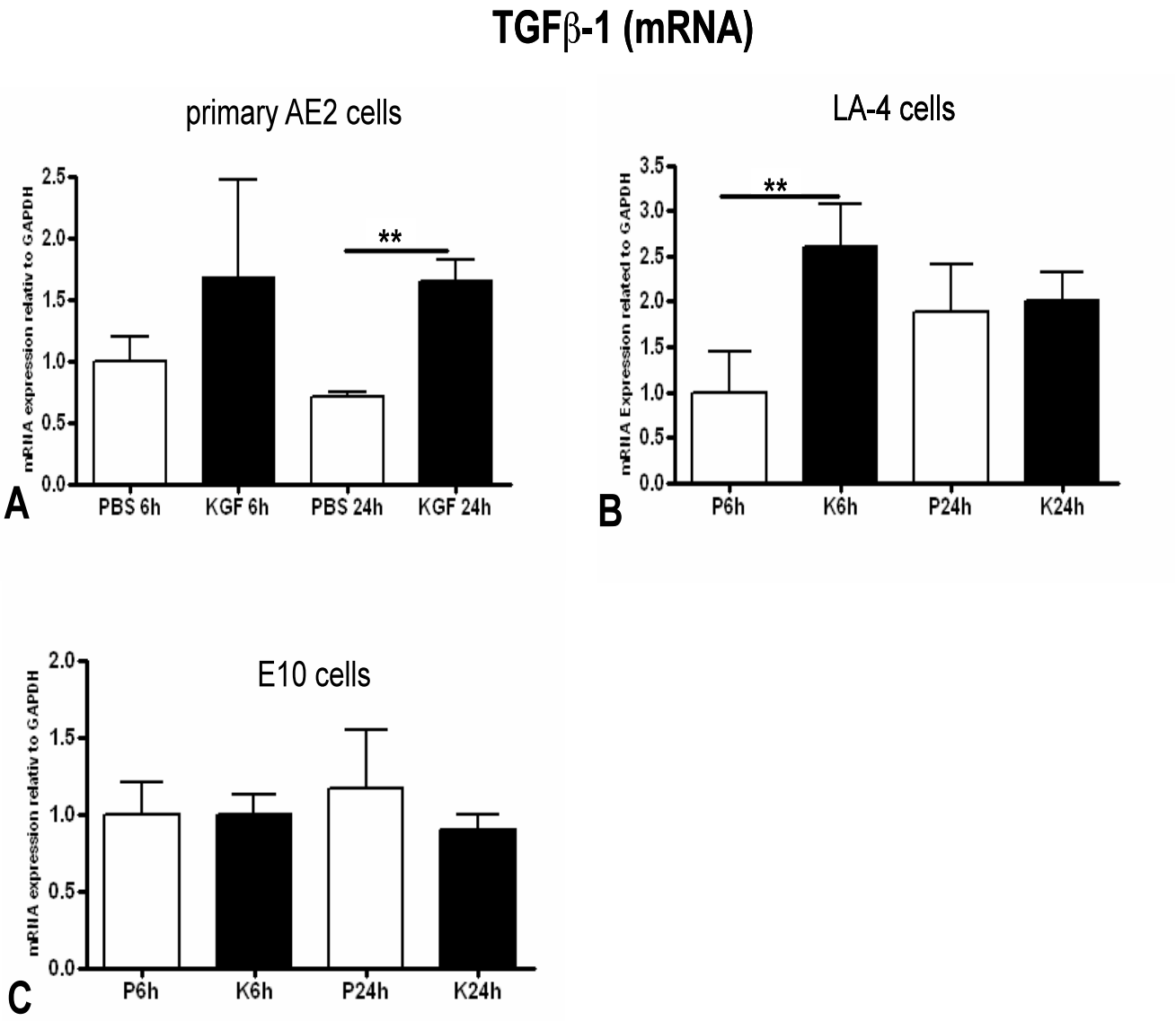


Figure 26 - *rHuKGF increased mRNA expression of TGF β-1 in primary AE2 cells and murine AE2-like LA-4 cells but not in murine AE1-like E10 cells in vitro. Quantitative real time RT-PCR revealed that incubation with rHuKGF significantly increased mRNA expression of TGF β-1 in mouse primary AE2 cells, which were isolated from C57BL/6 mice, after 24 hours (A), in LA-4 after 6 hours (B), but not in E10 cells (C) as compared to controls. **P<0.05 versus the respective control group*

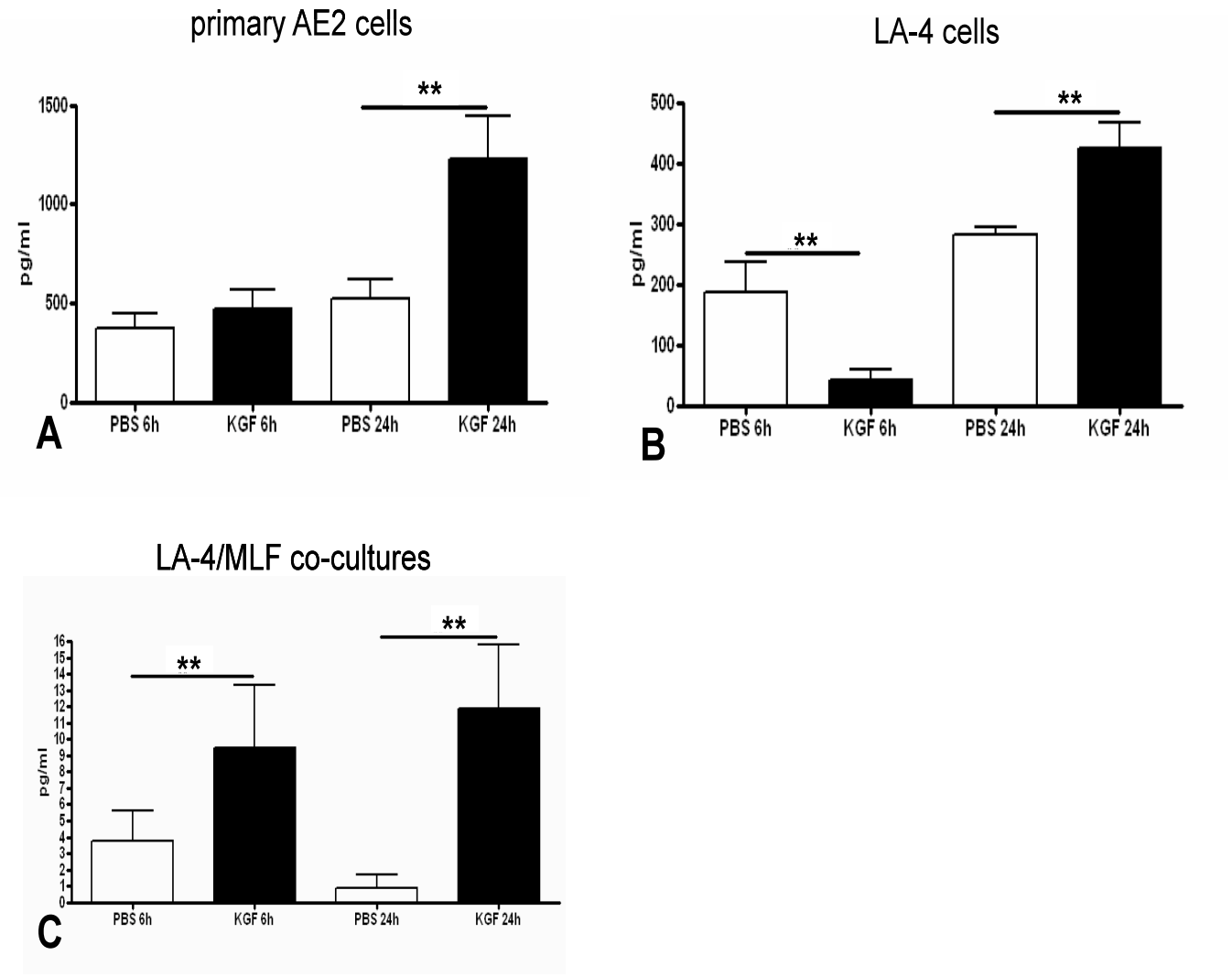


Figure 27 - *rHuKGF increased the release of active TGF β-1 in primary AE2 cells, murine AE2-like LA-4 cells, and LA4 cells cocultured with mouse lung fibroblast (MLF) cells in vitro. Incubation with rHuKGF significantly increased concentrations of activated TGF β-1 above control levels after 24 hours in primary AE2 cells (A), in LA-4 (B), and LA-4/MLF co-cultures after 6 as well as after 24 hours (C). **P<0.05 versus the respective control group.*

5.2.2 TGFβ-1 increased mRNA expression of genes characteristic of the TGFβ-1 pathway in murine fibroblasts.

In order to evaluate if the effects of rHuKGF on increased expression of elastin, were the result of a direct interaction with fibroblasts, MLF cells were incubated with 50 ng/ml of rHuKGF or 10 ng/ml of recombinant human TGFβ-1 for 6 and 24 hours,

of mRNA expression levels of SP1, TGF β-1, PAI-1, and elastin at both time-points, while incubation with rHuKGF did not alter the mRNA expression level of any of the all four genes (Fig.28A-D).

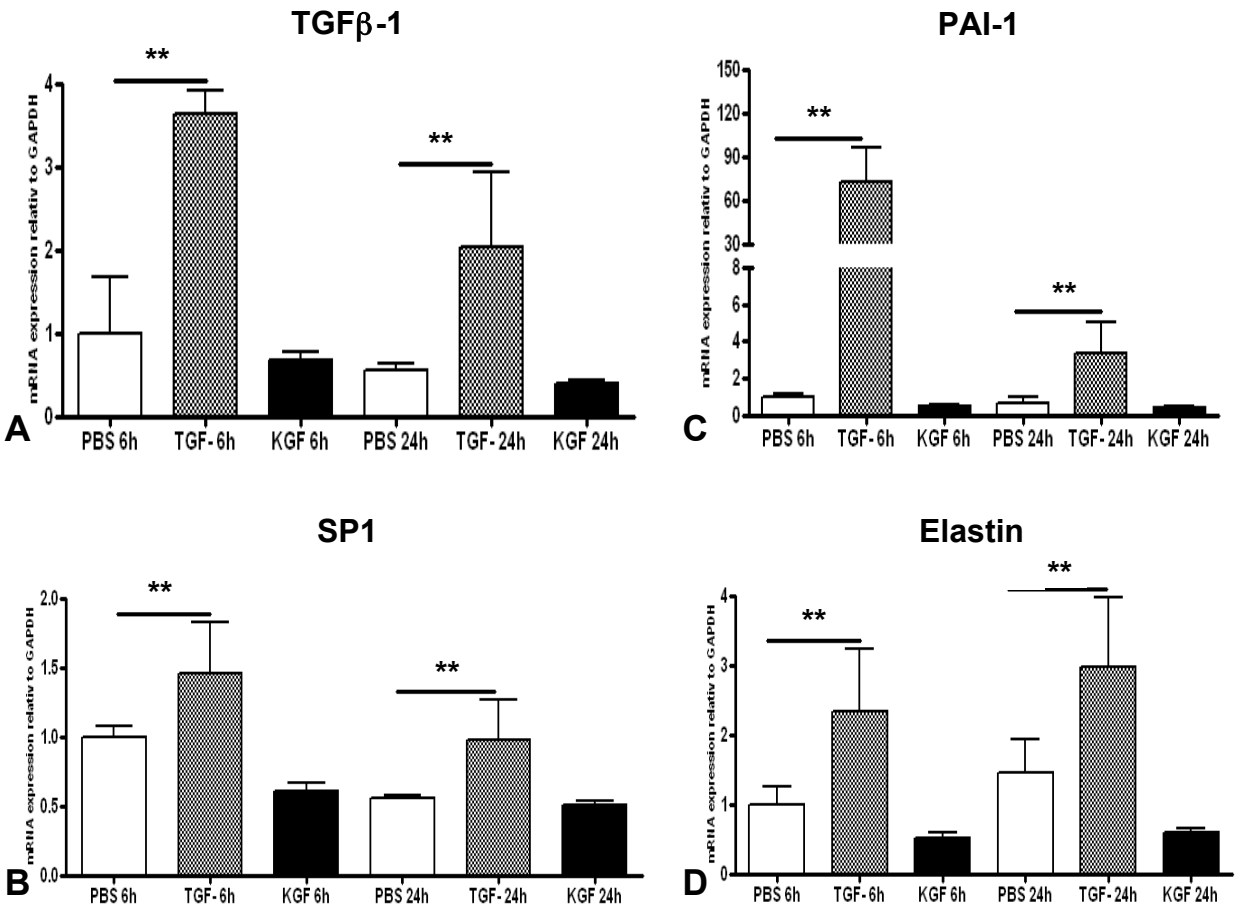


Figure 28 - *rHuKGF does not directly interact with fibroblasts to affect the mRNA expression of TGFβ-1 and its target genes. Quantitative real time RT-PCR revealed that incubation of mouse lung fibroblast (MLF) cells with TGFβ-1 (10 ng/ml) significantly increased mRNA expression levels of TGFβ-1 (A), SP-1 (B), PAI-1 (C) and elastin (D) after 6 and 24 hours in comparison with controls, whereas no effect was observed in cells incubated with rHuKGF (50 ng/ml). **P<0.05 versus the respective control group.*

5.2.3 Effects of neutralization of TGFβ in rHuKGF treated epithelial/fibroblast co-cultures.

To assess if the regenerative effects of rHuKGF on alveolar interstitial tissue

LA-4/MLF co-cultures were incubated with or without 50 ng/ml of rHuKGF in the presence of neutralizing anti-TGFβ antibody (clone 1D11; 20μg/ml) or control IgG1 (20μg/ml). In the absence of anti-TGFβ antibody, LA-4/MLF co-cultured cells incubated with rHuKGF for 6 or 24 hours showed a significant increase in mRNA expression levels of SP-1, PAI-1, and elastin. Interestingly, rHuKGF incubated LA-4/MLF co-cultured cells did not show upregulation of these genes when cells were treated with neutralizing anti-TGFβ antibody simultaneously, indicating that anti-TGFβ antibody abrogates rHuKGF induced SP-1, PAI-1 and elastin expression (Fig.29A-C).

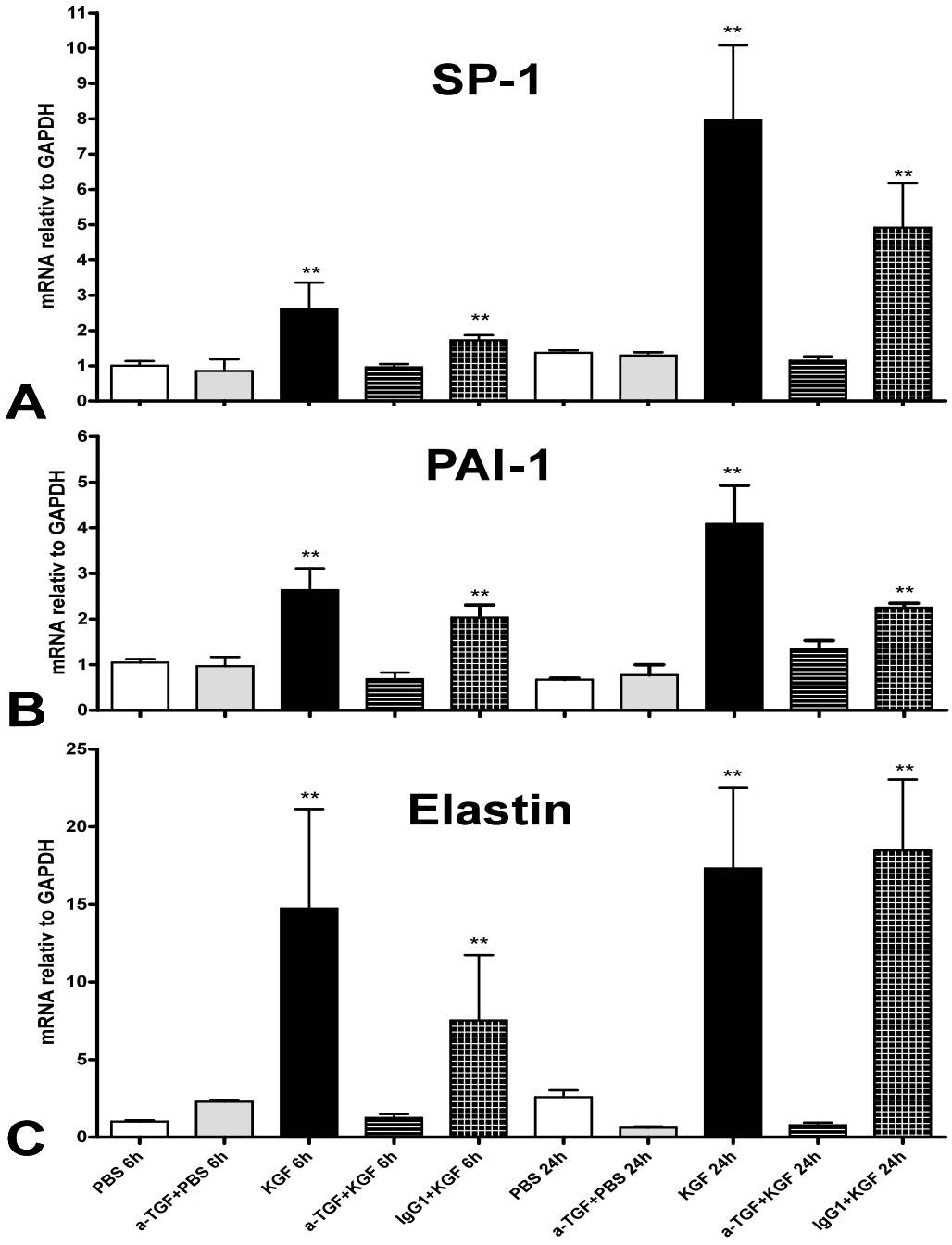


Figure 29 - *Murine AE2-like LA4 cells cocultured with mouse lung fibroblast (MLF) cells were incubated with rHuKGF (50 ng/ml) or PBS in the presence or absence of anti-TGFβ antibody (clone 1D11; 20 µg/ml) or control IgG1 (20 µg/ml) for 6 and 24 hours, respectively. In LA-4/MLF co-cultures incubated with rHuKGF, significantly increased mRNA expression of SP-1 (A), PAI-1 (B), and) elastin (C) were found at both time points which were almost completely blocked in the presence of neutralizing anti-TGFβ antibody (clone 1D11) but not in the presence of control IgG1 at 6 and 24 hours, respectively. **P<0.05 versus the respective control group.*

6 Discussion

Pulmonary emphysema is considered as a progressive disease related to cigarette smoking and characterized by significant airflow limitation, which is related to reduced elastic recoil of the lung through parenchymal destruction as well as reduced elastic load applied to the airways through destruction of alveolar attachments (6). Although inflammation, oxidative stress, elastinolytic activity, and apoptosis of lung parenchymal cells are believed to contribute to the pathogenesis of emphysema, the precise mechanisms behind these pathological changes remain unclear. Until today, no effective treatment is available to re-establish normal gas exchanging lung parenchyma after emphysematous changes have been established.

All-trans-retinoic acid (ATRA), a metabolite of retinol (vitamin A), which is a multifunctional modulator of cellular behaviour, may alter both extra-cellular matrix metabolism and normal epithelial differentiation in mammals such as the rat. Several reports have demonstrated that retinoic acid (RA), retinoic acid receptor (RAR), and retinoid X receptor (RXR) are intimately implicated in the process of differentiation of many types of cells, tissues, and organs including the lungs (189-190). Other than this, all-trans-retinoic acid enhances epithelial repairs and improves survival of alveoli in neonatal rats after lung injury by hyperoxic (191-192).

About 10 years ago, Massaro and Massaro reported that normal lung structure can be restored in emphysematous rat lungs by therapeutic supplementation with all-trans-retinoic acid (193). This report, therefore, dragged much attention and considerable interest on retinoic acid. In addition, a small study reported that supplementation of vitamin A can lead to improvement in pulmonary function in patients with moderate to severe COPD with reduced concentration of vitamin A in serum (194). Conversely, recent clinical assessments of the effect of all-trans-retinoic acid in emphysema patients were unable to present significant improvements (195). Retinoic acid failed to promote recovery from cigarette smoking induced emphysema in the guinea pig (196). In addition, retinoic acid has not been found to have a beneficial effect on the recovery from elastase induced emphysema in mice and Fischer 344 rat models (197-198). These studies raised concerns regarding a therapeutic role of retinoic acid in the

treatment of emphysema and suggest that the beneficial effect of retinoic acid treatment may be species specific.

Very recently, Takahashi et al reported that simvastatin reverses elastase induced emphysema in mice by promoting alveolar epithelial cell proliferation as shown by a decrease in mean linear intercept (Lm) and increased PCNA positively stained alveolar epithelial cells in statin treated emphysematous lungs (199). Statin is a 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor which is known to reduce cholesterol. Besides this, statins exert multiple effects including anti-inflammatory effects, improved pulmonary function and improvement of coronary endothelial cell function in humans (200-201). However, morphologically, Takahashi et al focused only on the mean linear intercept to assess air space enlargement which is only one characteristic of emphysema and did not focus on the reversal of loss of alveolar gas exchange area. In addition, after lung injury, restoration of alveolar epithelial integrity requires proliferation, migration and differentiation of alveolar epithelial type 2 (AE2) cells. These aspects were not addressed in their study except proliferation of alveolar epithelial cells. Therefore, the induction of alveolar regeneration in emphysema has been emerged as a crucial need in this field.

Repair and maintenance of an integer alveolar septal wall is achieved only if all tissue components of the lung are involved, the alveolar epithelium is particularly important as it interacts with various types of resident and mobile cells present in the alveolar interstitium including fibroblast cells and endothelium cells (202). Alveolar fibroblasts, which are the main cell type responsible for the synthesis and secretion of the main components of the alveolar extracellular matrix, seem to participate in the complex cellular interactions that lead to alveolar growth and multiplication both in the fetal lung (203) and in the adult lung. The interaction of AE2 cells with fibroblasts is important to the modeling of alveoles during lung morphogenesis (204) as well as during remodeling associated with alveolar repair following lung injury (205). In addition, AE2-cell-derived factors may affect extracellular matrix formation by fibroblasts, such as stimulation of collagen type I secretion by AE2-cell-derived insulin-like growth factor (IGF) type 1 (206). Sirianni and coworkers (207) showed that direct intercellular contacts between alveolar fibroblasts and alveolar epithelial cells or capillary

suggest that pulmonary emphysema fibroblasts may lack the capacity to participate in the direct intercellular communications which play an essential role in fetal lung development and probably during lung repair (203, 208-209).

Keratinocyte growth factor (KGF), one of the fibroblast-derived mitogen, promotes survival and induces proliferation, migration, and differentiation of cells that express its receptor, fibroblast growth factor receptor 2-IIIb (FGFR2-IIIb or KGFR). Hence, KGF has been shown to directly influence the morphologic appearance and phenotypic characteristics of a number of epithelial cell types during development and in adult life (103, 210-212). The therapeutic role of exogenous rHuKGF has shown favourable effects in dermal injury in porcine (105) and rabbit (210) models, in chemotherapy or irradiation-induced oral and gastrointestinal mucositis in mice (213), and in cyclophosphamide-induced ulcerative hemorrhagic cystitis in rats (214). Particulary, in lung, rHuKGF has been found to promote the proliferation of AE2 cell, which is a prominent feature associated with virtually all kinds of lung injury and is an essential element of lung repair (215).Thus, it was hypothesized that the supplementation of lung rHuKGF may represent a new therapeutic option for emphysema.

The present study intended to evaluate, whether rHuKGF is able to induce a regenerative response in distal lung parenchyma after induction of pulmonary emphysema in mice. Mice were therapeutically treated at three occasions by oropharyngeal aspiration of 10 mg rHuKGF per kg b.w. after induction of pulmonary emphysema by PPE. Our result demonstrated the following: 1) exogenous rHuKGF supplementation drastically reversed respiratory dysfunction in contrast to emphysematous lungs, which was assessed by non invasive head-out body plethysmography; 2) induced airspace enlargement was partially reversed and lost alveolar gas exchange area was partially regenerated by rHuKGF supplementation in emphysematous lungs as assessed by tools of stereology; 3) the regenerative capacity of rHuKGF therapy was reflected by proliferation and differentiation of the major components of the alveolar wall which was associated with, A) increased expression of genes implicated in the formation of alveolar epithelium including PCNA, Cyclin D1, Nrf2, SP-C, AQP-5, Foxa-2, HGF and FGF-7; B) increased expression of genes implicated in capillary endothelium formation including VEGF, VEGFR1,

maintenance including TGFβ-1, TGFβ-2, TGFβ-R-1, TGFβ-R-2, Smad 2, PAI-1, SP-1, elastin and PDGF-A as assessed by quantitative real-time RT-PCR, Western blotting and immunohistochemistry techniques; 4) rHuKGF (50 ng/ml) induced TGFβ-1 mRNA expression levels in and release of active TGFβ-1 protein from primary mouse AE2 cells, murine AE2-like cell line LA-4 and co-cultures of LA-4 and murine lung fibroblasts (MLF) in vitro but not in AE1-like cells E10 ; 5) recombinant TGFβ-1 protein (10 ng/ml) but not rHuKGF was able to induce elastin gene expression in MLF cells; 6) the induction of SP1, PAI-1, and elastin gene expression in LA-4/MLF co-cultures incubated with rHuKGF was completely blocked in the presence of neutralizing anti-TGFβ antibody 1D11 (20 µg/ml).
Taken collectively, our data demonstrate that exogenous supplementation of rHuKGF has the potential to induce alveolar maintenance programs in emphysematous mouse lungs.

6.1 Effects of rHuKGF in in-vivo animal model

As with the demonstration of a loss of alveolar structures in animal models of emphysema, the demonstration of alveolar regeneration can only be conclusive if appropriate parameters are chosen. Such quantitative morphological approach to assess emphysema has to comprise the estimation of both airspace enlargement and loss of the alveolar walls. Alveoli are composed of the alveolar airspace and the surrounding alveolar walls. As each alveolus has an opening towards the alveolar duct lumen and the alveolar wall typically separates adjacent alveoli. Therefore, a proper approach to assess airspace enlargement for each three-dimensional structure includes the estimation of mean chord length (MCL), absolute volume of air spaces (V_{air}) and alveolar surface area per unit volume (S_v). To assess the destruction of alveolar wall, quantification of absolute volume of septal tissue (V_{ast}), alveolar surface area (S_a) and arithmetic mean thickness of alveolar septal wall (τ_{ast}) are relevant (216).

Results obtained from this study demonstrated an increase in mean chord length (MCL) and in absolute volume of air spaces (V_{air}) accompanied by a loss of alveolar surface area per unit volume (S_v) in elastase treated lungs (Fig.11) indicating towards

area in emphysematous lungs suggested that alveolar gas exchange area was decreased. On the other hand, volume of alveolar septal tissue (V_{ast}) was not influenced by elastase treatment of lungs. Despite elastase treatment, this study showed that alveolar septal walls (τ_{ast}) became thicker in emphysematous lungs (Fig.12). It is probable that after the periods of elastolysis, elastin and other extracellular matrix components are remodeled perhaps in disordered state in the areas where elastolysis has initiated emphysematous lesions, and thus contributing to increased alveolar septal wall thickness in emphysematous lungs. An increase of alveolar septal wall thickness is a known factor in suppressing the efficiency of alveolar gas exchange in emphysema and there are some strong evidences of increased thickness of alveolar septal wall with increased mean chord length in emphysematous lesions (217).

Interestingly, rHuKGF significantly reduced enlargement of acinar air spaces (i.e. MCL) which was induced by elastase instillation. rHuKGF significantly induced alveolar surface area per unit volume (S_v) but did not alter the absolute volume of air spaces (V_{air}) in emphysematous lungs, which reflects that the reversal of air space enlargement was partial in emphysematous lungs (Fig.11). rHuKGF supplementation was associated with the improvement in alveolar gas exchange area as observed by a significant increase in absolute volume of alveolar septal tissue (V_{ast}) and alveolar surface area (S_a) (Fig.12). Arithmetic mean thickness of the alveolar septal wall (τ_{ast}) was also observed to be increased in rHuKGF treated emphysematous lungs (Fig.12). It is likely that after supplementation of rHuKGF in emphysematous lungs, there are periods of tissue repair and remodelling in which connective tissues undergo architectural rearrangement and thus contributing to an increase in thickness of the alveolar septal wall.

In the present study, significant alterations in body weight in any of the tested groups were not observed (Fig.9A). Several hypotheses have been formulated to explain the mechanism of body weight loss in patients with emphysema including chronic inflammation, altered levels of the fat hormone leptin and breathing workload leading to negative energy balance. Long term cigarette smoke exposure leads to marked accumulation of inflammatory cells in lungs thus causing body weight alterations. On

aspirations. Although kinetic studies and analysis of mutant mice lacking inflammatory mediators have demonstrated that PPE not only possesses proteolytic activity but also leads to subsequent inflammation (218), the degree of inflammation, however, might be less in order to produce a marked variation in body weight in the present study. The finding from this study is in agreement with previous studies that have suggested that weight loss occurs in only a minority of emphysematous patients (219-220). Furthermore, supplementation of rHuKGF provided no alteration in total volume of right lung (V_{RL}) in emphysematous lungs when compared to emphysematous lungs that received PBS (Fig.9B). On the other hand emphysematous lungs showed a significant increase in V_{RL} compared to PBS treated lungs, which reflects diminished elastic recoil, a characteristic feature of emphysema (Fig.9B). Using non invasive head-out body plethysmography, it was observed that supplementation of rHuKGF significantly reversed the airflow limitation (reflected by EF50) when compared with control-treated emphysematous lungs indicating that rHuKGF has potential beneficial influence on increased airway resistance and pulmonary compliance (Fig.10).

Alveolar repair events require alveolar cells of every type (epithelial, endothelial, and mesenchymal) to participate since the induction of equilibrated proliferation in a particular cell population of alveoli involves the simultaneous expansion of the each and every alveolar cell type. Re-epithelialization of airway and alveolar septal walls is one of the important components of each repair process in many types of lung injury. Results obtained from this study support the notion that rHuKGF has an important role in proliferate responses of epithelial cells by showing markedly up-regulated mRNA levels of PCNA, Cyclin D1 and Nrf2 (Fig.14). Proliferative cell nuclear antigen (PCNA), a marker for cell proliferation, is a cell cycle regulated nuclear protein whose rate of synthesis correlates with the proliferative rate of cells (221). In addition, PCNA immunoreactivity has been employed by many investigators as a tool to assess the proliferative potential of cell populations (222). Furthermore, significantly high induction of the expression of PCNA protein in emphysematous lungs which had received rHuKGF was observed (Fig.15) and could demonstrate by immunostaining for PCNA that the protein was highly expressed in alveolar and airway epithelial cells (Fig.13). In addition, Cyclin D1 is a nucleoprotein which controls the cell cycle

G1 phase is the central phase to the integration of signals that regulate exit from the cell division cycle to differentiation and the reactivation of cell proliferation. Therefore, in emphysematous lungs, rHuKGF maintained the alveolar structure by inducing the mRNA expression of two genes that are already known to be functionally associated, as Cyclin D1 is thought to regulate the function of PCNA in DNA repair and replication (178). In addition, studies have shown a crucial role of nuclear factor erythroid-related factor 2 (Nrf2) in lung epithelial cell proliferation and protection against oxidative stress where mice lacking Nrf2 in alveolar epithelial cells (AE2) showed hindrance in the proliferation of AE2 cells leading to the conclusion that Nrf2 is essential for proper proliferation on alveolar epithelial cells (179). Thus, the up-regulation of PCNA, Cyclin D1 and Nrf2 expression by rHuKGF suggests that it plays a role in the coordination of the cell cycle and proliferation of epithelial cells with DNA replication and repair (Fig.14).

The alveolar epithelium is composed of two morphologically and functionally distinct cell populations i.e. alveolar epithelial type 1 (AE1) and alveolar epithelial type 2 (AE2) cells. AE2 cells are cuboidal cells that are the primary sites of surfactant synthesis and secretion. AE1 cells are thin, flat cells that line 90-95% of the gas exchange surface of the lung. AE2 cells serve as progenitor cells for the alveolar epithelium (202). The process of one differentiated cell type, such as AE2, undergoing transition into another differentiated cell type, such as AE1, has been referred to as differentiation. After lung injury and damage to AE1 cells, AE2 cells proliferate (as described above) and then differentiate into AE1 cells, which is an important process during restoration of the alveolar epithelium after injury. rHuKGF has been shown to promote AE2 cell proliferation in rats both in vitro and in vivo (126, 164). rHuKGF has the unique ability to induce differentiation of AE2 cells in vitro suggesting a role for rHuKGF in repair of the alveolar epithelium following lung injury (141, 224). Similarly, in this study the important role of rHuKGF in promoting the alveolar epithelium differentiation was observed. The increase in whole lung mRNA for the surfactant protein-C (SP-C) (2.2x) was induced by rHuKGF (Fig.16A), which may relate to an increase in the number of AE2 cells expressing this protein (224). SP-C is a hydrophobic polypeptide which is involved in the regulation of the biophysical acitivity of the alveolar surfactant lining layer (225-226). Expression of

investigation comprises the expression of the AE1 cell specific marker, aquaporin (AQP)-5 (Fig.16B), which is a mercury-sensitive water channel in salivery, lacrimal and repiratory tissues (227) and is a member of the large family of aquaporin proteins, most of which are water channels. (228-229). In adult alveolar epithelium, in situ studies suggest that AQP-5 is expressed only on the apical surface of AE1 cells (230). AQP-5 contributes to alveolar fluid homeostasis and cell volume regulation via its role as a water channel (229). Results obtained from this study showed a significant induction in the expression of AQP-5 in rHuKGF treated emphysematous lungs (Fig.16B) which clearly indicates that rHuKGF promoted transdifferentiation of AE2 cells into AE1 cells. In this study, an increase in the expression of Foxa-2, a fork head transcription factor was observed in rHuKGF treated emphysematous lungs (Fi.16C). Foxa-2 was shown previously to regulate cell differentiation in many organs, including the lung, pancrease and liver (231-234). An electron microscopic study on fetal mouse lung morphology revealved that disruption of Foxa-2 in the fetal mouse lung leads to immaturation of AE2 cells with lack of their characteristic morphology such as lamellar bodies and sparse apical microvilli, as well as absence of AE1 cells suggesting its important role in the regulation of lung epithelial cells differentiation (184). In the same study, in Foxa-2 deleted fetal mouse lung, immunostaining for alveolar epithelial cells differentiation markers, SP-B and SP-C were found to be absent in immature AE2 cells indicating that Foxa-2 is essential for epithelial cell differentiation during lung morphogenesis (184). Recent studies showed that conditional deletion of Foxa-2 in the mouse lung epithelial cells leads to airspace enlargement reflecting its requirement for normal alveolarization and postnatal lung homeostasis (232). Ectopic expression of Foxa-2 in distal respiratory epithelial cells in the lungs of transgenic mice disrupts branching morphogenesis and arrests differentiation of peripheral epithelial cells, suggesting its potential role in lung formation (183). This finding suggests that the administration of rHuKGF in emphysematous lung has a potential role in the process of epithelial cells differentiation.

Hepatocyte growth factor (HGF) and fibroblast growth factors-7 (FGF-7) stimulate alveolar epithelial cells proliferation, migration and differentiation. It has been shown that HGF facilitates the repair of an experimental epithelial wound in vitro (235). In

lung, HGF and FGF-7 are relevant as both the factors have been demonstrated to be protective in a number of animal models of acute lung injury (236).

Moreover, it has been demonstrated that HGF plays a pulmotropic role in compensatory lung regeneration after pneumonectomy (237). A defect in HGF production by neighboring fibroblasts could contribute to the excessive apoptosis of epithelial and endothelial cells, which is now recognized as an important step in the constitution of emphysema. The role of HGF in COPD has been addressed by two previous studies. Plantier and colleagues (238) found that cultured fibroblasts harvested from patients with emphysema produced less HGF than controls, and Bonay and colleagues (239) found a direct relationship between the severity of airflow obstruction and HGF mRNA content in lung samples of smokers. Therefore, these two studies suggest that the pulmonary regulation of HGF may be abnormal in patients with COPD. Recently, Ishizawa et al (240) showed that the intraperitoneal recombinant human HGF abrogates elastase-induced emphysema in mice through the mobilization of endothelial precursor cells and their engraftment in the damaged alveoli. This study also reports the significant high levels of HGF and FGF-7 which are essential mediators of alveolar repair, by lung fibroblasts, in emphysematous lungs receiving rHuKGF therapy (Fig.17A-B).

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF) (241), is a 34–46 kDa glycoprotein that was first isolated from tumour cells. VEGF plays a fundamental role in physiological and pathophysiological forms of angiogenesis, which is the growth of new blood vessels from existing ones, and regulation of endothelial cell differentiation (158) $VEGF_{165}$ is the predominant isoform and the most highly expressed isoform in the human lung where it is produced mainly by epithelial cells (242). $VEGF_{165}$ is the most biologically active in the physiological state (243).

The lung contains the highest level of transcripts (244) amongst a wide range of organs that express VEGF. Lung VEGF is synthesized by alveolar epithelial cells, bronchial epithelial cells, smooth muscle cells, and alveolar macrophages (245-246).

VEGF is necessary for the formation of vascular beds of several organs during embryo development and coordinates essential interactions between airway and blood vessels that are required for the establishment of normal lung architecture, as

of the heart and large vessels with loss of only a single copy of the VEGF gene (247). VEGF signals through receptors VEGFR-1 and VEGFR-2. VEGFR-1 plays a role in the organization of development of embryonic blood vessels (248) and in enhanced monocyte adhesion to endothelial cells (249)

It has been shown that VEGF and VEGFR2 levels were decreased in the lung of emphysematous subjects compared to healthy controls (250) and were related to increased alveolar cell apoptosis. Inhibition of VEGFR-2 with chemical inhibitor SU5416 in rats leads to enlargement of the air spaces, alveolar septal cell apoptosis, and decreased capillary density, which are the features of emphysema (82). VEGF also plays an important role in postnatal lung growth, since blockade of VEGFR-2 and VEGFR-1 with DC101 (an antibody to VEGFR-2), and MF1 (an antibody to VEGFR-1) respectively, arrests lung growth and leads to an emphysematous mouse phenotype (251). These studies certainly support the known role of VEGF in vasculogenesis during embryonic and early life. In this study, the expression of VEGF and its receptors as well as protein expresson of VEGFR2 was significantly induced in rHuKGF received emphysematous lungs, suggesting its effects on pulmonary capillary endothelium formation (Fig.19A-C, Fig.20). This finding was also paralleled by the induced proliferation of capillary endothelium cells in rHuKGF administered emphysematous lungs as assessed by co-localization of Ki-67 and markers for endothelial cells (podocalyxin) (Fig.18A-C).

Extracellular matrix (ECM) plays a crucial role during the lung development as it regulates cellular growth, migration and differentiation processes (252). Transforming growth factorβ-1 (TGFβ-1) has been implicated as a key mediator to promote the synthesis, accumulation and repair of interstitial tissue (185). This action is exerted through two complementary pathways, one which reduces matrix degradation and anotherone which stimulates matrix accumulation (253-254). The TGFβ-signaling pathway is crucial in the regulation of the transcription of genes involved in interstitial tissue remodeling and repairs. The TGFβ-1 signal is transduced by the transmembrane serine-threonine kinase receptor TGFβ-R-1, TGFβ-R-2 and the cytosolic protein Smad 2 to the nucleus where this complex binds to DNA and regulates gene transcription (151). The TGFβ-1 signaling pathway also plays an important role in the maintenance of alveolar structure, promotes pulmonary

present study, it was observed that rHuKGF significantly induced expression of TGFβ-1, TGFβ-R-1, TGFβ-R-2 and Smad 2 in emphysematous lungs (Fig.22A-C, Fig.24B). This was associated with a high expression of ECM proteinase inhibitor PAI-1, which inhibits ECM degradation, and zinc finger transcription factor (SP-1) expression that regulates the transcription of TGFβ-1 in rHuKGF received emphysematous lungs (Fig.23A-B). It has been reported that TGFβ-1 up-regulates the expression of PAI-1 (186), which reflects that TGFβ-1 contributes to the inhibition of ECM degradation.

It was noticed that rHuKGF exerted a positive effect on interstitial tissue formation by inducing elastin expression in emphysematous lung tissue (Fig.22D). Elastin is a structural protein of the ECM which confers elastic properties on the pulmonary alveolar interstitium and remains distributed broadly within the alveoli, including the alveolar septae, septal junctions, and along the septal-free edges (257). Organisation of elastin is inextricably linked to the proper alveolar development. Destruction of elastin is implicated in thinning and weakening of the alveolar walls that leads to tissue destruction and abnormal expansion of air sacs. (258). Therefore, elastic fibre formation and deposition are critical for alveologenesis which is characterized by the development of septae that subdivide the terminal air sacs into mature alveoli. This process requires the elastin expression by alveolar myofibroblasts at the tips of the developing alveolar septae. (259-260). Mice deficient for elastin (Eln–/–) revealed arrested perinatal development of terminal airway branches, resulting in dilated distal air sacs with attenuated tissue septae, a condition characteristic of emphysema (261).

It has been demonstrated that bronchial epithelial cells can produce TGFβ-2 at levels that alter gene expression and migration of fibroblasts (262-263). Furthermore, it has been shown that epithelial derived TGFβ-2 plays a critical role in modulating the basal structure of interstitial tissue and maintains collagen homeostasis using in vitro model of epithelial-mesenchymal trophic unit in the human airways. The epithelial-mesenchymal trophic unit consists of opposing layers of epithelial and mesenchymal cells. The area between these two cell layers, the basement membrane zone, contains extracellular matrix and a network of nerve fibers.

The novel finding in this study is the increase in mRNA expression of TGFβ-2 by administration of rHuKGF in emphysematous lungs. Further support for a role for TGFβ-2 comes from the demonstration that there are increased numbers of TGFβ-2 expressing cells as assessed by immunohistochemical staining in emphysematous lungs receiving rHuKGF therapy (Fig.24A, D). Hence, this data indicates that TGFβ-2 may be contributing to the formation of interstitial tissue along with the contribution of TGFβ-1 to the process which is vital for the repair process after lung injury.

Platelet-derived growth factor (PDGF) was originally identified as a mitogen for smooth muscle cells, fibroblasts, and glia cells. Smooth muscle cells are situated at three locations in the lung, in vascular and bronchial walls, and in alveolar septae (264). Alveolar septal smooth muscle cells, which are also known as contractile interstitial cells or alveolar myofibroblasts (265), have the morphology of fibroblasts and they express alpha-smooth muscle actin (266). Study based on the alpha-smooth muscle actin stainings in the lung parenchyma of platelet-derived growth factor-alpha (PDGF-A) null mice revealed a profound deficiency in alveolar myofibroblast. Alveolar myofibroblasts are responsible for the deposition of alveolar septal elastin and are crucial for alveolar septal formation during lung development. Our results indicate that rHuKGF has a capacity to significantly induced transcript levels of PDGF-A in pulmonary emphysematous lungs that contribute to the alveolar repair (Fig.25).

Collectively, this study suggests that rHuKGF leads to alveolar maintenance programs in emphysematous lung through exerting a major positive effect on interstitial tissue formation and maintenance.

Earlier, protective effects of rHuKGF pre-treatment have been reported by Plantier and colleagues in elastase induced emphysema in mice (267). However, in contrast to the findings from the present study, these authors did not observe a curative effect of rHuKGF in the reversal of elastase induced emphysema when therapy was initiated after establishment of the disease. The discrepancy of their data to the results obtained from the present study may be related to the following reasons: Firstly, they supplemented mice with only 5 mg rHuKGF per kg b.w. whereas others

dose of 10 mg rHuKGF per kg b.w., which was used in the present study, has been proven to promote proliferation of AE2 cells to a maximal degree as assessed by SP-B immunohistochemical staining in mice (135). Secondly, Plantier and co-workers instilled rHuKGF via a subcutaneous route whereas intratracheal administration of 5 mg rHuKGF per kg b.w. has been confirmed to stimulate more potent and more lasting alveolar epithelial cell proliferation in rodents (268). Independently, our group demonstrated that the proliferation of AE2 cells was significantly increased via oropharyngeal aspiration compared with intratracheal instillation using 10 mg rHuKGF per kg b.w. in C57BL/6 mice (269). Third, Plantier and co-workers supplemented mice with rHuKGF continuously from day 21 till day 27 and sacrificed mice at day 27 after elastase instillation. The kinetics studies of the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into alveolar epithelial cells in rats showed that proliferation of AE2 cells peaks at ~2-3 days after treatment with intratracheally given rHuKGF (126). Additionally, Fehrenbach and co-workers showed that a significant portion of hyperplastic AE2 differentiated into AE1 cells after day 3 following rHuKGF instillation (127). Moreover, in vitro studies have revealed that the continous presence of rHuKGF to AE2 cells leads to hindrance in AE 2 cells differentiation to AE1 cells (182). Hence, the present study indicates that the therapeutic aspects of rHuKGF to favourably affect emphysematous related changes is attributable to the supplementation of 10 mg rHuKGF per kg b.w. at three occasions with the time gap of three days via oropharyngeal aspiration.

6.2 Effects of rHuKGF in in-vitro cell culture

The gene expression and protein synthesis for TGFβ-1 have been documented in embryonic and adult murine lung (154, 270), but relatively little is known about the precise cells expressing this gene. The previous study has shown that TGFβ-1 is localized to airway epithelial and AE2 cells in normal human and murine lung tissue (147).

Likewise, in the present study, it was observed that rHuKGF greatly increased the numbers of TGFβ-1 immunostained alveolar and airway epithelial cells (Fig.21). Interestingly, in-vitro cell culture, TGFβ-1 mRNA expression was found to be induced in primary mouse AE2 cells and in the murine AE2-like cell line LA-4 by incubation

with rHuKGF (Fig.26) as well as the release of active TGFβ-1 protein from primary mouse AE2 cells and LA-4 cells was found to be significantly increased (Fig.27).

Furthermore, TGFβ is a chemoattractant for fibroblasts and can exert mitogenic effects on pulmonary fibroblasts via cytokines secreted by alveolar macrophages and fibroblasts including platelet-derived growth factor (PDGF) (271). In addition, it induces the transcription and synthesis of various other components of the extracellular matrix by immature and mature pulmonary fibroblasts, such as fibronectin, glycosaminoglycans, and proteoglycans (272, 273).

It has been demostrated that in lung and dermal fibroblasts, TGFβ increases elastin mRNA levels (153, 274). Further, in cultured human lung fibroblast, TGFβ induced an increase in mRNA and protein levels of elastin (148). Besides, it has been demonstrated that mice deficient in Smad 3 or the β6-integrin develop air space enlargement which indicating the influence of TGFβ-signaling for the deposition of elastin in alveolar structures (152). Smad 3 is a component of the TGFβ signal transduction pathway and deficiency of Smad 3 leads to repression of tropoelastin (precursor of elastin) expression in lung, and the development of centrilobular emphysema (152). Moreover, β6-integrin is involved in TGFβ activation. β6-integrin converts latent TGFβ protein to an active form hence allowing it to bind to its cognate receptors (275). These observations suggest that lung fibroblasts under the influence of active TGFβ are required for elastin accumulation in interstitial tissue of alveolar structures.

Likewise, in the present study, an induction of elastin gene expression as well as induced mRNA levels of SP-1, PAI-1 and TGFβ-1 were observed in murine lung fibroblast (MLF) cells by recombinant TGFβ-1 protein. Whereas rHuKGF did not affect mRNA expression of TGFβ-1, SP1, PAI-1, and elastin in MLF cells cultured alone suggesting that it's TGFβ-1 but not rHuKGF which induces increased mRNA expression of genes implicated in TGFβ-1 pathway in MLF cells (Fig.28).

Interestingly, incubation of co-cultures of MLF cells and AE2-like murine LA-4 cells with rHuKGF for 24 hours resulted in an approximately 10-fold increased release of active TGFβ-1 into the supernatant suggest that the beneficial effect of rHuKGF in

This finding was further confirm by observing that if TGFβ-signaling was completely blocked in the presence of the anti-TGFβ neutralizing antibody 1D11, rHuKGF failed to increase mRNA expression levels of elastin, SP-1 and PAI-1 in the MLF/LA-4 co-culture system, thus highlighting the importance of TGFβ release into the medium to mediate the effect of rHuKGF on interstitial tissue maintenance (Fig.29).

7 Conclusions

The presented thesis work provides a broad and novel overview on the therapeutic effect of rHuKGF supplementation on elastase induced pulmonary emphysema in mice. This study obtained with lung function test and using tools of morphometry, clearly suggested that the exogenous rHuKGF supplemented emphysematous lungs had greatly improved lung function and regenerated alveolar gas exchange area, which were found to be lost in emphysematous lungs. Upon validation on the molecular level, genes that are associated with biological processes implicated in the repair of alveolar gas exchange area including alveolar epithelial, capillary endothelial and extracellular matrix (ECM) formation, were favourable expressed as per the exogenous rHuKGF supplementation in emphysematous lungs in comparison with control treated emphysematous lungs.

In in-vitro cell culture, rHuKGF induced the expression of TGFβ-1 in and release of active TGFβ-1 from primary mouse AE2 cells, murine AE2-like cells LA-4 and co-cultures of LA-4 and murine lung fibroblasts (MLF). Recombinant TGFβ-1 protein but not rHuKGF was found to increase elastin gene expression in MLF cells. The importance of TGFβ-signaling as a potential mediator of the AE2-linked effects of rHuKGF on the interstitial tissue compartment was investigated in LA-4/mouse lung fibroblast (MLF) co-cultures in the presence or absence of the neutralizing anti-TGFβ antibody 1D11. Blockade of TGFβ-signaling by neutralizing antibody abrogates rHuKGF induced SP-1, PAI-1 and elastin expression in LA-4/MLF co-cultures.

Taken together, firstly, the result from mice body weight measurement suggests that neither elastase nor rHuKGF had an influence on the health status of mice. Interestingly, results obtained from lung function test and quantitative morphologic methods suggest that exogenous supplementation of rHuKGF exerts therapeutic effects in pulmonary emphysema by promoting the regeneration of alveolar epithelium in lung. Furthermore, genes implicated in alveolar epithelial, capillary endothelial and intertitial tissue formation were found to be significantly induced because of the exposure of rHuKGF in emphysematous lungs suggests its

emphysematous lungs. The presented study further suggests that the regenerative effects of rHuKGF on the interstitial tissue compartment are linked to AE2 cell-derived TGFβ-1 signaling, which is well summarized in the figure 30.

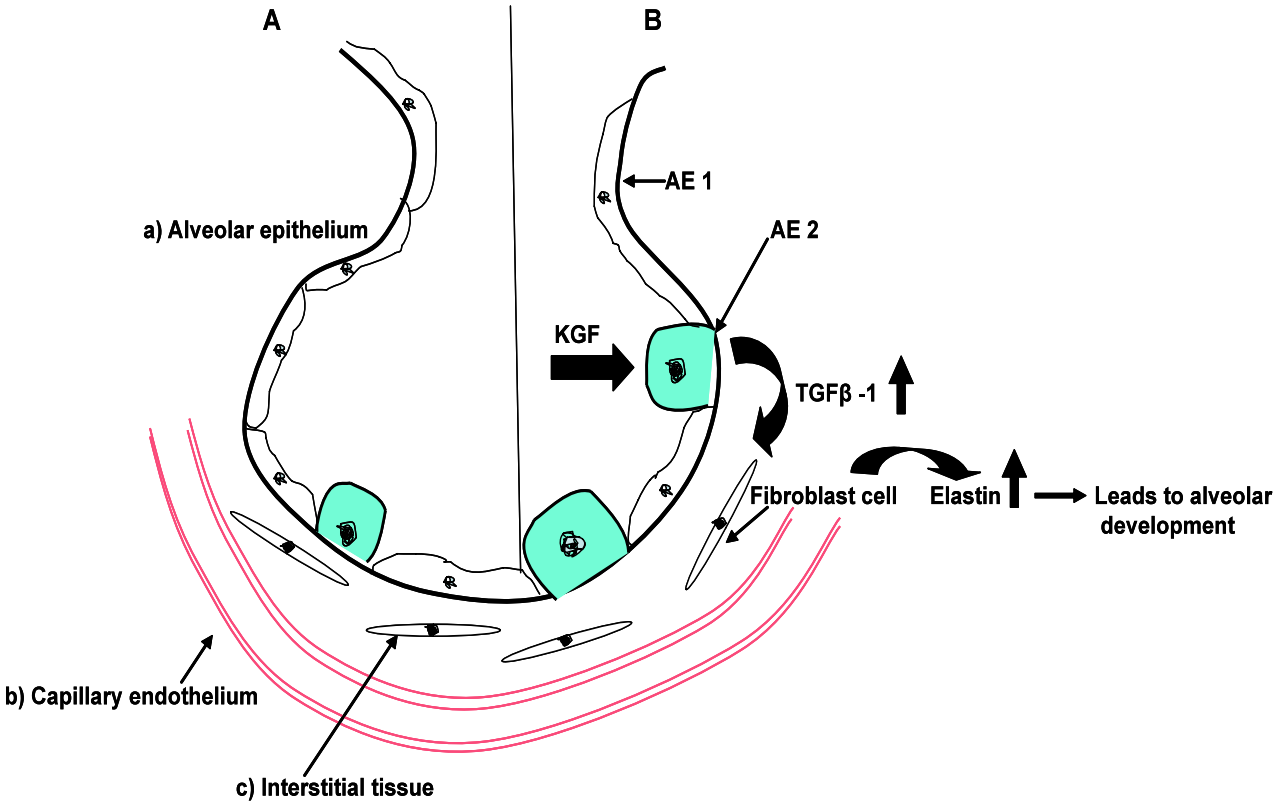


Figure 30 - *A. rHuKGF promotes alveolar structural maintenance programs in emphysematous lungs by inducing proliferation of a) alveolar epithelium, b) capillary endothelium, and c) interstitial tissue. B. The data suggests that rHuKGF has the potential to induce AE2 cell-derived TGFβ-1 being particularly important for maintenance of the elastin fiber scaffold.*

8 References

1. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. 2001. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/ WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) workshop summary. *Am J Respir Crit Care Med*. 163:1256–1276.
2. Lopez AD, Murray CC. 1998. The global burden of disease, 1990–2020. *Nat Med*. 4:1241–1243.
3. Murray CJL, Lopez AD, Mathers CD, Stein C. 2001. The Global Burden of Disease 2000 Project. Global programme on evidence for health policy discussion, paper #36. World Health Organization, Geneva, Switzerland, http:// www.who.int/whois/burden/papers/discussion paper revi sed.doc.
4. Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, Mannino DM. 2006. Global burden of COPD: systematic review and meta-analysis. *Eur Respir J*. 28: 523-532.
5. Barnes PJ. 1999. Molecular genetics of chronic obstructive pulmonary disease. *Thorax*. 54: 245–252.
6. Snider GL, Kleinerman J, Thurlbeck WM, Bengali Z H. 1985. The definition of emphysema. *Am Rev Respir Dis*. 132:181–185.
7. Bonet T. 1679. Sepulchretum sive anatonia pructica ex Cadaveribus Morbodenatis, proponens Histoa's Observations omnium pené humanicorporis affectuum, ipsarcomoue Causas recorditas revelans. Geneva.
8. Ruysch F. 1721. Observationes anatomica-chirurgicae.In: Tractatio anatomica Amsterdam.
9. Morgagni GB. 1769. The seats and causes of disease. Investigated byanatomy; in five books, containing a great variety of dissections, withremarks. Translated by Alexander B, Miller A, Caldwell T. London: Johnson and Payne.
10. Baillie M. 1799. A series of engravings, accompanied with explanation which are intended to illustrate the morbid anatomy of some of the most important parts of the human body divided into 10 fascicule. London: W. Blum R and Co.
11. Baillie M. 1807. The morbid anatomy of some of the most important parts of the

12. Laennec RT. 1819. De auscultation médiate; ou, Traité du diagnostic del maladies des poumons et du coeur. Paris, France: Brosson et Chaude.
13. Laennec RT. 1834. A treatise on the diseases of the chest. London, United Kingdom: Longman.
14. Osler W. 1892. The principles and practices of medicine: designed for theuse of practitioners and students of medicine. 8th ed. New York: D. Appleton and Co.
15. McClean K. 1956. The macroscopic anatomy of pulmonary emphysema. *Aust Ann Med.* 5:73-88.
16. World Health Organisation. 1961. Chronic cor pulmonare: Report of an expert committee. WHO Technical report series 213, 15.
17. American Thoracic Society. 1962. Statement on definitions and classification of chronic bronchitis, asthma, and pulmonary emphysema. *Am Rev Respir Dis*. 85:762-768.
18. American Thoracic Society. 1995. Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 152: S77-S121.
19. Thurlbeck WM. 1967. Internal surface area and other measurements in emphysema. *Thorax*. 22: 483–496.
20. Hogg JC, Wright JL, Wiggs BR, Coxson HO, Opazo SA, PD. 1994. Lung structure and function in cigarette smokers. *Thorax*. 49: 473–478.
21. Wiebe BM, Laursen H. 1998. Lung morphometry by unbiased methods in emphysema: bronchial and blood vessel volume, alveolar surface area and capillary length. *APMIS*. 106: 651–656.
22. Kim WD, Eidelman DH, Izqierdo JL, Ghezzo H, Saetta MP, Cosio MG. 1991. Centrilobular and panlobular emphysema in smokers. Two distinct morphologic and functional entities. *Am Rev Respir Dis*. 144:1385-1390.
23. Cosio MG, Cosio Piqueras MG. 2000. Pathology of emphysema in chronic obstructive pulmonary disease*. Monaldi Arch Chest Dis*. 55:124-129.
24. Erikkson S. 1964. Pulmonary emphysema and alpha-1-antitrypsin deficiency. *Acta Med Scand*. 175:197–205.
25. Saetta M, Finkelstein R, Cosio MG. 1994. Morphological and cellular basis for airflow limitation in smokers. *Eur Respir J*. 7:1505-1515.

26. Finkelstein R, Ma HD, Ghezzo H, WhittakerK, Fraser RS, Cosio MG. 1995. Morphometry of small airways in smokers and its relationship to emphysema type and hyperresponsiveness. *Am J Respir Crit Care Med*. 152:267–276.
27. Turato G, Zuin R, Saetta M. 2001. Pathogenesis and pathology of COPD. *Respiration*. 68:117–128.
28. Lacoste JY, Bousquet J, Chanez P, Van Vyve T, Simony-Lafontaine J, Lequeu N, Vic P, Enander I, Godard P, Michel FB. 1993. Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 92:537-548.
29. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, Pare PD. 2004. The nature of smallairway obstruction in chronic obstructive pulmonary disease. *N Engl J Med*. 350:2645–2653.
30. Retamales I, Elliott WM, Meshi B, Coxson HO, Pare PD, Sciurba FC, Rogers RM, Hayashi S, Hogg JC. 2001. Amplification of inflammation in emphysema and its association with latent adenoviral infection. *Am J Respir Crit Care Med*. 164:469-473.
31. Barnes PJ. 2004. Mediators of chronic obstructive pulmonary disease. *Pharmacol Rev*. 56:515-548
32. van Eeden SF, SinDD. 2008. Chronic obstructive pulmonary disease: a chronic systemic inflammatory disease. *Respiration*. 75:224-238.
33. Shapiro SD. 1999. The macrophage in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 160: S29-S32.
34. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallesco G, Tropeano G, Mapp CE, Maestrelli P, Ciaccia A, Fabbri LM. 1999. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 160:711–717.
35. Casolaro MA, Bernaudin JF, Saltini C, Ferrans VJ, Crystal RG. 1988. Accumulation of Langerhans' cells on the epithelial surface of the lower respiratory tract in normal subjects in association with cigarette smoking. *Am Rev Respir Dis*. 137: 406-411.
36. Soler P, Moreau A, Basset F, Hance AJ. 1989. Cigarette smoking-induced changes in the number and differentiated state of pulmonary dendritic

37. Black LF, Kueppers F. 1978. Alpha1-antitrypsin deficiency in nonsmokers. *Am Rev Respir Dis*. 117:421-428.
38. Hunninghake GW, Crystal RG. 1983. Cigarette smoking and lung destruction: accumulation of neutrophils in the lungs of cigarette smokers. *Am Rev Respir Dis*. 128:833-838.
39. Chung KF. 2001. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl*. 34:50s–59s.
40. Stockley RA. 2002. Neutrophils and the pathogenesis of COPD. *Chest*. 121:151S–155S.
41. Dubar V, Gosset P, Aerts C, Voisin C, Wallaert B, Tonnel AB. 1993. In vitro acute effects of tobacco smoke on tumor necrosis factor- and interleukin-6 production by alveolar macrophages. *Exp Lung Res*. 19:345–359.
42. Culpitt SV, Rogers DF, Shah P, De Matos C, Russell RE, Donnelly LE, Barnes PJ. 2003. Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 167: 24-31.
43. Morrison D, Strieter RM, Donnelly SC, Burdick MD, Kunkel SL, MacNee W. 1998. Neutrophil chemokines in bronchoalveolar lavage fluid and leukocyte-conditioned medium from non smokers and smokers. *Eur Respir J*. 12: 1067–1072.
44. Kuschner WG, D'Alessandro A, Wong H, Blanc PD. 1996. Dose-dependent cigarette smoking-related inflammatory responses inhealthy adults. *Eur Respir J*. 9: 1989–1994.
45. Agostini C, Facco M, Siviero M, Carollo D, Galvan S, Cattelan AM, Zambello R, Trentin L, Semenzato G. 2000. CXC chemokines IP-10 and mig expression and direct migration of pulmonary CD8+/CXCR3+ T cells in the lungs of patients with HIV infection and T-cell alveolitis. *Am J Respir Crit Care Med*. 162:1466–1473.
46. Lim S, Roche N, Oliver BG, Mattos W, Barnes PJ, Chung KF. 2000. Balance of matrix metalloprotease-9 and tissue inhibitor of metalloprotease-1 from alveolar macrophages in cigarette smokers: regulation by interleukin-10. *Am J Respir Crit Care Med*. 162:1355–1360.
47. Majo J, Ghezzo H, Cosio MG. 2001. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. *Eur Respir J*. 17: 946-

48. Maeno T, Houghton AM, Quintero PA, Grumelli S, Owen CA, Shapiro SD. 2007. CD8+ T Cells are required for inflammation and destruction in cigarette smoke-induced emphysema in mice. *J Immunol*. 178:8090-8096.
49. Huang Q, Liu D, Majewski P, Schulte LC, Korn JM, Young RA, Lander ES, and Hacohen N. 2001. The plasticity of dendritic cell responses to pathogens and their components. *Science*. 294: 870-875.
50. Zeid NA, Muller HK. 1995. Tobacco smoke induced lung granulomas and tumors: association with pulmonary Langerhans cells. Pathology. 27: 247-254.
51. Stoller JK, Aboussouan LS. 2005. Alpha 1-antitrypsin deficiency. *Lancet*. 365: 2225–2236.
52. Takubo Y, Guerassimov A, Ghezzo H, Triantafillopoulos A, Bates JH, Hoidal JR, Cosio MG. 2002. Alpha1-antitrypsin determines the pattern of emphysema and function in tobacco smoke-exposed mice: parallels with human disease. *Am J Respir Crit Care Med*. 166:1596-1603.
53. Cavarra E, Bartalesi B, Lucattelli M, Fineschi S, Lunghi B, Gambelli F, Ortiz LA, Martorana PA, Lungarella G. 2001. Effects of cigarette smoke in mice with different levels of E(1)-proteinase inhibitor and sensitivity to oxidants. *Am J Respir Crit Care Med*. 164:886-890.
54. Stockley RA. 1999. Neutrophils and protease/ antiprotease imbalance. *Am J Resp Crit Care Med*. 160:S49–S52
55. Shapiro SD, Goldstein NM, Houghton AM, Kobayashi DK, Kelley D, Belaaouaj A. 2003. Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice. *Am J Pathol*. 163:2329-2335.
56. Shapiro SD, Senior RM. 1999. Matrix metalloproteinases: matrix degradation and more. *Am J Respir Cell Mol Biol*. 20:1100–1102.
57. Takeyabu K, Betsuyaku T, Nishimura M. 1998. Cysteine proteinases and cystatin C in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Eur Respir J*.12:1033–1039.
58. Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C. 2000. Selman M. Upregulation of gelatinases A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. *Chest*. 117:684–694.
59. Stamenkovic I. 2003. Extracellular matrix remodelling: the role of matrix metallo-proteinases. *J Pathol*. 200: 448-464.

60. Finlay GA, O'Driscoll LR, Russell KJ, D'Arcy EM, Masterson JB, FitzGerald MX, O'Connor CM. 1997. Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am J Respir Crit Care Med*. 156:240-247.
61. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, Kawakami Y. 1999. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med*. 159:1985-1991.
62. Culpitt SV, Nightingale JA, Barnes P. 1999. Effect of high dose inhaled steroid on cells, cytokines and proteases in induced sputum in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 160:1635-1639.
63. Demedts IK, Morel-Montero A, Lebecque S, Pacheco Y, Cataldo D, Joos GF, Pauwels RA, Brusselle GG. 2006. Elevated MMP-12 protein levels in induced sputum from patients with COPD. *Thorax*. 61:196–201.
64. Molet S, Belleguic C, Lena H, Germain N, Bertrand CP, Shapiro SD, Planquois JM, Delaval P, Lagente V. 2005. Increase in macrophage elastase (MMP-12) in lungs from patients with chronic obstructive pulmonary disease. *Inflamm Res*. 54: 31–36.
65. Caramori G, Di Gregorio C, Carlstedt I, Casolari P, Guzzinati I, Adcock IM, Barnes PJ, Ciaccia A, Cavallesco G, Chung KF, Papi A. 2004. Mucin expression in peripheral airways of patients with chronic obstructive pulmonary disease. *Histopathology*. 45:477–484.
66. Kang MJ, Homer RJ, Gallo A, Lee CG, Crothers KA, Cho SJ, Rochester C, Cain H, Chupp G, Yoon HJ, Elias JA. 2007. IL-18 is induced and IL-18 receptor alpha plays a critical role in the pathogenesis of cigarette smoke-induced pulmonary emphysema and inflammation. *J Immunol*. 178:1948-1959.
67. Punturieri A, Filippov S, Allen E, Caras I, Murray R, Reddy V, Weiss SJ. 2000. Regulation of elastinolytic cysteine proteinase activity in normal and cathepsin K-deficient human macrophages. *J Exp Med*. 192:789–99.
68. Cantin A, Crystal RG. 1985. Oxidants, antioxidants and the pathogenesis of emphysema. *Eur J Respir Dis Suppl*. 139:7-17
69. Laurent P, Janoff A, Kagan HM. 1983. Cigarette smoke blocks cross-linking of elastin in vitro. *Am Rev Respir Dis*. 127:189-192.

70. Pryor WA, Stone K. 1993. Oxidants in cigarette smoke: radicals, hydrogen peroxides, peroxynitrate, and peroxynitrite. *Ann N Y Acad Sci*. 686:12-27.
71. MacNee W. 2001. Oxidative stress and lung inflammation in airways disease. *Eur J Pharmacol*. 429:195-207.
72. Taggart C, Cervantes-Laurean D, Kim G, McElvaney NG, Wehr N, Moss J, and Levine RL. 2000. Oxidation of either methionine 351 or methionine 358 in α1-antitrypsin causes loss of anti-neutrophil elastase activity. *J Biol Chem*. 275: 27258-27265.
73. Hubbard RC, Ogushi F, Fells GA, Cantin AM, Jallat S, Courtney M, Crystal RG. 1987. Oxidants spontaneously released by alveolar macrophages of cigarette smokers can inactivate the active site of alpha 1-antitrypsin rendering it ineffective as an inhibitor of neutrophil elastase. *J Clin Invest*. 80:1289-1295.
74. Kramps JA, Rudolphus A, Stolk J, Willems LN, Dijkman JH. 1991. Role of antileukoprotease in the lung. *Ann N Y Acad Sci*. 624:97-108.
75. Kramps JA, van Twisk C, Klasen EC, Dijkman DH. 1988. Oxidative inactivation of antileukoprotease is triggered by polymorphonuclear leukocytes. *Clin Sci*. 75:53-62.
76. Rahman I, MacNee W. 1998. Role of transcription factors in inflammatory lung diseases. *Thorax*. 53:601-612.
77. Rahman I, Marwick J, Kirkham P. 2004. Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem Pharmacol*. 68:1255-1267.
78. Liebow AA. 1959. Pulmonary emphysema with special reference to vascular changes. *Am Rev Respir Dis*. 80:67–93.
79. Aoshiba K, Nagai A. 1999. Apoptosis in chronic obstructive pulmonary disease. *Nippon Rinsho*. 57:1972–1975.
80. Degterev A, Boyce M, Yuan J. 2003. A decade of caspases. *Oncogene*. 22:8543–67.
81. Aoshiba K, Yokohori N, Nagai A. 2003. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol*. 28:555–562.
82. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. 2000. Inhibition of VEGF receptors causes

83. Tsao PN, Su YN, Li H. 2004. Overexpression of placenta growth factor contributes to the pathogenesis of pulmonary emphysema. *Am J Respir Crit Care Med*. 169: 505-511.
84. Matsuzaki Y, Xu Y, Ikegami M, Besnard V, Park KS, Hull WM, Wert SE, Whitsett JA. 2006. Stat3 is required for cytoprotection of the respiratory epithelium during adenoviral infection. *J Immunol*. 177: 527–537.
85. Barry M, Bleackley RC. 2002. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol*. 2:401–409.
86. Liu AN, Mohammed AZ, Rice WR, Fiedeldey DT, Liebermann JS, Whitsett JA, Braciale TJ, Enelow RI. 1999. Perforin-independent CD8 (+) T-cell-mediated cytotoxicity of alveolar epithelial cells is preferentially mediated by tumor necrosis factor-alpha: relative insensitivity to Fas ligand. *Am J Respir Cell Mol Biol*. 20:849–858.
87. Campbell EJ. 1986. Preventive therapy of emphysema: lessons from the elastase model. *Am Rev Respir Dis*. 134:435-437.
88. Snider GL, Lucey EC, Stone PJ. 1986. Animal models of emphysema. *Am Rev Respir Dis*. 133:149-69.
89. Snider GL. 1992. Emphysema: The first two centuries; and beyond. A historical overview, with suggestions for future research: Part 1. *Am Rev Respir Dis*. 146:1334-44.
90. Wright JL, Churg A. 1990. Cigarette smoke causes physiologic and mophologic changes of emphysema in the guinea pig. *Am Rev Respir Dis*. 142:1422–1428.
91. Shapiro SD. 2000. Animal models for chronic obstructive pulmonary disease: age of klotho and marlboro mice. *Am J Respir Cell Mol Biol*. 22:4–7.
92. March TH, Barr EB, Finch GL, Hahn FF, Hobbs CH, Menache MG, Nikula KJ. 1999. Cigarette smoke exposure produces more evidence of emphysema in B6C3F1 mice than in F344 rats. *Toxicol Sci*. 51:289–299.
93. Guerassimov A, Hoshino Y, Takubo Y, Turcotte A, Yamamoto M, Ghezzo H, Triantafillopoulos A, Whittaker K, Hoidal JR, Cosio MG. 2004. The development of emphysema in cigarette smoke-exposed mice is strain dependent. *Am J Respir Crit Care Med*. 170: 974-80.
94. Fabbri L, Pauwels RA, Hurd SS, and GOLD Scientific Committee. 2003. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive

Pulmonary Disease: GOLD Executive Summary updated 2003. *COPD*. 1: 105-141.

95. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J. 2007. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med*. 176: 532-555.
96. Gross P, Pfitzer E, Tolker M, Babyak MA, Kaschak M. 1965. Experimental emphysema: its production with papain in normal and silicotic rats. *Arch Environ Health*. 11:50-58.
97. Lieberman J. 1976. Elastase collagenase emphysema and alpha-1-antitrypsin deficiency. *Chest.* 70: 62-67.
98. Janoff A, White R, Carp H, Harel S, Dearing R, Lee D. 1979. Lung injury induced by leukocytic proteases. *Am J Pathol*. 97: 111-136.
99. Snider GL. 1992. Emphysema: The first two centuries-and beyond: A historical overview, with suggestions for future research: Part 2. *Am Rev Respir Dis*. 146:1615–1622.
100. Lesser M, Padilla ML, Cardozo C. 1992. Induction of emphysema in hamsters by intratracheal instillation of cathepsin B. *Am Rev Respir Dis*. 145: 661-668.
101. Kuhn C, Yu SY, Chraplyvy M, Linder HE, Senior RM. 1976. The induction of emphysema with elastase. II. Changes in connective tissue. *Lab Invest*. 34:372-380.
102. Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. *Proc Natl Acad Sci U.S.A*. 86: 802–806.
103. Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA. 1989. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science.* 245: 752-755.
104. Wen J, Hsu E, Kenney WC, Philo JS, Morris CF, Arakawa T. 1996. Characterization of keratinocyte growth factor binding to heparin and dextran sulfate. *Arch Biochem Biophys*. 332: 41-46.
105. Staiano-Coico L, Krueger JG, Rubin JS, D'Limi S, Vallat VP, Valentino L, Fahey T 3rd, Hawes A, Kingston G, Madden MR, Mathwich M, Gottlieb A, Aaronson

SA. 1993. Human keratinocyte growth factor effects in a porcine model of epidermal wound healing. *J Exp Med*. 178:865–878.

106. Sotozono C, Inatomi T, Nakamura M, Kinoshita S. 1995. Keratinocyte growth factor accelerates corneal epithelial wound healing in vivo. *Invest Ophthalmol Vis Sci*. 36: 1524–1529.
107. Zeeh JM, Procaccino F, Hoffmann P, Aukerman SL, McRoberts JA, Soltani S, Pierce GF, Lakshmanan J, Lacey D, Eysselein VE. 1996. Keratinocyte growth factor ameliorates mucosal injury in an experimental model of colitis in rats. *Gastroenterology*. 110: 1077–1083.
108. Winkles JA, Alberts GF, Chedid M, Taylor WG, Demartino S, Rubin JS. 1997. Differential expression of the keratinocyte growth factor (KGF) and KGF receptor genes in human vascular smooth muscle cells and arteries. *J Cell Physiol.* 173:380–386.
109. Smola H, Thiekötter, Fusenig NE. 1993. Mutual induction of growth factor gene expression by epidermal-dermal cell interaction. *J Cell Biol*. 122:417–429.
110. Boismenu R, Havran WL. 1994. Modulation of epithelial cell growth by intraepithelial γδT cells. *Science*. 266:1253–1255.
111. Yamashita T, Yoshioka M, Itoh N. 2000. Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochem Biophys Res Commun*. 277:494-498.
112. Klagsbrun M. 1989. The fibroblast growth factor family: structural and biological properties. *Prog. Growth Factor Res*. 1: 207-235.
113. Thomas K A. 1987. Fibroblast growth factors. *FASEB J*. 1:434-440.
114. Basilico C, Moscatelli D. 1992. The FGF family of growth factors and oncogenes. *Adv. Cancer Res*. 59:115-165.
115. Martin GR. 1998. The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* 12:1571-1586.
116. Peters KG, Werner S, Chen G, Williams LT. 1992. Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissues during limb formation and organogenesis in the mouse. *Development*. 114: 233-43.
117. Orr-Urtreger A, Bedford MT, Burakova T, Arman E, Zimmer Y, Yayon A, Givol D, Lonai P. 1993. Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). *Dev Biol*. 158:475-86.

119. McKeehan WL, Wang F, Kan M. 1998. The heparan sulfate-fibroblast growth factor family: diversity of structure and function. *Prog. Nucleic Acids Res. Mol*. Biol. 59:135-176
120. Johnson DE, Lu J, Chen H, Werner S, Williams LT. 1991. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. *Mol Cell Biol*. 11: 4627-4634.
121. Igarashi M, Finch PW, Aaronson SA. 1998. Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). *J Biol Chem*. 273:13230-13235.
122. Aaronson SA, Bottaro DP, Miki T, Ron D, Finch PW, Fleming TP, Ahn J, Taylor WG, Rubin JS. 1991. Keratinocyte growth factor: a fibroblast growth factor family member with unusual target cell specificity. *Ann NY Acad Sci*. 638: 62-77.
123. Miki T, Fleming TP, Bottaro DP, Rubin JS, Ron D, Aaronson SA. 1991. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. *Science*. 251:72–75.
124. Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA. 1990. Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. *J Biol Chem*. 265:12767-12770.
125. Miki T, Bottaro DP, Fleming T P, Smith C L, Burgess WH, Chan AM, Aaronson SA. 1992. Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. *Proc Natl Acad Sci U. S. A*. 89:246-250.
126. Ulich TR, Yi ES, Longmuir K, Yin S, Biltz R, Morris CF, Housley RM, Pierce GF. 1994. Keratinocyte growth factor is a growth factor for type II pneumocytes in vivo. *J Clin Invest*. 93:1298-1306.
127. Fehrenbach H, Kasper M, Tschernig T, Pan T, Schuh D, Shannon JM, Muller M, Mason RJ. 1999. Keratinocyte growth factor-induced hyperplasia of rat alveolar type II cells in vivo is resolved by differentiation into type I cells and apoptosis. *Eur Respir J*. 14:534-544.
128. Michelson PH, Tigue M, Panos RJ, Sporn PH. 1999. Keratinocyte growth factor stimulates bronchial epithelial cell proliferation in vitro and in vivo. *Am J Physiol Lung Cell Mol Physiol*. 277: L737-L742.

129. Panos R J, PM Bak, WS Simone, JS Rubin, LJ Smith. 1995. Intratracheal instillation of KGF decreases hyperoxia-induced mortality in rats. *J Clin Invest*. 96: 2026-2033.
130. Deterding RR, Havill AM, Yano T, Middleton SC, Jacoby CR, Shannon JM, Simonet WS, Mason RJ. 1997. Prevention of bleomycin-induced lung injury in rats by keratinocyte growth factor. *Proc Assoc Am Physicians*. 109:254–268.
131. Sugahara K, Iyama K, Kuroda MJ, Sano K. 1998. Double intratracheal instillation of keratinocyte growth factor prevents bleomycin-induced lung fibrosis in rats. *J Pathol*. 186:90–98.
132. Viget NB, Guery BPH, Ader F, Neviere R, Alfandari S, Creuzy C, Roussel-Delvallez M, Foucher C, Mason CM, Beaucaire G, Pittet JF. 2000. Keratinocyte growth factor protects against Pseudomonas aeruginosa-induced lung injury. *Am J Physiol Lung Cell Mol Physiol*. 279: L1199-L1209. .
133. Yano T, Deterding RR, Simonet WS, Shannon JM, Mason RJ. 1996. Keratinocyte growth factor reduces lung damage due to acid instillation in rats. *Am J Respir Cell Mol Biol*. 15:433–442.
134. Nemzek JA, Ebong SJ, Kim J, Bolgos GL, Remick DG. 2002. Keratinocyte growth factor pretreatment is associated with decreased MIP-2 concentrations and reduced neutrophil recruitment in acid aspiration lung injury. *Shock*.18:501–506.
135. Ulrich K, Stern M, Goddard ME, Williams J, Zhu J, Dewar A, Painter HA, Jeffery PK, Gill DR, Hyde SC, Geddes DM, Takata M, Alton EW. 2005. Keratinocyte growth factor therapy in murine oleic acid-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 288:L1179-L1192.
136. Yi ES, Williams ST, Lee H, Malicki DM, Chin EM, Yin S, Tarpley J, Ulich TR. 1996. Keratinocyte growth factor ameliorates radiation- and bleomycin-induced lung injury and mortality. *Am J Pathol*. 149:1963-1970.
137. Takeoka M, Ward WF, Pollack H, Kamp DW, Panos RJ. 1997. KGF facilitates repair of radiation-induced DNA damage in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 272: L1174–L1180.
138. Wu KI, Pollack N, Panos RJ, Sporn PHS, Kamp DW. 1998. Keratinocyte growth factor promotes alveolar epithelial cell DNA repair after H2O2 exposure. *Am J Physiol Lung Cell Mol Physiol*. 275: L780–L787.

139. Guery BPH, Mason CM, Dobard EP, Beaucaire G, Summer WR, Nelson S. 1997. Keratinocyte growth factor increases transalveolar sodium reabsorption in normal and injured rat lungs. *Am J Respir Crit Care Med*. 155:1777-1784.
140. Borok Z, Danto SI, Dimen LL, Zhang XL, Lubman RL. 1998. Na+-K+-ATPase expression in alveolar epithelial cells: upregulation of active ion transport by KGF. *Am J Physiol Lung Cell Mol Physiol*. 274:L149-L158.
141. Sugahara K, Rubin JS, Mason RJ, Aronsen EL, Shannon JM. 1995. Keratinocyte growth factor increases mRNAs for SP-A and SP-B in adult rat alveolar type II cells in culture. *Am J Physiol*. 269:L344-L350.
142. Waters CM, Savla U. 1999. Keratinocyte growth factor accelerates wound closure in airway epithelium during cyclic mechanical stain. *J Cell Physiol*. 181:424-432.
143. Post M, Souza P, Liu J, Tseu I, Wang J, Kuliszewski M, Transwell AK. 1996. Keratinocyte growth factor and its receptor are involved in regulating early lung branching. *Development*. 122:3107-3115.
144. Wessells NK. 1979. Mammalian lung development: interactions in formation and morphogenesis of tracheal buds. *J. Exp. Zool*. 175:445-460.
145. Padela S, Yi M, Cabacungan J, Shek S, Belcastro R, Masood A, Jankov RP, Tanswell AK. 2008. A critical role for fibroblast growth factor-7 during early alveolar formation in the neonatal rat. *Pediatr Res*. 63:1-7.
146. Matsumoto K, Nagayasu T, Hishikawa Y, Tagawa T, Yamayoshi T, Abo T, Tobinaga S, Furukawa K, Koji T. 2009. Keratinocyte growth factor accelerates compensatory growth in the remaining lung after trilobectomy in rats. *J Thorac Cardiovasc Surg*. 137:1499-1507.
147. Coker RK, Laurent GJ, Shahzeidi S, Hernandez--Rodriguez NA, Pantelidis P, du Bois RM, Jeffery PK, McAnulty RJ. 1996. Diverse cellular TGF-beta 1 and TGF-beta 3 gene expression in normal human and murine lung. *Eur Respir J.* 9:2501-2507.
148. Kuang PP, Zhang XH, Rich CB, Foster JA, Subramanian M, Goldstein RH. 2007. Activation of elastin transcription by transforming growth factor beta in human lung fibroblasts. *Am J Physiol Lung Cell Mol Physiol*. 292:L944-L952.
149. Shifren A, Mecham RP. 2006. The stumbling block in lung repair of emphysema: elastic fiber assembly. *Proc Am Thorac Soc*. 3:428-433

150. Wendel DP, Taylor DG, Albertine KH, Keating MT, Li DY. 2000. Impaired distal airway development in mice lacking elastin. *Am J Respir Cell Mol Biol*. 23:320-326.
151. Morris DG, Huang X, Kaminski N, Wang Y, Shapiro SD, Dolganov G, Glick A, Sheppard D. 2003. Loss of integrin alpha(v)beta6-mediated TGF-beta activation causes Mmp12-dependent emphysema. *Nature.* 422:169-173.
152. Chen H, Sun J, Buckley S, Chen C, Warburton D, Wang XF, Shi W. 2005. Abnormal mouse lung alveolarization caused by Smad3 deficiency is developmental antecedent centrilobular emphysema. *Am J Physiol Lung Cell Mol Physiol*. 288:L683-L691.
153. Kucich U, Rosenbloom JC, Abrams WR, Rosenbloom J. 2002. Transforming growth factor-stabilizes elastin mRNA by a pathway requiring active Smads, Protein Kinase C-, and p38. *Am J Respir Cell Mol Biol*. 26: 183–188.
154. Schmid P, Cox D, Bible G, Maier R, Mcmaster GK. 1991. Differential expression of TGFβ1, β2, and β3 genes during mouse embryogenesis. *Development*. 111:117–130.
155. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. 1997. TGF2 knockout mice have multiple developmental defects that are non-overlapping with other TGFβ knockout phenotypes. *Development.* 124(13):2659–2670.
156. Ferrara N. 2004. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 25: 581–611.
157. Zeltner TB, Caaduff JH, Gehr P, Pfenninger J, Burri PH. 1987. The postnatal development and growth of the human lung. *I. Morphometry. Respir Physiol*. 67: 247–267.
158. Cross MJ, Claesson-Welsh L. 2001. FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition. *Trends Pharmacol Sci.* 22:201–7.
159. Matsumoto T. Claesson-Welsh L. 2003. VEGF receptor signal transduction. *Trends Biochem Sci*. 2003:488–494.
160. Fong GH, Rossant J, Gertsenstein M. 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature.* 376(6535):66–70.

161. Brown KR, England KM, Goss KL. 2001. VEGF induces airway epithelial cell proliferation in human fetal lung in vitro. *Am J Physiol Lung Cell Mol Physiol.* 281(4):L1001–L1010.
162. Nakamura T, Nawa K, Ichihara A. 1984. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun*. 122:1450–1459.
163. Mason RJ. 2002. Hepatocyte growth factor. The key to alveolar septation? *Am J Respir Cell Mol Biol*. 26:517–20.
164. Panos RJ, Rubin JS, Csaky KG, Aaronson SA, Mason RJ. 1993. Keratinocyte growth factor and hepatocyte growth factor/scatter factor are heparin-binding growth factors for alveolar type II cells in fibroblast-conditioned medium. *J. Clin. Invest.* 92:969–977.
165. Ohmichi H, Matsumoto K, Nakamura T. 1996. In vivo mitogenic action of HGF on lung epithelial cells: pulmotrophic role in lung regeneration. *Am J Physiol.* 270:L1031–L1039.
166. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM. 1992. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol*. 119: 629–641.
167. Boström H, Willetts K, Pekny M, Levéen P, Lindahl P, Hedstrand H, Pekna, M, Hellström M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Törnell J, Heath JK, Betsholtz C. 1996. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell*. 85:863-873.
168. Kapanchi Y, Gabbiani G. 1997. Contractile cells in pulmonary alveolar tissue. In The Lung. Scientific Foundations, 2nd edition (ed. Crystal RG, West JB, Weibel ER and Barnes PJ), pp. 697-707. Philadelphia: Lippincott-Raven Publishers.
169. Vijayaraghavan R, Schaper M, Thompson R, Stock MF, Alarie Y. 1993. Characteristic modifications of the breathing pattern of mice to evaluate the effects of airborne chemicals on the respiratory tract. *Arch Toxicol*. 67:478-490.
170. Scherle W. 1970. A simple method for volumetry of organs in quantitative stereology. *Mikroskopie*. 26:57-60.
171. Fehrenbach H, Ochs M. 1998. Studying lung ultrastructure. In: Uhlig S, Taylor AE, eds. Methods in pulmonary research. Birkhäuser, Basel. 429–54.

172. Dunnill MS. 1962. Quantitative methods in the study of pulmonary pathology. *Thorax*. 17:320–328.
173. Stoner GD, Kikkawa Y, Kniazeff AJ, Miyai K, Wagner RM. 1975. Clonal isolation of epithelial cells from mouse lung adenoma. *Cancer Res*. 35:2177-2185.
174. Kathuria H, Cao Y, Hinds A, Ramirez MI, Williams MC. 2007. ERM is expressed by alveolar epithelial cells in adult mouse lung and regulates caveolin-1 transcription in mouse lung epithelial cell lines. *J Cell Biochem*. 102:13-27.
175. Müller B, Garn H, Hochscheid R. 2003. Impaired recycling of surfactant-like liposomes in type II pneumocytes from injured lungs. *Thorax*. 58:127-134.
176. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature*. 326: 515-517.
177. Shivji KK, Kenny MK, Wood RD. 1992. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell.* 69:367-374.
178. Xiong Y, Zhang H, Beach D. 1992. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell*. 71:505-514.
179. Reddy NM, Kleeberger SR, Cho HY, Yamamoto M, Kensler TW, Biswal S, Reddy SP. 2007. Deficiency in Nrf2-GSH signaling impairs type II cell growth and enhances sensitivity to oxidants. *Am J Respir Cell Mol Biol*. 37:3-8.
180. Kalina M, Mason RJ, Shannon JM. 1992. Surfactant protein C is expressed in alveolar type II cells but not in clara cells of the rat lung*. Am J Respir Cell Mol Biol*. 6: 594–600.
181. Hicks SM1, Vassallo JD, Dieter MZ2, Lewis CL, Whiteley LO3, Fix AS, Lehman-McKeeman LD. 2003. Immunohistochemical analysis of Clara cell secretory protein expression in a transgenic model of mouse lung carcinogenesis. *Toxicology*.187: 217-28.
182. Borok Z, Lubman RL, Danto SI, Zhang XL, Zabski SM, King LS, Lee DM, Agre P, Crandall ED. 1998. Keratinocyte growth factor modulates alveolar epithelial cell phenotype in vitro: expression of aquaporin-5. *Am J Respir Cell Mol Biol.* 18:554–561.
183. Zhou L, Dev CR, Wert SE, Yan C, Costa RH, Whitstt JA. 1997. Hepatocyte nuclear factor-3beta limits cellular diversity in the developing respiratory epithelium and alters lung morphogenesis in vivo. *Dev Dyn*. 210:305-14.

184. Wan H, Dingle S, Xu Y, Besnard V, Kaestner KH, Ang SL, Wert S, Stahlman MT, Whitstt JA. 2005. Compensatory roles of Foxa1 and Foxa2 during lung morphogenesis. *Biol Chem*. 280:13809-16.
185. Roberts AB, McCune BK, Sporn MB. 1992. TGF-beta: regulation of extracellular matrix. *Kidney Int.* 41:557–559.
186. Laiho M, Saksela O, Andreasen PA, Keski-Oja.1986. Enhanced production and extra cellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-beta. *J Cell Biol.* 103:2403-10.
187. Selman M, King TE, Pardo A. 2001. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann. Intern. Med*. 134:136.
188. Geiser AG, Busam KJ, Kim SJ, Lafyatis R, O'Reilly MA, Webbink R, Roberts AB, Sporn MB. 1993. Regulation of the transforming growth factor-beta 1 and beta 3 promoters by transcription factor Sp1. *Gene.* 129:223-228.
189. Chinoy MR. 2003. Lung growth and development. *Front Biosci*. 8:D392-D415.
190. Kimura Y, Suzuki T, Kaneko C, Darnel AD, Moriya T, Suzuki S, Handa M, Ebina M, Nukiwa T, Sasano H. 2002. Retinoid receptors in the developing human lung. *Clin Sci*. 103:613-621.
191. Veness-Meehan KA, Bottone FG Jr, and Stiles AD. 2000. Effects of retinoic acid on air space development and lung collagen in hyperoxia-exposed newborn rats. *Pediatr Res*. 48: 434-444.
192. Randell SH, Mercer RR, Young SL. 1989. Postnatal growth of pulmonary acini and alveoli in normal and oxygen-exposed rats studied by serial section reconstructions. *Am J Anat*. 186:55-68.
193. Massaro GDC, Massaro D. 1997. Retinoic acid treatment abrogates elastase induced pulmonary emphysema in rats. *Nature Med*. 3:675-7.
194. Paiva SA, Godoy I, Vannucchi H, Favaro RM, Geraldo RR, Campana AO. 1996. Assessment of vitamin A status in chronic obstructive pulmonary disease patients and healthy smokers. *Am J Clin Nutr*. 64: 928–34.
195. Mao JT, Goldin JG, Dermand J, Ibrahim G, Brown MS, Emerick A, McNitt-Gray MF, Gjertson DW, Estrada F, Tashkin DP, Roth MD. 2002. A pilot study of all-trans-retinoic acid for the treatment of human emphysema. *Am J Respir Crit*

196. Meshi B, Vitalis TZ, Ionescu D, Elliott WM, Liu C, Wang XD, Hayashi S, and Hogg JC. 2002. Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response. *Am J Respir Cell Mol Biol*. 26:52-57.
197. Fujita M, Ye Q, Ouchi H, Nakashima N, Hamada N, Hagimoto N, Kuwano K, Mason RJ, Nakanishi Y. 2004. Retinoic acid fails to reverse emphysema in adult mouse models. *Thorax*. 59:224–230.
198. March TH, Cossey PY, Esparza DC, Dix KJ, McDonald JD, Bowen LE. 2004. Inhalation administration of all-trans-retinoic acid for treatment of elastase inducd pulmonary emphysema in Fischer 344 rats. *Exp Lung Res*. 30:383-404.
199. Takahashi S, Nakamura H, Seki M, Shiraishi Y, Yamamoto M, Furuuchi M, Nakajima T, Tsujimura S, Shirahata T, Nakamura M, Minematsu N, Yamasaki M, Tateno H, Ishizaka A. 2008. Reversal of elastase-induced pulmonary emphysema and promotion of alveolar epithelial cell proliferation by simvastatin in mice. *Am J Physiol Lung Cell Mol Physiol*. 294:L882-890.
200. Johnson BA, Iacono AT, Zeevi A, McCurry KR, Duncan SR. 2003. Statin use is associated with improved function and survival of lung allografts. *Am J Respir Crit Care Med*. 167: 1271–1278.
201. Weis M, Pehlivanli S, Meiser BM, von Scheidt W. 2001. Simvastatin treatment is associated with improvement in coronary endothelial function and decreased cytokine activation in patients after heart transplantation. *J Am Coll Cardiol*. 38: 814–818.
202. Fehrenbach H. 2001. Alveolar epithelial type II cell: defender of the alveolus revisited. *Respir Res*. 2:33-46.
203. Warburton D, Bellusci S. 2004. The molecular genetics of lung morphogenesis repair. *Paediatr Respir Rev*. 5 Suppl A:S283–7.
204. Shannon JM, Deterding RR. 1997. Epithelial-mesenchymal interactions in lung development. In Lung growth and development. Edited by McDonald JA. New York; Marcel Dekker, Inc.,81-118
205. O'Reilly MA, Stripp BR, Pryhuber GS. 1997. Epithelial-mesenchymal interactions in the alteration of gene expression and morphology following lung injury. *Microsc Res Tech*. 38: 473-479.

206. Griffin M, Bhandari R, Hamilton G, Chan YC, Powell JT. 1993. Alveolar type II cell–fibroblast interactions, synthesis and secretion of and type I collagen. *J Cell Sci*. 105:423–432.
207. Sirianni FE, Milaninezhad A, Chu FS, Walker DC. 2006. Alteration of fibroblast architecture and loss of Basal lamina apertures in human emphysematous lung. *Am J Respir Crit Care Med*. 173:632–8.
208. Parker MA, Bell ML, Barlow LA. 2004. Cell contact-dependent mechanisms specify taste bud pattern during a critical period early in embryonic development. *Dev Dyn*. 230:630–42.
209. Warburton D, Tefft D, Mailleux A, Bellusci S, Thiery JP, Zhao J, Buckley S, Shi W, Driscoll B. 2001. Do lung remodeling, repair, and regeneration recapitulate respiratory ontogeny? *Am J Respir Crit Care Med*. 164:S59–62.
210. Pierce GF, Yanagihara D, Klopchin K, Danilenko DM, Hsu E, Kenney WC, Morris CF. 1994. Stimulation of all epithelial elements during skin regeneration by keratinocyte growth factor. *J Exp Med*. 179:831–840.
211. Rubin JS, Bottaro DP, Chedid M, Miki T, Ron D, Cheon HG, Taylor WG, Fortney E, Sakata H, Finch PW, LaRochelle JW. 1995. Keratinocyte growth factor. *Cell Biol Int*. 19:399–411.
212. Werner S, Smola H, Liao X, Longaker MT, Krieg T, Hofschneider PH, Williams LT. 1994. The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science*. 266:819–822.
213. Farrell CL, Bready JV, Rex KL, Chen JN, DiPalma CR, Whitcomb L, Yin S, Hill DC, Wiemann B, Starnes CO, Havill AM, Lu ZN, Aukerman SL, Pierce GF, Thomason A, Potten CS, Ulich TR, Lacey DL. 1998. KGF protects mice from chemotherapy and radiation-induced gastrointestinal injury and mortality. *Cancer Res*. 58: 933-939.
214. Ulich TR, Whitcomb L, Tang W, O'Connor Tressel P, Tarpley J, Yi ES, Lacey DL. 1997. Keratinocyte growth factor ameliorates cyclophosphamide-induced ulcerative hemorrhagic cystitis. *Cancer Res*. 57:472-475.
215. Witschi H. 1976. Proliferation of type II alveolar cells: a review of common responses in toxic lung injury. *Toxicology*. 5:267-277.
216. Fehrenbach H. 2006. Animal models of pulmonary emphysema: a stereologist's perspective. *Eur Respir Rev*. 15:136-147.

217. Vlahovic G, Russell ML, Mercer RR, Crapo JD. 1999. Cellular and connective tissue changes in alveolar septal walls in emphysema. *Am J Respir Crit Care Med*. 160:2086-2092.
218. Lucey EC, Keane J, Kuang PP, Snider GL, Goldstein RH. 2002. Severity of elastase-induced emphysema is decreased in tumor necrosis factor-alpha and interleukin-1 beta receptor-deficient mice. *Lab Invest*. 82: 79–85.
219. Muers MF, Green JH. 1993. Weight loss in chronic obstructive pulmonary disease. *Eur Respi J*. 6:729-734.
220. Wilson DO, Donahoe M, Rogers RM, Pennock BE. 1990. Metabolic rate and weight loss in chronic obstructive lung disease. *J Parenteral Enteral Nutr*. 14:7-11.
221. Boton WE, Mikulka WR, Healy CG, Schmittling RJ, Kenyon NS. 1992. Expression of proliferation associated antigens in the cell cycle of synchronized mammalian cells. *Cytometry.* 13:117-26.
222. Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R. Waseem NH, Lane DP. 1990. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol*. 162:285-94.
223. Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. 1993. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev*. 7:812–821.
224. Yano T, Mason RJ, Pan T, Deterding RR, Nielsen LD, Shannon JM. 2000. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in the adult rat lung. *Am J Physiol Lung Cell Mol Physiol*. 279:L1146-L1158.
225. Takahashi A, Fujiwars T. 1986. Proteolipid in bovine lung surfactant: its role in surfactant function. Biochem Biophys. *Res Commun*. 135: 527–532.
226. Hawgood S, Benson BJ, Schliling J, Damm D, Clements JA, White T. 1987. Nucleotide and amino acid sequences of pulmonary surfactant protein SP 18 and evidence for cooperation between SP 18 and SP 28-36 in surfactant lipid adsorption. *Proc Natl Acad Sci.* 64:66–70.

227. Raina S, Preston GM, Guggino WB, Agre P. 1995. Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. *J. Biol. Chem*. 270:1908-1912.
228. Williams MC. 2003. Alveolar type I cells: molecular phenotype and development. *Annu Rev Physiol*. 65: 669–695.
229. Verkman AS, Matthay MA, Song Y. 2000. Aquaporin water channels and lung physiology. *Am J Physiol Lung Cell Mol Physiol*. 278:L867–L879.
230. Nielsen S, King LS, Christensen BM, Agre P. 1997. Aquaporins in complex tissues: II. Subcellular distribution in respiratory and glandular tissues of rats. *Am J Physiol*. 42:C1549–C1561.
231. Zhou L, Dey CR, Wert SE, Yan C, Costa RH, Whitsett JA. 1997. Hepatocyte nuclear factor-3beta limits cellular diversity in the developing respiratory epithelium and alters lung morphogenesis in vivo. *Dev Dyn*. 210:305-314.
232. Wan H, Kaestner KH, Ang SL, Ikegami M, Finkelman FD, Stahlman MT, Fulkerson PC, Rothenberg ME, Whitsett JA. 2004. Foxa2 regulates alveolarization and goblet cell hyperplasia. *Development.* 131: 953–964.
233. Duncan SA, Navas MA, Dufort D, Rossant J, Stoffel M. 1998. Regulation of a transcription factor network required for differentiation and metabolism. *Science.* 281: 692-695.
234. Sund NJ, Ang SL, Sackett SD, Shen W, Daigle N, Magnuson MA, Kaestne KH. 2000. Hepatocyte nuclear factor 3beta (Foxa2) is dispensable for maintaining the differentiated state of the adult hepatocyte. *Mol Cell Biol*. 20:5175–5183.
235. Furuyama A, Mochitate K. 2004. Hepatocyte growth factor inhibits the formation of the basement membrane of alveolar epithelial cells in vitro. *Am J Physiol Lung Cell Mol Physiol*. 286: L939–L946.
236. Ware LB, Matthay MA. 2002. Keratinocyte and hepatocyte growth factors in the lung: roles in lung development, inflammation, and repair. Am *J Physiol Lung Cell Mol Physiol*. 282: L924–L940.
237. Sakamaki Y, Matsumoto K, Mizuno S, Miyoshi S, Matsuda H, Nakamura T. 2001. Hepatocyte growth factor stimulates proliferation of respiratory epithelial cells during postpneumonectomy compensatory lung growth in mice. *Am J Respir Cell Mol Biol*. 26:525-533.
238. Plantier L, Marchand-Adam S, Marchal-Somme J, Leseche G, Foumier M,

production by fibroblasts in human pulmonary emphysema. *Am J Physiol Lung Cell Mol Physiol*. 288:L641–7.

239. Bonay M, Boutten A, Lecon-Malas V, Marchal J, Soler P, Foumier M, Leseche G, Dehoux M, Crestani B. 2005. Hepatocyte and keratinocyte growth factors and their receptors in human lung emphysema. *BMC Pulm Med*. 5:13.
240. Ishizawa K, Kubo H, Yamada M, Kobayashi S, Suzuki T, Mizuno S, Nakamura T, Sasaki H. 2004. Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells. *Biochem Biophys Res Commun*. 324: 276–280.
241. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*. 219:983–985.
242. Park JE, Keller GA, Ferrara N. 1993. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell*. 4: 1317–1326.
243. Robinson CJ, Stringer SE. 2001. The splice variants of vascular endothelial growth factor (VEGF) and their receptors. *J Cell Sci*. 114:853–65.
244. Monacci WT, Merrill MJ, Oldfield EH. 1993. Expression of vascular permeability factor/vascular endothelial growth factor in normal rat tissues. *Am J Physiol*. 264:C995–C1002.
245. Mura M, dos Santos CC, Stewart D, Liu M. 2004. Vascular endothelial growth factor and related molecules in acute lung injury. *J Appl Physiol*. 97:1605–1617.
246. Tuder RM, Flook BE, Voelkel NF. 1995. Increased gene expression for VEGF and the VEGF receptors KDR/Flk and Flt in lungs exposed to acute or to chronic hypoxia. Modulation of gene expression by nitric oxide. *J Clin Invest*. 95:1798–1807.
247. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. 380:435–439.
248. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-

249. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. 1996. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood*. 87:3336–3343.
250. Marwick JA, Stevenson CS, Giddings J, MacNee W, Butler K, Rahman I, Kirkham PA. 2006. Cigarette smoke disrupts $VEGF_{165}$-VEGFR-2 receptor signaling complex in rat lungs and patients with COPD: morphological impact of VEGFR-2 inhibition. *Am J Physiol Lung Cell Mol Physiol*. 290:L897–908.
251. McGrath-Morrow SA, Cho C, Cho C, Zhen L, Hicklin DJ, Tuder RM. 2005. Vascular endothelial growth factor receptor 2 blockade disrupts postnatal lung development. *Am J Respir Cell Mol Biol*. 32:420–427.
252. McGowan SE. 1992. Extracellular matrix and the regulation of lung development and repair. *FASEB J*. 11:2895-2904
253. Massague´, J. 1990. The transforming growth factor-beta family. *Annu Rev Cell Biol*. 6: 597–641.
254. Slack JL, Liska DJ, Bornstein P. 1993. Regulation of expression of the type I collagen genes. *Am J Med Genet*. 45: 140–151.
255. Verrecchia F, Mauviel A. 2002. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. *J Invest Dermatol*. 118:211–215.
256. Sterner-Kock A, Thorey IS, Koli K, Wempe F, Otte J, Bangsow T, Kuhlmeier K, Kirchner T, Jin S, Keski-Oja J, von Melchner H. 2002. Disruption of the gene encoding the latent transforming growth factor-β binding protein 4 causes abnormal lung development, cardiomyopathy, and colorectal cancer. *Genes Dev*. 16:2264–2273.
257. Starcher BC. 2000. Lung elastin and matrix. *Chest*. 117:229S–234S.
258. Hoidal JR, Niewoehner DE. 1983. Pathogenesis of emphysema. *Chest*. 83:679-685.
259. Emery JL. 1970. The postnatal development of the human lung and its implication for lung pathology. *Respiration*. 27:41–50.
260. Lindahl P, Karlsson L, Hellstrom M, Gebre-Medhin S, Willetts K, Heath JK, Betsholtz C. 1997. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. *Development*. 124: 3943-3953.

261. Wendel DP, Taylor DG, Albertine KH, Keating MT, Li DY. 2000. Impaired distal airway development in mice lacking elastin. *Am J Respir Cell Mol Biol*. 23:320–32.
262. Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST, Davies DE. 2000. Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J*. 14: 1362–1374.
263. Tschumperlin DJ, Shively JD, Kikuchi T, and Drazen JM. 2003. Mechanical stress triggers selective release of fibrotic mediators from bronchial epithelium. *Am J Respir Cell Mol Biol*. 28: 142–149.
264. Noguchi A, Reddy R, Kursar JD, Parks WC, Mecham RP. 1989. Smooth muscle isoactin and elastin in fetal bovine lung. *Exp Lung Res*. 4:537–552.
265. Kapanchi Y, Assimacopoulos A, Irle C, Zwahlen A, Gabbigrants G. 1974. Contractile interstitial cells in pulmonary alveolar septa: a possible regulator of ventilation/perfusion ratio? *J Cell Biol*. 60:375–392.
266. Leslie K, Mitchell JJ, Woodcock-Mitchell JL, Low RB. 1990. a Smooth muscle actin expression in developing and adult lung. *Differentiation.* 44:143–149.
267. Plantier L, Marchand-Adam S, Antico VG, Boyer L, De CC, Marchal J, Bachoual R, Mailleux A, Boczkowski J, Crestani B. 2007. Keratinocyte growth factor protects against elastase-induced pulmonary emphysema in mice. *Am J Physiol Lung Cell Mol Physiol*. 293:L1230-L1239.
268. Guo J, Yi ES, Havill AM, Sarosi I, Whitcomb L, Yin S, Middleton SC, Piguet P, Ulich TR. 1998. Intravenous keratinocyte growth factor protects against experimental pulmonary injury. *Am J Physiol*. 275:L800-L805.
269. Yaldirim AO, Muyal V, John G, Müller B, Seifart C, Kasper M, Fehrenbach H. 2010. Palifermin induces alveolar maintenance programs in emphysematous mice. *Am J Respir Crit Care med*. 181(7):705-717.
270. Pelton RW, Johnson MD, Perkett EA, Gold LI, Moses HL. 1991. Expression of transforming growth factor-β1, -β2 and -β3 mRNA and protein in the murine lung. *Am J Respir Cell Mol Biol*. 5: 522–530.
271. Leof EB, Proper JA, Goustin AS, Shipley GD, DiCorleto PE, Moses HL. 1986. Induction of c-sis mRNA and platelet-derived growth factor like activity by transforming growth factor-β: a proposed model for indirect mitogenesis involving autocrine activity. *Proc Natl Acad Sci U S A*. 83:2453–2457.

272. Ignotz RA, Massague J. 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J Biol Chem*. 261:4337–4345.
273. Dubaybo BA, Thet LA. 1990. Effect of transforming growth factor β on synthesis of glycosaminoglycans by human lung fibroblasts. *Exp Lung Res*. 16:389–403.
274. Kahari VM, Olsen DR, Rhudy RW, Carillo P, Chen YQ, Uitto J. 1992. Transforming growth factor-beta upregulates elastin gene expression in human skin fibroblasts. Evidence for a posttranscriptional modulation. *Lab Invest*. 66: 580–588.
275. Giancotti FG, Tarone G. 2003. Positional control of cell fate through joint integrin/receptor protein kinase signalling. *Annu Rev Cell Dev Biol*. 19:173-206.

9 Abbreviations

ABC	AVIDIN-BIOTIN-COMPLEX
ALI	Acute lung injury
AM	Alveolar macrophage
ANOVA	One-way analysis of variance
A(p)	Area per point
ATRA	All-trans-retinoic acid
A1AT	Alpha1-antitrypsin
ATS	American Thoracic Society
AQP-5	Aquaporin-5
BAL	Bronchoalveolar lavage
Bp	Base pair(s)
b.w.	Body weight
C.A.S.T.	Computer assisted stereological toolbox
°C	Degree celcius
cDNA	Complementary DNA
CLE	Centrilobular (or centriacinar) emphysema
COPD	Chronic obstructive pulmonary disease
Ct	Cycle threshold
DAB	3, 3`-diaminobenzidintetrahydrochloride
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates mix
EDTA	Ethylendinitrilo-N, N, N', N',-tetra-acetate
ECM	Extracellular matrix
EF50	Mid expiratory airflow
ELISA	Enzyme-linked immunosorbent assay
ENA-78	Epithelial cell-derived neutrophil-activating peptide-78
FGFR	Fibroblast growth factor receptor
FGFR2	Fibroblast growth factor receptor 2

FGF-7	Fibroblast growth factor-7
FOXA-2	Forkhead transcription factor-2
G	Gram, unit of weight
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GOLD	Global Initiative on Chronic Obstructive Lung Disease
HCL	Hydrochloric acid
H2O	Water
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IP-10	Interferon-gamma inducible protein
Kb	Kilobase(s)
kDa	Kilodalton
Kg	Kilogram
L(p)	Length per point
M	Molar
MCL	Mean chord length
MCP-1	Monocyte chemotactic protein-1
Mig-gamma	Monokine induced by interferon-gamma
MIP-1α	Macrophage inflammatory protein-1α
Mg	Milligram
Ml	Millilitre
MLF	Mouse lung fibroblast
mm	Millimeter
MMPs	Matrix metalloproteinases
M.O.M.	Mouse on mouse
mRNA	Messenger RNA
NE	Neutrophil elastase
NFκB	Nuclear factor kappa B
NHLBI	National Heart, Lung, and Blood Institute
NO	Nitric oxide

Nrf2	Nuclear factor erythroid-related factor 2
OD	Optical density
Oligos	Oligodeoxynucleotides
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCNA	Proliferative cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
pH	Potential of hydrogen
PLE	Panlobular (or panacinar) emphysema
PPE	Porcine pancreatic elastase
PVDF	Polyvinylidene fluoride
rHuKGF	recombinant human keratinocyte growth factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
Rtase	Reverse transcriptase
S_a	Alveolar surface area
SDS	Sodium dodecylsulfate
Smad 2	Mothers against decapentaplegic homologue 2
SP-C	Surfactant protein-C
STAT3	Signal transducer and activator of transcription 3
S_v	Alveolar surface area per unit volume
TGFβ-1	Transforming growth factor β-1
TGFβ-R-1	TGFβ receptor-1
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumor necrosis factor-α
Tris	Tris-(hydroxymethyl)-aminomethane

UV	Ultraviolet
V_{air}	Absolute volume of alveolar air spaces
V_{ast}	Absolute volume of alveolar septal tissue
V_{RL}	Total volume of right lung
Vv	Volume of parenchyma per volume of lung
WHO	World Health Organization
ΔCt	Delta cycle threshold
τ_{ast}	Mean thickness of alveolar septum
β-ME	Beta-mercaptoethanol
µg	Microgram
µl	Microlitre
µm	Micrometer

10 Summary

Pulmonary emphysema is a major manifestation of chronic obstructive pulmonary disease (COPD), which is characterized by persistent inflammation and progressive alveolar destruction. Such destruction in the distal respiratory tract is thought to be irreversible. Despite promising initial experiments using all-trans retinoic acid (ATRA) supplementation to reverse emphysema in rats, contradictory results were obtained by others. A number of studies have shown that the instillation of recombinant human keratinocyte growth factor (rHuKGF) into the lung protects animals from acute lung injury and favourably influences alveolar maintenance and repair. The mechanism of protection, however, is not completely understood.

This study aimed at investigating the potential of rHuKGF to induce alveolar regeneration. Two sets of experiments were performed: 1) an in-vivo study to evaluate the therapeutic effects of rHuKGF in the elastase-induced pulmonary emphysema mouse model; 2) an in-vitro study to elucidate the basic molecular mechanism underlying the therapeutic effect of rHuKGF.

In-vivo experiments:
Lung emphysema was induced in C57BL/6 male mice by two oropharyngeal aspirations of porcine pancreatic elastase (PPE, 2.25 mg/kg b.w., day 0, 10) versus saline (PBS-group) into the lungs. To evaluate the regenerative effect of rHuKGF, PPE treated mice received oropharyngeal aspirations of rHuKGF (PPE+KGF, 10 mg/kg b.w.) versus saline (PPE+PBS) at day 31, 34, and 37, respectively. Initially, lung function was assessed at day 31 (PBS versus PPE) or day 40 (PPE+PBS versus PPE+KGF) by non invasive head-out body plethysmographic measurement of mid-expiratory airflow (EF50). At day 31 or 40, lungs were prepared for analyses of immunohistochemistry, quantitative morphometry, quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blotting techniques. The statistical significance of differences between groups was assessed by using t-test or non-parametric U-test.

Regeneration is a complex process involving periods of cellular proliferation, differentiation and extracellular matrix (ECM) synthesis and remodeling. Interestingly, rHuKGF therapy significantly reversed respiratory dysfunction induced by PPE application: PBS-group: 100%; PPE-group: 66%; control treatment (PPE+PBS)-group: 53%; therapeutic treatment (PPE+KGF)-group: 98%. This was accompanied by partial reversal of airspace enlargement and partial regeneration of alveolar gas exchange area as assessed by design based stereology.

Analyses in whole lung tissue homogenate demonstrated that the beneficial effect of rHuKGF was associated with increased mRNA expression of genes involved in: a) proliferation of epithelium, i.e. PCNA, Cyclin D1 and Nrf2; b) differentiation of alveolar epithelium, i.e. SP-C; AQP-5 and Foxa-2 as well as HGF and FGF-7 which possess mitogenic effect on alveolar epithelium; c) formation of capillary endothelium, i.e. VEGF and its specific receptors VEGFR1 and VEGFR2; d) maintenance of interstitial tissue, i.e. TGFβ-1, TGFβ-2, TGFβ-R-1, TGFβ-R-2, Smad 2, PAI-1, SP-1, elastin and PDGF-A.

Furthermore, Western blot analysis and immunohistochemistry for PCNA revealed that therapeutic application of rHuKGF resulted in a prominent increase of the proliferation of alveolar and airway epithelial cells in emphysematous lungs. Therapeutic administration of rHuKGF was associated with increased capillary endothelial cell proliferation as assessed by double immunohistochemistry for Ki-67 and podocalyxin. Besides, an increase in the protein level of VEGFR2 was revealed by Western blot analysis.

In addition, rHuKGF therapy induced the expression of TGFβ-1 and TGFβ-2 proteins in alveolar as well as airway epithelial cells as assessed by immunohistochemistry. TGFβ-1 and TGFβ-2 are implicated in the regulation of interstitial tissue formation.

In-vitro experiments:
In order to proof that the stimulating effects of rHuKGF on the expression of elastin observed in vivo were mediated via alveolar epithelial type 2 cells (AE2) derived TGFβ-1, primary AE2 cells isolated from C57BL/6 mouse lungs, murine AE2-like cell

and LA-4/MLF co-cultures were incubated with rHuKGF (50 ng/ml), recombinant TGFβ-1 protein (10 ng/ml) or PBS in the presence or absence of anti-TGFβ-neutralizing antibody (clone 1D11, 20 µg/ml)) or control IgG. After 6 hrs and 24 hrs, cells were collected for mRNA expression analysis by means of quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR). Supernatants were analysed by ELISA for activated TGFβ-1 protein.

TGFβ-1 was evaluated as a potential mediator of AE2-linked regenerative effect of rHuKGF on the interstitial tissue compartment in the lung. rHuKGF induced the expression of TGFβ-1 in and release of active TGFβ-1 from primary mouse AE2 cells, murine AE2-like cells LA-4 and co-cultures of LA-4/MLF but not in murine AE1-like cells E10. Recombinant TGFβ-1 protein but not rHuKGF was found to induce elastin gene expression in MLF cells. Additionally, blockade of TGFβ-signaling by neutralizing anti-TGFβ-antibody 1D11 abrogates rHuKGF induced mRNA expression levels of SP-1, PAI-1 and elastin in LA-4/MLF co-cultures.

Taken collectively, the results from this study suggest that therapeutic application of exogenous rHuKGF into emphysematous lungs is able to induce alveolar maintenance programs in the emphysematous mouse lung and that alveolar epithelial type 2 cells (AE2) derived TGFβ-1 is involved in these processes. Therefore, it is suggested that rHuKGF has a potential as therapeutic agent in the treatment of pulmonary emphysema.

11 Zusammenfassung

Das Lungenemphysem ist eine der wichtigsten Ausprägungen chronisch obstruktiver Lungenerkrankungen (COPD), die durch eine persistierende Entzündung und eine fortschreitende Zerstörung der Lungenalveolen gekennzeichnet sind. Eine solche Zerstörung des für den Gasaustausch zuständigen distalen Atemwegtraktes wurde bislang als irreversibel eingestuft. Trotz initial viel versprechender Experimente, bei denen eine Supplementierung mit All-trans-Retinolsäure (ATRA) eingesetzt wurde, um ein Lungenemphysem bei der Ratte umzukehren, erbrachten Folgestudien anderer Arbeitsgruppen widersprüchliche Resultate. Durch zahlreiche Untersuchungen konnte belegt werden, dass eine intratracheale Instillation von rekombinantem humanen Keratinozyten-Wachstumsfaktor (rHuKGF) in die Lunge vor einem akuten Lungenschaden schützt und sich günstig auf die Aufrechterhaltung und Reparatur alveolärer Strukturen auswirkt. Dieser Schutzmechanismus ist jedoch noch nicht gänzlich entschlüsselt.

Das Ziel dieser Untersuchung war es, dass Regenerationspotential von rHuKGF in alveolärem Gewebe zu erforschen. Zwei experimentell Ansätze wurden durchgeführt: 1) eine in-vivo Studie zur Beurteilung des therapeutischen Effektes von rHuKGF im Mausmodell beim Elastase-induzierten Emphysem; 2) eine in-vitro Studie zur Charakterisierung der molekularen Mechanismen, die dem therapeutischen Effekt von rHuKGF zugrundeliegen.

In-vivo Experimente:
Ein Lungenemphysem wurde bei männlichen C57BL/6-Mäusen durch zweimalige oropharyngeale Aspiration von Pankreaselastase des Schweins (PPE-Gruppe; 2,25 mg/kg Körpergewicht; Tag 0, 10) in die Lunge ausgelöst; eine Kontrollgruppe erhielt eine entsprechende Gabe von Kochsalzlösung (PBS-Gruppe). Um einen therapeutischen Effekt von rHuKGF zu prüfen, erhielten mit PPE behandelte Mäuse oropharyngeale Applikationen von rHuKGF (PPE+KGF-Gruppe, 10 mg/kg KG) bzw. Kochsalzlösung (PPE+PBS-Gruppe) an den Tagen 31, 34 und 37. Zunächst wurde ein Lungenfunktionstest vorgenommen, was an Tag 31 (PBS versus PPE) bzw. an

plethysmographischer Messung des mittleren exspiratorischen Atemflusses (EF50) erfolgte. Anschließend wurden die Lungen an Tag 31 bzw. 40 für Analysen mittels Immunhistochemie, quantitativer Morphometrie, quantitativer Reverse Transkriptase-Polymerase-Kettenreaktion (RT-PCR) und Western Blotting aufbereitet. Unterschiede zwischen den experimentellen Gruppen wurden mittels t-Test oder nicht-parametrischem U-Test auf Signifikanz geprüft.

Regeneration ist ein komplexer Vorgang, der Phasen der Zellproliferation und Differenzierung sowie der Synthese und des Umbaus extrazellulärer Matrix (ECM) umfasst. Bemerkenswerterweise führte die therapeutische Gabe von rHuKGF zu einer signifikanten Verbesserung der nach PPE-Gabe festgestellten, respiratorischen Dysfunktion: PBS-Gruppe: 100%; PPE-Gruppe: 66%; Kontrollbehandlungs- (PPE+PBS-) Gruppe: 53%; Therapie- (PPE+KGF-) Gruppe: 98%. Dieser Effekt ging einher mit einer partiellen Aufhebung der Vergrößerung der distalen Gasaustauschräume und einer partiellen Regeneration der alveolären Gasaustauschfläche, was mit Hilfe von Methoden der *design-based stereology* beurteilt wurde.

Die molekularbiologischen Analysen an Lungengewebehomogenat ergaben, dass der regenerative Effekt von rHuKGF mit einer erhöhten mRNA-Expression von Genen assoziiert war, die an folgenden Prozessen beteiligt sind: a) Proliferation, z.B. PCNA, Cyclin D1, Nrf2); b) Epithelzelldifferenzierung, z.B. SP-C, AQP-5 und Foxa-2, sowie HGF und FGF-7; c) Kapillarendothel-Bildung, z.B. VEGF und seine spezifischen Rezeptoren VEGFR1 und VEGFR2; d) Bildung extrazellulärer Matrix, z.B. TGFβ-1, TGFβ-2, TGFβ-R-1, TGFβ-R-2, Smad2, PAI-1, SP-1, Elastin und PDGF-A.

Außerdem konnte mittels Western Blotting und Immunhistochemie für PCNA gezeigt werden, dass die therapeutische Gabe von rHuKGF bei emphysematösen Lungen zu einem ausgeprägten Anstieg der Proliferation von Alveolar- und Atemwegsepithel führt. Die therapeutische Gabe von rHuKGF war einerseits mit einer erhöhten Zellproliferation von Kapillarendothel verbunden, wie mit Immunhistochemie für Ki-67 und Podocalyxin gezeigt wurde. Andererseits bewirkte sie eine erhöhte

war. Zusätzlich erbrachten immunhistochemische Untersuchungen, dass durch die Therapie mit rHuKGF die Expression von TGFβ-1- und TGFβ-2-Proteinen, welche die Bildung der extrazellulären Matrix steuern, in Alveolar- und Atemwegsepithelzellen induziert wird.

In vitro Experimente:
Um zu überprüfen, ob die stimulierende Wirkung von rHuKGF auf die Expression von Elastin, die in den in-vivo Experimenten gesehen wurde, über Alveolarzellen vom Typ 2 (AE2) mittels TGFβ-1 vermittelt werden, wurden AE2-Zellen aus C57BL/6 Mäuselungen, murine AE2-ähnliche Zelllinie LA-4, die AE1-ähnliche Zelllinie E10, die murine embryonale Lungenfibroblasten-Linie MLF und LA-4/MLF Co-Kulturen mit rHuKGF (50 ng/ml), rekombiniertem TGFβ-1 Protein (10 ng/ml) oder PBS in Anwesenheit oder Abwesenheit von anti-TGFβ-neutralisierendem Antikörper (Klon 1D11, 20 µg/ml)) bzw. Kontroll-IgG inkubiert. Nach sechs und 24Stunden wurden die Zellen für die mRNA Expressionsanalyse mit Hilfe quantitativer Reverse Transkriptase-Polymerase-Kettenreaktion (RT-PCR) gesammelt.

Des Weiteren wurde geprüft, ob TGFβ als potentieller Mediator des regenerativen Effekts von rHuKGF auf interstitielles Lungengewebe gewertet werden kann. Mehrere Hinweise unterstützen die Annahme, dass TGFb in diesem Zusammenhang eine wichtige Rolle zukommt. rHuKGF induziert die Expression von TGFβ-1 in und die Ausschüttung von aktivem TGFβ-1 aus AE2-Mauszellen, murinen AE2-ähnlichen Zellen LA-4 und den Co-Kulturen von LA-4/MLF, nicht aber aus murinen AE1-ähnlichen Zellen E10. Rekombinantes TGFβ-1, nicht aber rHuKGF, induzierte die Genexpression von Elastin in MLF Zellen. Zusätzlich hebt die Blockade des TGFβ-Signalweges mittels neutralisierendem anti-TGFβ-Antikörper 1D11, die rHuKGF-induzierte mRNA-Expression auf der Ebene von SP-1, PAI-1 und Elastin in LA-4/MLF Co-Kulturen auf.

Zusammengefasst zeigen die Ergebnisse dieser Studie, dass die therapeutische Gabe von exogenem rHuKGF in emphysematösen Lungen Mechanismen induzieren kann, die für die Erhaltung alveolärer Strukturen von wesentlicher Bedeutung sind und dass Alveolarepithelzellen vom Typ2 über die Freisetzung von TGFβ-1 in diesen

Prozess involviert sind. Aus der Gesamtdatenlage lässt sich ableiten, dass rHuKGF ein Potenzial als Therapeutikum bei der Behandlung des Lungenemphysems hat.

12 Curriculum Vitae

Name:	Vandana Muyal, geborene Bhatgain
Date of Birth:	30-07-1981
Address:	34/4, Block No.4, Teg Bahadur Road, Dalanwala, Dehradun, Uttarakhand, India
Martital status:	Married
Husband's name:	Dr. Jai Prakash Muyal
Son's name:	Daksh Muyal
E-mail:	vmuyal@gmail.com
Telephone No:	+91-135 –2676904 (In India) +91-9582136682 (Mobile)

SCHOOL EDUCATION:

1988-96	From 1st standard to 9 th standard	GGIC Gopeshwar, Chamoli, Uttarakhand, India.
1996–97	Class 10 th (High School)	GGIC Gopeshwar, Chamoli, Uttarakhand, India. Final marks: 66 %
1997– 99	Class 12 th (Intermediate)	MKP, Dehradun, Uttarakhand, India. Subject: Biology, Hndi, Physics, Chemistry and English. Final marks: 66 %

UNIVERSITY EDUCATION:

1999 – 2003	Bachelors in Science (B.Sc.), DBS PG Collage, Dehradun, HNB Garhwal University Srinagar, Uttarakhand, India. Final marks: 64.2%
2003 – 2005	Masters of Science in Zoology (M.Sc.), DBS PG Collage, Dehradun, HNB Garhwal University Srinagar

	Uttarakhand, India. Final marks: 56 %
2003 – 2005	During Masters programmed, I had done one year's complete lab project work on "DNA microarray technology and its application in Biomedical Research" under the supervision of Prof. S.P. Singh, Department of Zoology, DBS PG collage, Dehradun, HNB Garhwal University Srinagar, Uttarakhand, India.
Aug 2005 – Dec 2005	Learned Molecular Biology based techniques, in particulars, RNA Isolation, cDNA synthesis, DNA isolation, protein extraction, Agrose gel electrophoresis, Polymerase Chain Reaction from Molecular Biology and Genetic Engineering Center, GB Pant Agriculture and Technology University, Pantnagar, Uttarakhand, India.

POSTGRADUATE EDUCATION:

June 2006 – Dec 2008	Done Ph.D. thesis work on " Therapeutic Effects of recombinant human Keratinocyte Growth Factor (rHuKGF) in an Elastase-Induced Emphysema Model in the Mouse " under the supervision of Prof. Heinz Fehrenbach, head of Experimental Pneumology Research Center Borstel, Germany.

Place: Greater Noida, India

Date: Signature:

13 Verzeichnis der akademischen Lehrer

Meine akademischen Lehrer waren Damen / Herren

In Marburg

1. Prof. Dr. Heinz Fehrenbach
Old address- Head, Clinical Research Group "Chronic Airway Diseases"
Clinic of Internal Medicine, Div. of Respiratory Medicine
University Hospital Giessen & Marburg, Germany (2002-2008)
New address- Head, Experimental Pneumology
Research Center Borstel, Germany (2008-today)

2. PD Dr. Carola Seifart
Ethikkomission, FB Medizin
Philipps-Universität Marburg
Geschäftsführung Baldingerstrasse
35043 Marburg, Germany

In Indien

1. Dr. Shakti Sahi, PhD
Assistant Professor
School of Biotechnology
Gautam Buddha University
Greater Noida, India

14 Acknowledgments

First and foremost, I express my deep sense of gratitude and indebtedness to my supervisor, Prof. Dr. Heinz Fehrenbach, head of Clinical Research Group "Chronic Airway Diseases" Clinic of Internal Medicine, Div. of Respiratory Medicine, University Hospital Giessen & Marburg (2002-2008); head of Experimental Pneumology Research Center Borstel, Germany (2008-today) for providing invaluable advice and giving me the first impulse to take up this work and for providing the facilities for laboratory work and other resources.

I am grateful to Dr. Ali Önder Yildirim for his constant helpful discussions and encouragements. I would like to thanks Dr. Gerrit John for providing his support in performing in vito cell culture experiments.

I wish to acknowledge my special thanks to our technicians Tanja Rausch, and Larissa Greif for their help and suggestions.

I address my deep cordially thanks to my husband Dr. Jai Prakash Muyal for providing me his care and encouragement during the compilation of this thesis. Moreover, I very much appreciate his continuous interest in the progress of my work, his high degree of steady willingness for helpful discussions.

Above all, I tender my heartfelt thanks to my son, my parents, in-laws, and family for their love, care and encouragement during the compilation of this thesis.

15 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel ‘’Therapeutic Effects of recombinant human Keratinocyte Growth Factor (rHuKGF) in an Elastase-Induced Emphysema Model in the Mouse’’ am Klinikum der Philipps-Universität, Innere Medizin, Schwerpunkt Pneumologie unter Leitung von Prof. Dr. Heinz Fehrenbach mit Unterstützung durch die unten genannten Personen ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Eine Unterstützung der Arbeit fand durch folgende Personen statt:

Prof. Dr. Heinz Fehrenbach:

Dr. Ali Önder Yildirim:

Prof. Bernd Müller:

Dr. Gerrit John:

Tanja Rausch: Technische Unterstützung

Larissa Greif: Technische Unterstützung

Greater Noida (India), May 2010

16 List of Publications

The major results of the thesis have been published in:

- Ali Ö. Yildirim[#], **Vandana Muyal[#]**, Gerrit John, Bernd Müller, Carola Seifart, Heinz Fehrenbach. [#] These authors equally contributed to this work**.**
  Palifermin induces alveolar maintenance programs in emphysematous mice. Am J Respir Crit Care Med. 2010 Apr 1; 181(7):705-17.

The results from experiments performed in Marburg that are not included in this thesis were published in:

- Jai Prakash Muyal*****, **Vandana Muyal***, Brajesh Pratap Kaistha, Carola Seifart, Heinz Fehrenbach. * These authors equally contributed to this work.
  Systematic comparison of RNA extraction techniques from frozen and fresh lung tissues; Checkpoint towards gene expression studies. Diagn Pathol. 2009 Mar 24; 4:9.