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## Fibroblasts as matrix modulating cells in asthma and COPD

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From the department of Experimental Medical Science  
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# Fibroblasts as matrix modulating cells in asthma and COPD

by

Kristian Nihlberg



LUND UNIVERSITY  
Faculty of Medicine

Lund 2009  
Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds  
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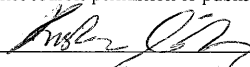
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Abstract <p>Chronic lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are two lung diseases that are continuously increasing worldwide. Despite extensively research to find curative treatment, no so such therapy exists today. New hypothesis suggests that an aberrant chronic wound healing process takes place which involves both the classical inflammation and more chronic changes of the structural environment where the mesenchymal cell phenotypes such as the fibroblasts play a key role. This phenomenon is defined as airway remodeling and is characterized by a deposition of extracellular matrix (ECM) molecules such as collagens and proteoglycans. Importantly, hitherto available studies have only studied fibroblast from central airways from humans where the role of the fibroblast might be different compared to the distal lung. The aim of the present thesis was to study the different fibroblast phenotypes and their biological role from central and distal localizations from human lung in controls, rhinitis, asthmatics and CODP subjects. Another aim was to investigate the possible origin (circulating progenitor cells) of these cells and thereby highlight the heterogeneity of fibroblast phenotypes resident within the human lung. This investigation demonstrated the novel finding that different fibroblast phenotypes are present in human adult lung. Moreover importantly, they are different in asthma and COPD which suggest disease related fibroblasts and could be of fundamental importance in lung disease</p>		
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# Fibroblasts as matrix modulating cells in asthma and COPD

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From:  
The department of Experimental Medical Science



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Faculty of Medicine

Lund 2009



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“Life will guide you home”

*Kristian Nihlberg*



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- I. **Kristian Nihlberg**, Kristoffer Larsen, Anna Hultgårdh-Nilsson, Anders Malmström, Leif Bjermer and Gunilla Westergren-Thorsson. Tissue fibrocytes in patients with mild asthma: A possible link to thickness of reticular basement membrane? *Respiratory Research* 2006,7:50
- II. Kristoffer Larsen, David Macleod, **Kristian Nihlberg**, Eylem Gürcan, Leif Bjermer György Marko Varga and Gunilla Westergren-Thorsson, *Journal of Proteome Research*, 2006, 5 (6) 1479-1483
- III. **Kristian Nihlberg**, Annika Andersson-Sjöland, Ellen Tufvesson, Leif Bjermer and Gunilla Westergren-Thorsson, Altered matrix production in the distal airways of asthmatic and atopic individuals, *Manuscript to be submitted in may 2009 to American Journal of Respiratory Critical Care and Medicine*
- IV. Oskar Hallgren, **Kristian Nihlberg**, Magnus Dahlbäck, Leif Bjermer, Leif Eriksson, Jonas S Erjefält, Claes-Göran Löfdahl and Gunilla Westergren-Thorsson, *Submitted to Thorax*

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## Additional papers not included in the thesis

Annika Anderson-Sjöland, Carolina García de Alba, **Kristian Nihlberg**, Carina Becerril, Remedios Ramirez, Annie Pardo, Gunilla Westergren-Thorsson, Moises Selmán, Fibrocytes are a potential source of lung fibroblasts in idiopathic pulmonary fibrosis, *International Journal of Biochemistry and Cell Biology*, 2008;40(10):2129-40

Gunilla Westergren-Thorsson, Kristoffer Larsen, **Kristian Nihlberg**, Annika Andersson-Sjöland, Oskar Hallgren, György Marco-Varga, Leif Björner, Pathological airway remodeling in airway inflammation, *Clinical Respiratory Journal*, 2009

# Abbreviations

AHR	Airway hyperresponsiveness
$\alpha$ -SMA	Alpha-smooth muscle actin
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
COPD	Chronic obstructive pulmonary disease
CS/DS	Chondroitin/Dermatan Sulphate
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
EMTU	Epithelial mesenchymal trophic unit
Endotype	A subtype of disease defined functionally and pathologically by a molecular mechanism or treatment response
FEV <sub>1</sub>	Forced expiratory volume in 1s
FVC	Forced vital capacity
GAG	Glycosaminoglycan
IPF	Idiopathic pulmonary fibrosis
IL	Interleukin
MSC	Mesenchymal stem cells
PG	Proteoglycan
SMC	Smooth muscle cells
SLRPs	Small leucine-rich proteoglycans
TGF- $\beta$ <sub>1</sub>	Transforming growth factor beta-1
Th1	T-helper cell type 1
Th2	T-helper cell type 2
TLR	Toll like receptor
VEGR	Vascular endothelial growth factor





# Introduction

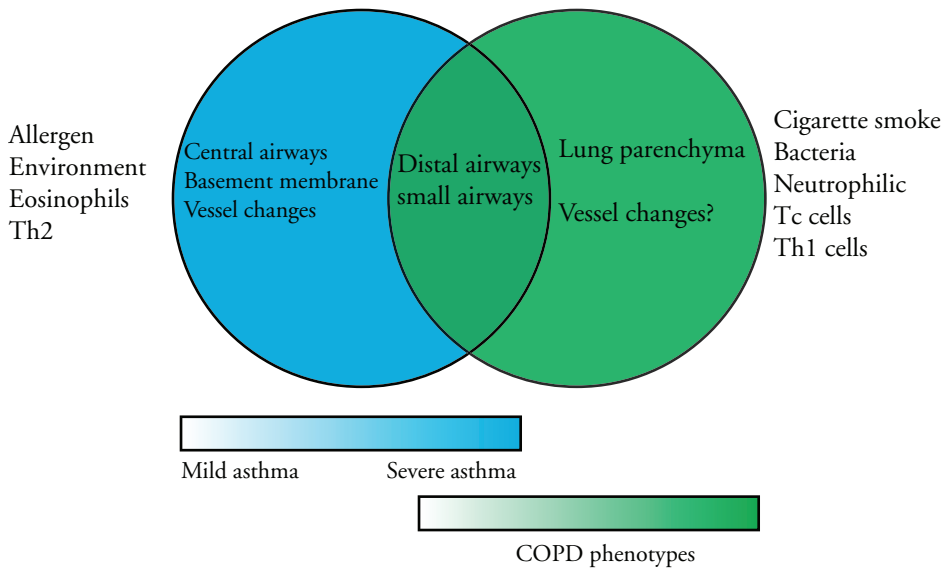
Chronic lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) are two lung diseases that are continuously increasing worldwide. Despite extensively research to find curative treatment, no such therapy exists today. The primary goal from both academic institutions and the pharmaceutical industry has thus been to try to diminish or reduce inflammation. This approach has to some extent been successful in patients with mild asthma but unsuccessful in moderate/severe asthma phenotypes. In COPD the efforts to treat and to prevent further deterioration of the disease have been even more disappointing. However, new hypothesis and data are being generated that may reveal new treatment targets. This could provide tools to explain and help to develop therapies that not only stop the decline in lung function but also may improve and restore the lung function back to normal. The new hypothesis suggests that an aberrant chronic wound healing process takes place which involves both the classical inflammation and more chronic changes of the structural environment where the mesenchymal cell phenotypes such as the fibroblasts play a key role. This phenomenon is defined as airway remodeling and is characterized by a deposition of extracellular matrix (ECM) molecules such as collagens and proteoglycans. It is generally accepted that fibroblasts play a key role in this pathological process due to their ability to change their phenotype and produce high levels of the ECM molecules and change ECM composition. Importantly, hitherto available studies have only studied fibroblast from central airways from humans where the role of the fibroblast might be different compared to the distal lung. The aim of the present thesis was to study the different fibroblast phenotypes and their biological role from central and distal localizations from human lung in controls, rhinitis, asthmatics and CODP subjects. Another aim was to investigate the possible origin (circulating progenitor cells) of these cells and thereby highlight the heterogeneity of fibroblast phenotypes resident within the human lung.



# Background

## Asthma and COPD

Both asthma and COPD are characterized by abnormal lung function and structure; however they involve different subsets of inflammatory and structural cells in combination with mediators which exhibit disease specific mechanisms<sup>1</sup>. Despite intensive research, little progress in identification of new treatments has been made since the introduction of new  $\beta_2$ -adrenoreceptor selective agonist and the inhaled glucocorticosteroids in the late 60s and the 70s respectively. Although inhaled steroids have been successful to achieve control in many asthmatics, asthma is still a problem with estimated 300 million affected individuals in 2004<sup>2</sup>. World health organization estimated 2007 that 210 million people had COPD and 3 million died in 2005. They predict that COPD will be the third largest cause of death worldwide by 2030. While smoke exposure is a main risk factor for COPD development, the causative factor for asthma development is less clear. However it is a multifactorial disease and includes allergens, virus and environmental triggerfactors. At the same time COPD can coexist with asthma as individuals with asthma who are exposed to chemical agents, particularly cigarette smoke, may also develop airflow limitation and a mixture of asthma and COPD-like symptoms. Therefore; asthma and COPD show similarities, common features and substantial differences<sup>1;3</sup> (figure 1). One structural important difference between these diseases is the involvement of the lung parenchyma. The impaired network of fibers in the COPD emphysema lung results from a destruction of the elastic fiber network of the lung with time<sup>4</sup>. A major challenge for the respiratory science community is to identify and to define molecular mechanisms and markers that help to predict the outcome of the diseases. In the clinic, pulmonary function testing has proven to be a reasonable reliable method in to maintain asthma control but this methodology has not been that successful in predicting the outcome for COPD. Furthermore, the attempts to find non-invasive techniques which could help to phenotype patients and to investigate the disease pathophysiology have been far more difficult than predicted. Despite the current problems to improve asthma and COPD treatment the scientific community have the last years come together to discuss and update the clinical guidelines for both asthma and COPD, trying to elucidate the similarities and differences.



**Figure 1** Asthma and COPD have different disease mechanisms however there are structural and inflammatory similarities. Most overlap is between severe asthmatics and COPD individuals where Th1 and neutrophilia are common features.

## Asthma and asthma phenotypes

The clinical definition of asthma includes two major parts- symptoms and variable airway obstruction- that can be clinically evaluated. Another key criterion is presence of airway inflammation and airways hyperresponsiveness (AHR) which are more difficult to access in clinical practice<sup>5,6</sup>. Asthma are present in all ages from young individuals to older subjects. Most asthma patients at younger ages are atopic (extrinsic asthma) but a few are non atopic (intrinsic asthma). By age the number of non-allergic asthmatics increases. Moreover asthmatic individuals with concomitant rhinitis and/or rhinosinusitis are known to be at risk of having a disease less easy to control<sup>7</sup>. Chronic inflammation may eventually lead to structural changes in the airways resulting in increased airway hyperresponsiveness and decline in lung function. Asthma has been regarded as a disease where eosinophils play the major role. Indeed, treatment with inhaled corticosteroids reduced number of eosinophils in airway biopsies as well as in induced sputum. The importance of eosinophils have however been questioned in some studies. The clinical trials where an anti IL-5 antibody (chemotactic agent in eosinophil recruitment) was administered turned out to be a failure, despite 80% reduction in both sputum and blood eosinophils<sup>8,9</sup>.

Thus other cells other than eosinophils have been proposed to be of importance in asthma. Mast cells may have a role in some patients with asthma as it was found that mast cells infiltrating the airway bronchial mucosa layer was the single most important factor differentiating asthmatic from those with eosinophilic bronchitis without asthma<sup>10</sup>. In one study with more severe asthma it was found that the patients had increased numbers of mast cells in the peripheral airways<sup>11</sup>. These findings may be of special importance since as they pinpoint mechanisms not responsive to corticosteroid therapy, standard anti-inflammatory treatment in asthma. In addition, a specific group of patients have high numbers of neutrophils which also suggest a different disease mechanism, also non-responsive to corticosteroid therapy. Thus asthma is a complex disease involving several different mechanisms which only partly may be controlled by corticosteroid therapy. The level of remodeling is also believed to be important in the different asthma phenotypes. In this process, activated fibroblasts, and circulating fibroblast progenitor cells (fibrocytes) are believed to play a key role in the process of airway remodeling associated with the different asthma phenotypes.

These asthma subtypes strongly suggests that asthma collectively is a symptom and that we have to investigate these symptoms more in detail since it is likely that they have distinct clinical features which requires different treatments<sup>12</sup>

### *Clinical classification of Asthma*

Asthma is associated with airway obstruction and AHR that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the morning. The airflow obstruction is often reversible, either spontaneously or after treatment<sup>6</sup>. The preferred method for objective measurement of the functional limitation of the lung capacity is the characterization of the lung function with spirometry, e.g. forced expiratory volume in 1 s ( $FEV_1$ ), forced vital capacity (FVC) and the  $FEV_1$ /FVC ratio. A significant reversibility is usually defined as an improvement of  $FEV_1 > 12\%$  after administration of a bronchodilator (such as  $\beta_2$ -agonist salbutamol). Another indicator of airway obstruction is also a reduced ratio of  $FEV_1$ /FVC. The normal ratio is greater than 0.75-0.8 and any value below this suggest airflow limitation<sup>6</sup>. Measurement of AHR to methacholine or histamine reflects both structural changes and to some degree presence of inflammation in the lower airways. The readout generally expressed as the “provocative concentration (PC), or cumulative dose, (PD) of the agonist inducing a 20% reduction in FEV compared to baseline. Hyperresponsiveness may be useful in diagnosing asthma but presence of AHR may not always be related to degree of lower airway inflammation<sup>13</sup>. A non-invasive method to measure lower airway inflammation is the measurements of the nitric oxide levels (NO) in exhaled breath air. Nitric oxide has been shown to be elevated in active, preferably atopic asthma where eosinophils and epithelial cells are believed to be the primary source. When NO is elevated in active asthma, inhaled corticoidsteroids has shown to restore the nitric oxide levels back to normal.

However, not all patients with elevated levels respond to inhaled corticosteroid treatment indicating a high constitutive production in some individuals. The reason for

these differences are not yet fully understood<sup>14</sup>. Measurements are used in combination with clinical parameters as day-time symptoms, limitations of activity, need for rescue/reliever drugs, exacerbations and treatment responses<sup>6</sup>. Briefly, the frequency of the symptoms, exacerbations and lung function test are used as major guidelines in the classification of asthma severity as shown in table 1.

<b>Intermittent</b>	<b>Mild persistent</b>	<b>Severe persistent</b>	<b>Moderate persistent</b>
<b>Symptoms less than once a week</b>	<b>Symptoms more than once a week but less than once a day</b>	<b>Symptoms daily</b>	<b>Symptoms daily</b>
<b>Rare exarcebations</b>	<b>Occasionally exarcebations may affect activity and sleep</b>	<b>Frquent Exarcebations may affect activity and sleep</b>	<b>Frequent exarcebations Limitation of physical activities</b>
<b>FEV<sub>1</sub> ≥ 80% predicted</b>	<b>FEV<sub>1</sub> ≥ 80% predicted</b>	<b>FEV<sub>1</sub> ≤ 60-80% predicted</b>	<b>FEV<sub>1</sub> ≤ 60% predicted</b>
<b>AHR</b>	<b>AHR</b>	<b>AHR</b>	<b>AHR</b>
<b>Increased nitric oxide</b>	<b>Increased nitric oxide</b>	<b>Increased nitric oxide</b>	<b>Increased nitric oxide</b>

**Table 1** Basic classification of the asthma severity according to the GINA guideline. (Global initiative for Asthma). FEV<sub>1</sub>=forced expiratory volume in 1 s. AHR= airway hyperresponsiveness, measured after administration of an agonist (usually metacholine) causing a given fall in FEV<sub>1</sub> (20%). Exarcebation=suddenly worsen of symptoms.

## COPD and COPD phenotypes

COPD is clinically characterized by airflow limitation that is not fully reversible. In addition to the abnormal inflammatory responses other classifications such as chronic bronchitis (chronic productive cough for 3 months in each of 2 years where other explanations that causes the chronic cough have been excluded) and emphysema which is defined as “presence of permanent enlargement of the airspaces distal to the terminal bronchioles accompanied by destruction of their walls” are present a various degrees. Simultaneously, there could be areas that have extensive fibrosis (deposition of ECM molecules) and fully normal areas, and these morphological differences have shed light to COPD as a multi heterogeneous disease. A possible future way to further phenotype the patients could be to include the pathophysiological variable such as connective tissue changes into the diagnostic criteria. This would enable the physician to estimate the levels of the structural changes. The mixture of obstructive bronchiolitis, emphysema and locally fibrotic areas ultimately cause the airflow limitation. Tobacco smoke is the

dominant risk factor for COPD, but other risk factors such as  $\alpha$ 1-antitrypsin deficiency, gene/environment interactions, occupational exposure, lung growth, gender, age, respiratory infections and nutrition contribute individually or in combination to the development of COPD

### *Clinical classification of COPD*

The symptoms of COPD are cough, sputum production and chronic progressive dyspnea as guided by the GOLD standards<sup>15</sup>. The prevalence of COPD is higher in men than in women and increased by age. Early signs of COPD may be the chronic cough and sputum production as a physiologic response of the tobacco exposure. As the disease progress there is a more rapid decline in lung function over year and many patients are not aware of the loss until a substantial worsening of lung function have occurred. Commonly they seek medical attention when FEV<sub>1</sub> is around 50% of predicted.

Unfortunately, it is often too late as most of the changes are irreversible and the lungs cannot be restored. Thus it is of most importance to detect the disease as early as possible. COPD can be classified in 4 different groups as judged by table 2 (GOLD-1, -2, -3, 4) where gold 4 is the most severe form of COPD.

<b>GOLD I Mild</b>	<b>GOLD II Moderate</b>	<b>GOLD III Severe</b>	<b>GOLD IV Very Severe</b>
FEV <sub>1</sub> /FVC < 0.70 FEV <sub>1</sub> 80% predicted <sup>1</sup>	FEV <sub>1</sub> /FVC < 0.70 50% FEV <sub>1</sub> 80% predicted <sup>1</sup>	FEV <sub>1</sub> /FVC < 0.70 30% FEV <sub>1</sub> 50% predicted <sup>1</sup>	FEV <sub>1</sub> /FVC < 0.70 FEV <sub>1</sub> 30% predicted FEV <sub>1</sub> or <50% predicted <sup>1</sup> plus chronic respiratory failure
presence of chronic cough and sputum	presence of chronic cough and sputum	presence of chronic cough and sputum	presence of chronic cough and sputum
	chronic respiratory symptoms	repeated exacerbations, chronic respiratory symptoms	repeated exacerbations, chronic respiratory symptoms

**Table 2** Basic classification of COPD severity according to GOLD standard (Global initiative for chronic obstructive lung disease). FEV<sub>1</sub>=forced expiratory volume in 1 s. FVC= Forced vital capacity. Exacerbation=suddenly worsen of symptoms. <sup>1</sup>Post broncho dilating.





# The united airways

The respiratory system is design to ensure that all our cells have access to the life needed oxygen. The upper (nose and throat) and lower airways (lungs) are anatomically and functionally integrated in normal physiological and host defending responses. The united airway theory state that the “respiratory disease is reflected throughout the airways even though symptoms appear to be confined to a specific or isolated segment” Briefly, the airways are constituted by nose and throat (nasopharynx), the trachea and the lung. The lungs are then further subdivided into bronchus (central airways), bronchioles (small airways) and alveolus (distal airways). Throughout the conductive airways epithelial cells, structural cells (fibroblasts, smooth muscle cells), inflammatory cells (t-cells, b-cells, dendritic cells, macrophages, eosinophils, mast cells etc) are distributed at various density depending of their role (Fig 2). Classically, asthma has been regarded to be a bronchial derived disease. Common features are infiltration and activation of inflammatory cells, subepithelial fibrosis, smooth muscle hyperplasia, AHR and mucous production. However, accumulating data now suggest that it is time to modify this view and include the whole lung when studying lung diseases<sup>16-19</sup>.

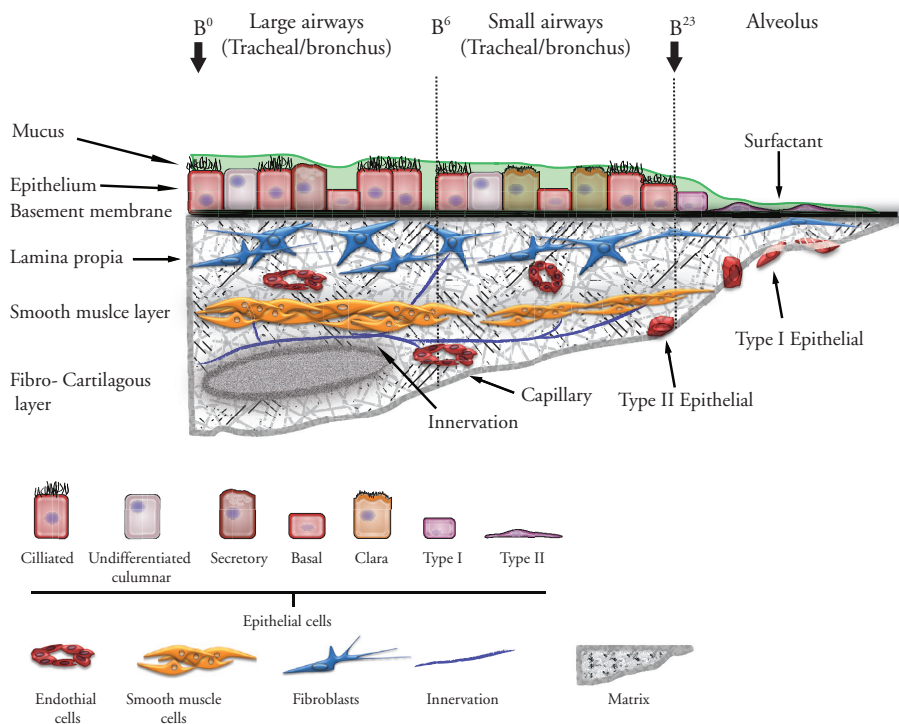
## Upper airways vs. central airways

Structurally, the upper airways have similarities to the central airways. They consist of a mucosa composed of a ciliated epithelium, a connective tissue lamina, a smooth muscle cell area and cartilage to maintain the stability of the airways. Continuous basement membrane, mucosal transport, goblet cells, cartilage and innervations are also structures present in both localizations. Acute and late phase inflammation is comparable in both localizations and immune responses could potentially be triggered by cold, cigarette smoke and allergens. It is therefore reasonable to conclude that close interactions and crosstalk between upper and central airways take place during both normal and pathological conditions.

## Central airways vs. distal airways

Interestingly, from the central airways, the cellular composition e.g. smooth muscle cells and connective tissue density change when reaching the bronchioles and further

more in the alveolus. Smooth muscle density decreases and in the small bronchioles the smooth muscle layer is replaced by a small layer of connective tissue that is direct contact with the alveoli. The epithelial cells are primarily type I cells which are closely localized to the capillaries. The resident interstitial cells in the alveolar septum (distal airways) comprises mainly of fibroblasts. Fibroblasts cells produce elastic fibers, bundles of collagen fibrils and proteoglycans to support and maintain lung elasticity and mechanical properties throughout life (Figure 2) Thus, the cellular and molecular environment in the distal airways is completely different compared to the central and upper airways. This could be of fundamental importance in lung diseases.



**Figure 2** Schematic view of the structural cells and their presence in the central-, small- and distal airways. In central airways the epithelium are composed of ciliated, basal, undifferentiated columnar and secretory cells. Fibroblasts are spindle shaped and smooth muscle layer is obvious. The cartilage layer protects the large airways. In the small airway the important clara cells are more prominent. The numbers of the smooth muscle cells are decreased compared to central airways. In the alveolus, epithelial cells are type I and type II where type I produce the important surfactant protein. The numbers in the picture is not representative in the human lung but more to visualize the different phenotypes. B=Branch. Modified from ref 132

# Structural cells in the lung

The entire lung is maintained by a continuously turnover of cells and matrix components where the structural cells including fibroblasts, smooth muscle cells and epithelial cells preserves the three-dimensional structure<sup>20</sup>. Evidence now emerge that pathogenesis of asthma and COPD can not only be explained on the basis of a dysregulated immune- response and that the homeostasis of the ECM contributes significantly to the pathology in both diseases<sup>21-25</sup>.

In addition, it is now clear that fibroblast progenitor cells which are recruited from the circulation are involved in the ECM processes, epithelial progenitor cells derived from the bone marrow play a role in the maintenance and repair of the epithelium and endothelial progenitor cells participate in the vascular changes in the lung. Although we know that these cells exist, we still know very little regarding their biological activity, cellular turnover, and their relevance for disease development and pathology.

## Overview of the fibroblast role in the wound healing processes

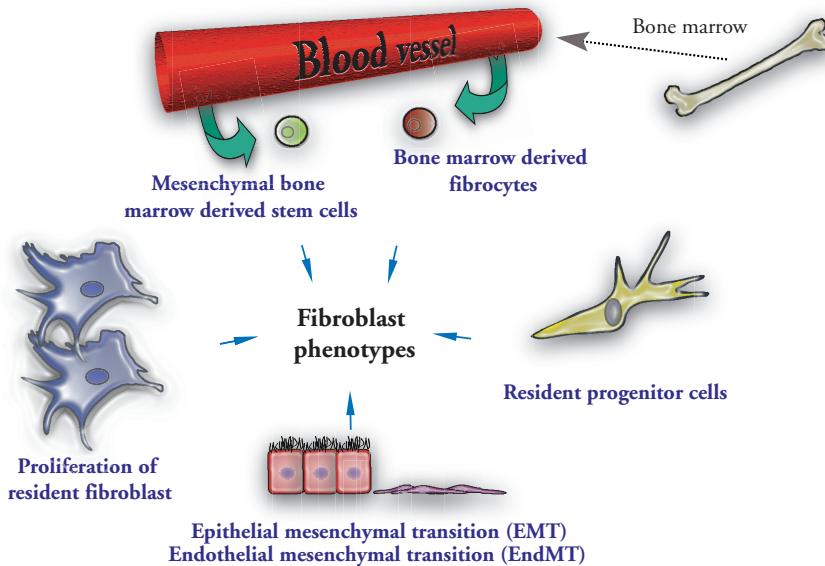
Lung injury and normal repair of the central airways and the alveolar-capillary environment results in restoration of tissue integrity and function depending of the nature of the insult. After an injury, the process of intrinsic and extrinsic coagulation cascades in combination with plasma extravasations in the lung tissue takes place. This eventually leads to fibrin deposition and establishment of a provisional matrix<sup>26</sup>. Fibroblasts, leukocytes and endothelial cells are then recruited and activated by lipids, growth factors and cytokines released from the platelets. Stimulated by growth factors such as transforming growth factor beta- 1 (TGF- $\beta$ 1, discussed later) the fibroblast differentiate to a more contractile myofibroblast phenotype. This myofibroblast starts to produce high levels of ECM molecule and contract the tissue<sup>26,27</sup>. Fibroblast growth factors, particular fibroblast growth factor- 2 (FGF2) and vascular endothelial growth factor (VEGF) have been suggested to play a major role in the remodeling process. FGF2 induce proliferation of fibroblasts, myofibroblasts and smooth muscle cells<sup>28-30</sup>. The enhanced production of VEGF from the endothelial and smooth muscle cells induces vascular remodeling and thereby contributes to the wound healing process<sup>31</sup>. This process results in ECM reorganization and finally wound closure where the tissue stiffness seems to be the crucial signal due to the fibroblast ability to recognize TGF- $\beta$ <sub>1</sub> in stiff tissue<sup>32</sup>.

## Fibroblast phenotypes and origin

Fibroblasts are widely distributed in all lung structures and play a key role in matrix homeostasis. For example, when exposed to growth factors or mechanical stimuli, these cells are capable of generating more than 5,000 molecules of procollagen per cell per minute and its primary genes are involved in connective tissue production in both fibrosis and inflammation<sup>33;34</sup>. They are normally found in the stroma of many tissues and in the normal adult lung. Furthermore, they are also present in the adventitia of vascular structures and airways. Fibroblasts are regarded to be spindle shaped, and produce various amounts of ECM molecules such as collagen I and III.

The differentiation to an active fibroblast/myofibroblast is very important for the inflammatory and remodeling response and is not restricted to the lung but to variety of organs<sup>35</sup>. However, fibroblast exists in different phenotypes. In one report, the loss of the mesenchymal/fibroblast marker Thy-1 has more fibrotic properties compared to its positive counterpart and the myofibroblasts have lost their expression of Thy-1. Importantly, Thy-1 negative cells are present in lung fibrotic disease such as idiopathic pulmonary fibrosis (IPF). This and other studies suggest that the different fibroblast phenotypes are presence in human lung<sup>36-41</sup>.

The origins of the different fibroblasts phenotypes are today not known. Several hypotheses describe the possible origin of fibroblasts, their subpopulations and how they renew in the lung<sup>42-48</sup>. Briefly, fibroblast could derived from (1) proliferation of already tissue present fibroblasts/myofibroblasts, (2) mesenchymal stem cells (MSC) derived from the bone marrow, (3) fibrocytes which are cells with both hematopoietic and mesenchymal properties, (4) from epithelial mesenchymal transition (EMT) and from (5) tissue-resident stem cells which differentiate upon local activation (Fig 3). The different origins are discussed in more detail below.



**Figure 3** Fibroblast phenotypes could be derived from 1) proliferation from resident fibroblast, 2) mesenchymal bone marrow derived stem cells (MSC), 3) Bone marrow derived fibrocytes, 4) epithelial mesenchymal transition (EMT) and/or endothelial mesenchymal transition (EnMT) and 5) specific resident progenitor cells.

### 1) *The myofibroblast*

Despite the different suggested fibroblast phenotypes, the primary mesenchymal cell that modulates the matrix turnover and the crosstalk with inflammatory cells is suggested to be the myofibroblast. Characteristic for myofibroblast is the increased collagen production and it is suggested that the differentiation of a fibroblast to a myofibroblast is necessary for the increased production<sup>49</sup>. Myofibroblast are activated via a variety of mechanisms, including signals from lymphocytes, macrophages and pathogen-associated molecular pattern (PAMPs) that interact with its receptors (such as Toll like receptors, TLRs) on fibroblasts<sup>26;27;43;46;50</sup>. Cytokines such as IL-13, IL-21, TGF- $\beta_1$ , chemokines (MCP-, MIP-1 $\beta$ ), VEGF, platelet derived growth factor, peroxisome proliferator activated receptors, acute phase protein, caspases and components of the rennin-angiotensin-aldosterone system have been identified as important regulators of myofibroblast activation, fibrosis and inflammation<sup>43</sup>.

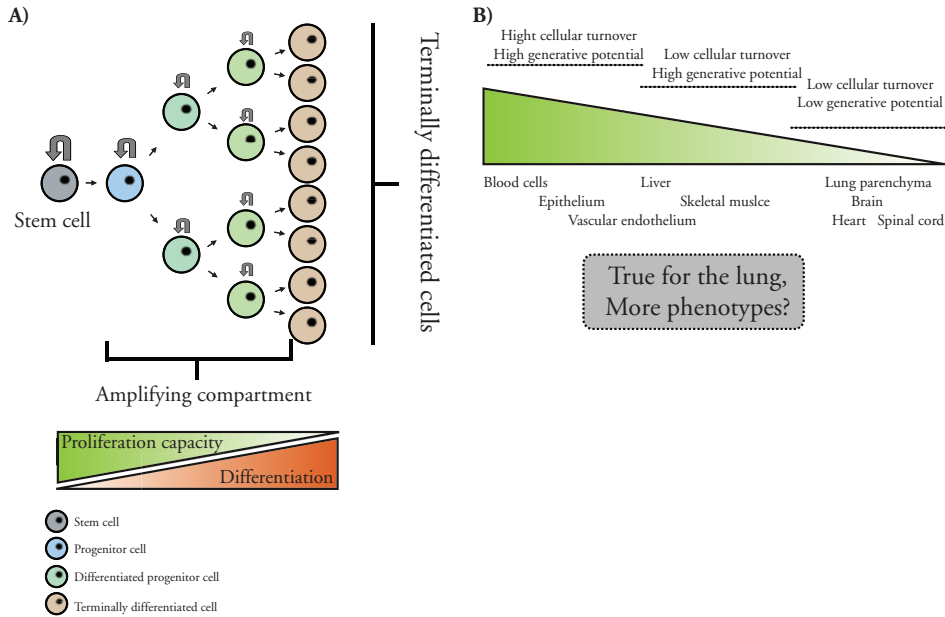
The expression of alpha- smooth muscle actin ( $\alpha$ -SMA) protein is regarded to be a representative marker of a myofibroblast and that polymerization of the  $\alpha$ -SMA protein results in increased collagen production as the suppression of  $\alpha$ -SMA results in reduction in collagen gene expression<sup>51</sup>. However it has been proposed that there are subtypes of myofibroblasts, so called “proto- myofibroblasts” that do not express  $\alpha$ -SMA but in-

stead cytoplasmic actins. The role of the proto-myofibroblast is to migrate and start the wound closure process. Importantly, the proto-myofibroblast is less contractile compared to the myofibroblast. This subtype is present in early granulation tissue and once the proto-myofibroblast have developed in response to mechanical stress they can be stimulated by platelet derived growth factor, ED-A fibronectin and TGF- $\beta_1$  to further differentiate into myofibroblasts<sup>26;52</sup>. On the other hand, stimulation with biglycan redistributes  $\alpha$ -SMA to focal adhesion sites. These cells are also migratory which suggests that they are proto-myofibroblast as well<sup>53</sup>. Differentiation to myofibroblast *in vitro* is commonly induced by administration of TGF- $\beta_1$ . Importantly it has been shown that TGF- $\beta_1$  promotes the formation of stress fibers, focal adhesions and fibronectin fibrils. In addition, when these fibroblasts are cultured in stressed collagen lattices their stress fiber expression correlates with increased generation of contractile force<sup>54</sup>. Activation of  $\alpha$ -SMA requires activation of Smad3- binding element and additional transcription factors including C/EBP, Notch signaling, c-myc have been implicated to regulate this gene. Following wound closure, it is believed that the myofibroblast undergo apoptosis but this has not been proven yet.

Expression of  $\alpha$ -SMA is regulated differently in myofibroblasts and smooth muscle cells (SMC). In SMCs, DNA specific elements such as CArGs A and B are required for SMC function. However these elements do not seem to be necessary for the increased expression of  $\alpha$ -SMA in cultured myofibroblasts<sup>55;56</sup>. Today, it is unclear whether myofibroblasts and SMC represent a step of a continuous differentiation between the fibroblast and the SMCs.

## 2) Bone marrow derived mesenchymal stem cells

MSC are heterogeneous subsets of stromal stem cells that can be isolated from many adult tissues. They have the capacity to self-renewal and differentiation to different cell lineages. These cells can differentiate into mesodermal lineages, such as bone, fat, and cartilage but they also have neuroendodermic<sup>57;58</sup> (epithelial, neuron) and endoderm (muscle, gut epithelial and lung cells) potential<sup>59</sup>. The stem cells have the capacity to proliferate and amplify into terminal differentiated cells. True stem cells differentiate into progenitor cells and this process is believed to be non- reversible (Fig 4a). As the cells differentiate, they lose their capacity to be true stem cells. Organs have different cellular turnover and generative potential. For example, blood cells have high cellular turnover and high generative potential whereas the lung is considered to have low cellular turnover and low regenerative potential (Fig 4b). Maybe the debate on the possible origin of the fibroblast could change this view?



**Figure 4** Traditional view of cell lineage in renewing tissue. The stem cells renew themselves through mitotic cell division and differentiate into progenitor cells. Simultaneously as proliferation take place the differentiation into a fully differentiated cells take place. Different organs host different stem cells according to the organs cellular turnover and generative potential.

After transplantation of the mice MSC into to the bone marrow of non-obese diabetic-severe combined immunodeficiency mice, MSC have the ability to differentiate into pericytes, myofibroblasts, bone-marrow stromal cells, osteocytes, osteoblasts and endothelial cells. All these cell types of which constitute the components of the hematopoietic stem cell niche that support haematopoiesis<sup>60</sup>. There is a general consensus that human MSCs cultured *in vitro* do not express the hematopoietic markers CD45, CD34, CD14 or the co-stimulatory molecule CD86, CD86 and CD40, whereas they express variable levels of CD105 (endoglin), CD73 (ecto-5' nucleotidase), CD44, CD90 (Thy-1), CD71 (transferrin receptor), the ganglioside GD2 and CD271 (low-affinity nerve growth factor receptor) and they are recognized by the monoclonal antibody STRO-1. *In vitro*, MSCs proliferate as adherent cells, have fibroblast morphology, form colonies and can differentiate into bone, cartilage and fat cells<sup>61;62</sup>.

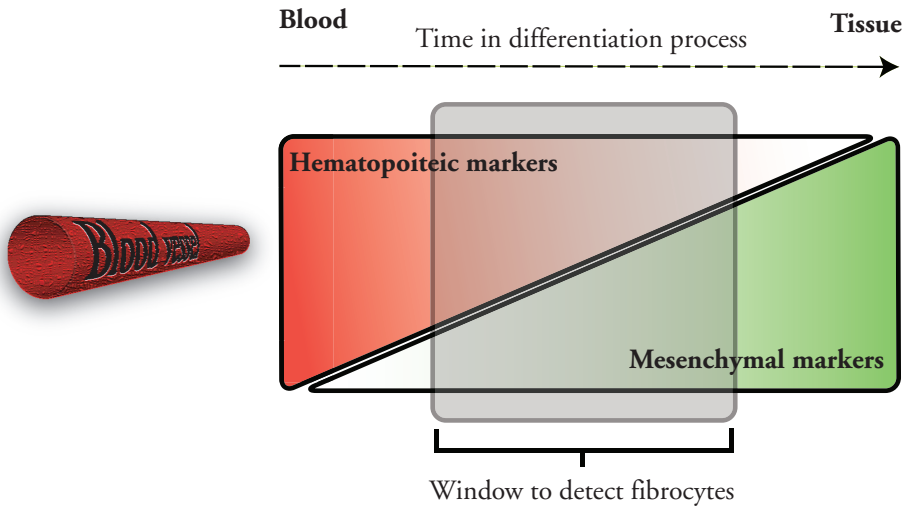
In general, MSC are not that well characterized compared to hematopoietic stem cells and they are also suggested to have immunomodulatory effects. This has been investigated in studies where bone-marrow derived MSC suppressed T- cell proliferation; inhibited differentiation/function of monocyte derived dendritic cells. Furthermore, MSC inhibited *in vitro* B-cell proliferation, differentiation to plasma cells and sub-



sequent antibody secretion<sup>63-65</sup>. The role of traditional bone marrow derived MSCs in lung disease remains to be identified. Researchers have tried to prove the potential of MSCs to engraft the lung after lung injury but they have in general not been very successful. In a study by Ortiz *et al*, presence of bone marrow MSC were enhanced in response bleomycin (BLM) exposure<sup>66</sup>. Localization of the male Y chromosome by fluorescence *in situ* hybridization revealed that engrafted male cells were localized to areas of BLM- induced injury and exhibited an epithelium-like morphology. Importantly, administration of the MCS reduced the degree of inflammation and collagen deposition within the lung. The authors therefore suggested that the MSC engraftment ameliorated the fibrotic effect of the bleomycin<sup>66</sup>. Nevertheless, it is likely that the mesenchymal progenitor cells have specific markers depending of their final destination. In the murine adult lung, Sca-1 positive cells have been identified as selectable marker for isolation of non-hematopoietic CD45 (neg), non-endothelial CD31 (neg) bronchioalveolar stem cells. Sca-1 is not expressed in humans and no homolog has been found. However mouse Sca-1 over-expression affect human as well as mouse stem/progenitor cell activity, suggesting the possibility of a functional human Sca-1 homolog<sup>67</sup>.

### 3) Fibrocytes

The fibrocytes are bone marrow derived progenitor cells that coexpress cell antigens from the hematopoietic lineage and mesenchymal products. Their ability to constitutively produce ECM components and capacity to further differentiate into myofibroblast both *in vivo* and *in vitro* is of significant importance. Accumulating data suggest that fibrocytes are important cells in diseases such as asthma (paper II), hypertrophic scars, interstitial pulmonary fibrosis, systemic fibrosis, atherosclerosis, chronic pancreatitis and in tumor induced stromal reaction<sup>41;42;65;68-75</sup>. Importantly, these diseases have a persistent inflammation in combination with ongoing matrix remodeling which suggests that fibrocytes are important in such environments. The most frequently used markers to identify fibrocytes in the tissue are the combination of one hematopoietic marker (CD45, CD34, CD14 and CXCR4) with one mesenchymal marker (pro-collagen, collagen I, vimentin, prolyl-4-hydroxylase). Differentiations of fibrocytes are believed to occur in the tissue and upon tissue activation, fibrocytes downregulate their hematopoietic markers and upregulate expression of the mesenchymal markers. The window to detect the fibrocytes is therefore limited suggesting that the number of fibrocytes in a measured area is underestimated (Figure 5).



**Figure 5** Circulating fibrocytes derived from the bone marrow have high expression of hematopoietic markers such as CD45 and CD34. Upon differentiation, these cells lose their hematopoietic markers and increase their expression of mesenchymal markers. When fully differentiated into a fibroblast like phenotype, it is believed that no hematopoietic markers are expressed and only mesenchymal such as collagen and prolyl-4-hydroxylase are expressed. The fibrocytes could therefore be underestimated since they could only be detected in a small window.

Furthermore, there is no standardized way to identify the cells and this cause confusion. Fibrocytes are present in the peripheral blood and has been estimated to comprise 0.1-0.5% of the “circulating” non erythrocytic cells on the basis of the number fibrocytes isolated from peripheral blood<sup>75</sup>. However Bellini *et al* suggests that fibrocytes may not be present as such but rather as a subset of CD14<sup>+</sup>/CD16<sup>-</sup> human mononuclear cells that are positive for the CC chemochine receptor CCR2<sup>76;77</sup>. These cells are immature, are normally present in the circulation and have been termed “inflammatory monocytes”. The authors suggest that this cell population in absence of inflammation is likely to replenish the tissue-resident macrophage- and dendritic- cell populations. Following and inflammatory response, they are released in high numbers from the bone marrow to the peripheral blood and directly migrate to site of inflammation where they differentiate fibroblast/myofibroblast like cells. The evidence that fibrocytes are derived from monocyte lineages are supported by the expression of major histocompatibility complex class I and II and the co- stimulatory molecule CD80 and CD86. On the contrary they lack specific monocyte-derived markers such as CD10 and CD83<sup>42</sup>. Furthermore, fibrocytes can be distinguished from the circulating or tissue-resident mesenchymal stem cells/multipotent mesenchymal stromal since fibrocytes do not express CD90 (Thy-1) but CD34, CD45 or the monocyte marker<sup>42;78</sup>. When recruited in the tissue, they have the capacity to differentiate into mesenchymal and also adipocytes<sup>70;79</sup>. Fibrocytes have been demonstrated to migrate to the tissue by chemotaxis towards specific chemokines gradient. In fluorescein isothiocyanate (FITC) induces fibrosis there was a significant reduction in the fibrotic changes in the lung tissue from mice lacking

CCR2 (CCR2<sup>-/-</sup>). Administration of bone marrow cells from CCR2<sup>+/+</sup> positive cells into lethally irradiated CCR2<sup>-/-</sup> induced the fibrotic changes<sup>80</sup>. In other animal models of lung fibrosis and renal fibrosis the role of the CXCR4 and CCR7 receptors and their corresponding ligands, CXCL12 and CC21 have been shown to be of importance for fibrocytes recruitment<sup>81;82</sup> (Fig 6). In patients with idiopathic pulmonary fibrosis, accumulation of fibrocytes could be correlated with number fibroblastic foci. Furthermore, CXCL12 levels in the bronchoalveolar fluid (BALF) was increased and CXCL12 was strongly expressed by alveolar epithelial cells which suggests a role of the fibrocytes in human lung fibrosis<sup>83</sup>.

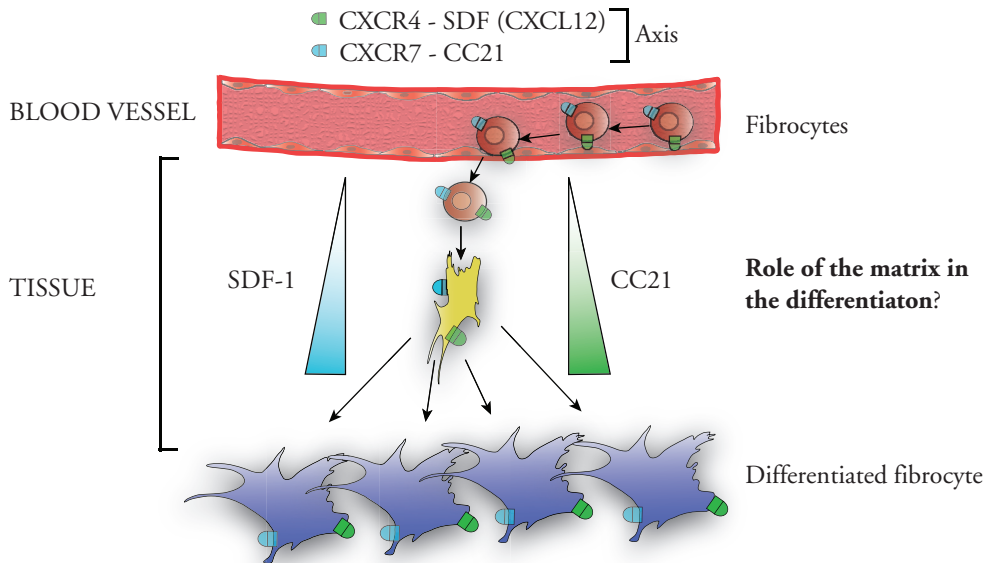


Figure 6. Recruitment of the fibrocytes to areas needed to be restored. Locally produced cytokines such as SDF-1 and CC21 forms a gradient towards the blood vessel which activates the endothelial cells. The fibrocytes adhere and migrate through the endothelium (diapedesis). When entering tissue the fibrocytes starts to differentiate upon factors such as TGF- $\beta_1$  to a fully matured mesenchymal cell.

What are the evidences that fibrocytes could acquire fibroblast-like properties? In a mouse model of wound healing, Bellini et al isolates CD45<sup>+</sup>/ CD13<sup>+</sup>/Collagen I<sup>+</sup> fibrocytes between 4 and 7 days post wounding. These cells expressed collagen I, measured by pro-collagen I mRNA expression. Simultaneously as the cell increased their expression of  $\alpha$ -SMA, they decreased their expression of CD34 and CD46. At 7 days post wounding, 59% cells expressed  $\alpha$ -SMA<sup>70</sup>. Culturing of BALF fibroblasts derived from patients with mild asthma 17% of the cells expressed fibrocyte markers further confirmed presence of fibrocytes in human lung<sup>41</sup>.

#### 4) *Epithelial and endothelial mesenchymal transition (EMT and EndMT)*

Recent studies suggest that in response to lung injury both epithelial and endothelial cells have the capacity to change their phenotypes into matrix producing like phenotypes via EMT or EndMT. The true definition of both epithelial and endothelial mesenchymal transition is “a process when fully differentiated epithelial/endothelial cells undergo phenotypic transition to fully differentiated mesenchymal cells (fibroblast/myofibroblasts)<sup>84</sup>. These transitions appear to contribute to the pathological processes in cancer, kidney, heart and lungfibrosis<sup>85-87</sup>. EMT and the mesenchymal epithelial transition also play a central role in embryogenesis. Mesoderm generated by EMTs develops into multiple tissue types and late in development mesodermal cells generate epithelial organs such as the kidney and ovary via mesenchymal epithelial transition<sup>84</sup>.

The transition from an epithelial cell in to a mesenchymal cell includes alteration of morphology (from a cuboidal cell shape to an elongated or spindles-shaped front), cellular architecture, adhesion, migration capacity. It also includes the loss of characteristic epithelial markers such as E-cadherin, cytokeratin and the acquisition of fibroblast- or myfibroblast specific markers such as fibroblast specific protein-1 and  $\alpha$ -SMA, respectively. The transition could further be validated by studying transcription factors such as Snail1 (snail) and Snail2 (slug) that inhibit E-cadherin production. TGF- $\beta_1$  has shown to be a strong inducer of both EMT and EndMT<sup>85;87</sup>. It has been suggested that the balance of TGF- $\beta_1$  and the bone morphogenic protein-7 (BMP-7) is of major importance to maintain or induce EMT and EndMT in kidney and heart<sup>86;87</sup>. BMP-7 acts as a competitor to TGF- $\beta_1$  and diminishes the effect of TGF- $\beta_1$  but the levels of TGF- $\beta_1$  in the tissue is likely to be the same. BMP-7 is a member of the transforming growth factor beta superfamily and signal through Smad-1, Smad-5 and Smad-8 whereas TGF- $\beta_1$  primarily signals through Smad-2 and Smad-3. The possibility that EMT and EndMT take place in mild, moderate or severe asthma or if these processes play a role in COPD development remains to be elucidated. Borok *et al* propose that alveolar epithelial type I cells (ATII), may also have the capacity to undergo transition to fibroblasts and myofibroblasts through the process of EMT<sup>44</sup>. In COPD this would be of specific interest since the parenchyma is exposed to tobacco smoke and this chronic exposure might induce EMT.

#### 5) *Tissue- resident mesenchymal stem cells*

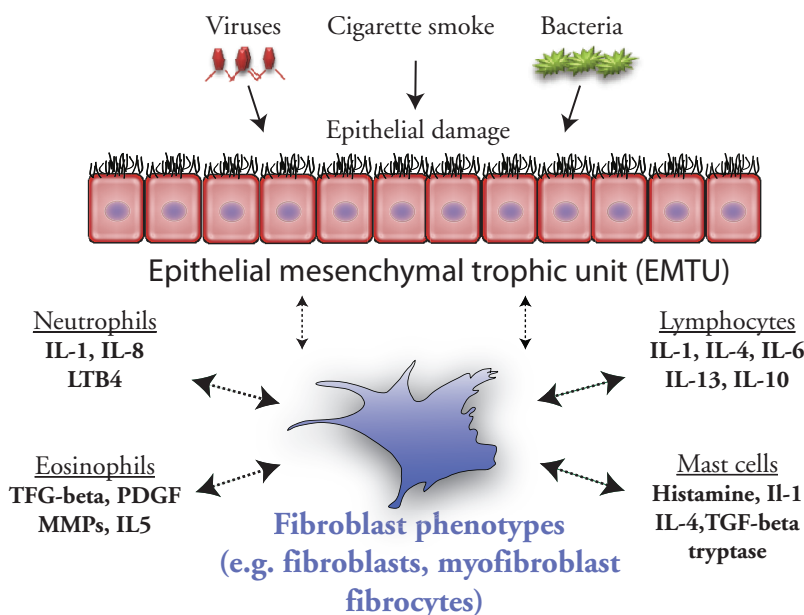
This hypothesis has been difficult to prove but in a recent published study in the journal of clinical investigation this was investigated. By examination of plastic-adherent cell populations in bronchoalveolavage samples obtained from 76 human lung transplants, fibroblast like cells were obtained in 62% of the samples. These cells expressed vimentin and prolyl-4-hydroxylase. Importantly, by multiparametric flow cytometric analyzes revealed expression of the cell surface markers CD73, CD90 and CD105 which are markers commonly found of bone marrow MSC. Furthermore, these cells were negative for the hematopoietic markers CD14, CD34 and CD45. What was the evidence that these cells were tissue- resident? Cytogenetic analysis of cells from 7 sex-mismatched

lung transplants recipients harvested up to 11 years after transplantation revealed that the sex genotype were expressed in the donor.

In addition when compared the BAFL derived cells versus the MSCs from the same subjects, affymetrix analyses revealed that several chemokines were more highly expressed (such as IL-8, CXCL2 VEGF, FGF2) in the MSC. This suggests that MSCs derived from these different tissue environments reflect distinct gene expression profiles consistent with their origin and functional roles<sup>88</sup>

## Fibroblasts and the crosstalk with immune cells

Pathological changes that occur in chronic diseases involve the adaptive and innate immune system in combination with connective tissue alterations. It was recently suggested that conserved pathogen-associated molecular pattern (PAMPs) found in viruses, bacteria and fungi help to maintain myofibroblasts in a activated state<sup>89;90</sup>. Furthermore, activation of the epithelial-mesenchymal trophic unit (EMTU) causes cytokines such as IL-4, IL-5, IL-13 and CXCL8 (IL-8) to be generated as part of the inflammatory response. This environment promotes fibroblasts to stay activated which further contribute to the pathogenesis of asthma and COPD<sup>1;91;92</sup> (Fig 7).



**Figure 7** Activation of the epithelial mesenchymal trophic unit (EMTU). Viruses, bacteria and pollution are factors that potentially could activate the epithelium at different localization in the lung. This starts an extensive crosstalk between the epithelium and the mesenchymal cells beneath the epithelium. In this process, inflammatory cells contribute and communicate with to fibroblast activation by releasing growth factors such as TGF- $\beta_1$ , IL-13 and PDGF. Different fibroblast phenotypes (fibroblasts, myofibroblasts and fibrocytes) all participates in this crosstalk

*Interleukin 4 and IL-13 crosstalk*

IL-4 and IL-13 are molecules that affect both the structural cells (fibroblasts and epithelial cells) and inflammatory responses. Both cytokines are produced by activated CD4<sup>+</sup> cells and are critical for the promotion of allergic and remodeling responses. IL-4 is primarily involved in promoting the differentiation of T helper 2 cells (Th2) and which leads to activation of B-cell and subsequently synthesis of immunoglobulin E (IgE). It has also been shown that GAGs bind and store IL-4 and could therefore modulate cellular responses of this cytokine<sup>93</sup>. IL-13 has a critical role in mediating AHR, mucus hypersecretion and remodeling<sup>94</sup>.

Despite different mechanism of action, there are important features of these cytokines. One difference between the two molecules is that IL-4 binds to two receptor complexes whereas IL-13 only binds one of these complexes<sup>95</sup>. IL-4 bind type I receptor which is a heterodimer of IL-4R $\alpha$  chain and the  $\gamma_c$  chain and the type II receptor, which is a heterodimer of IL-4R $\alpha$  and IL-13R $\alpha$ 1. IL-13 does not bind IL-4R $\alpha$  but only IL-13R $\alpha$ 1 and can therefore only activate type II IL-4 $\alpha$ . Overexpression of IL-13 in the lung triggered subepithelial fibrosis where additional inflammatory stimulus was absent<sup>96</sup>. One hypothesis suggest that IL-13 signals through the IL-4R $\alpha$ /Stat6 pathway, but studies by Blease *et al* and Fichtner-Feigl *et al* suggests that IL-13 could instead signal through IL-4 $\alpha$  independent pathways in models describing fungal asthma and bleomycin induced fibrosis<sup>97;98</sup>. In the latter study, the authors, found that IL-13 induce TGF- $\beta$ <sub>1</sub> in macrophages through induction of the IL-13R $\alpha$ <sub>2</sub>. This mechanism was dependent of IL-13 (or IL-4) and tumor necrosis factor (TNF)- $\alpha$ .

*Granulocytes and macrophages*

In allergic and acute inflammatory situations, granulocytes such as eosinophils, mastcells and neutrophils play a role in the fibroblast/inflammatory cell interaction. Eosinophils which are prominent cells of in allergic asthma are released from the bone marrow as CD34 precursors and recruited to the airways by prostaglandin D<sub>2</sub>, cysteinyl leukotrienes and cytokines such as IL-5. They are an important source of fibrogenic cytokines, including TGF- $\beta$ <sub>1</sub> and IL-13<sup>43;92;99</sup> (Fig 7). The capacity of the IL-5 to bind to the HS chains on the GAGs enables the ECM to function as a reservoir for sustained eosinophilic presence, activation and survival which could promote fibrotic events<sup>100</sup>. Although the eosinophils role in allergic asthma may be less important, their role in airway remodeling may be of importance. It has been suggested to play a role in pathological remodeling where IL-5 can amplify fibrotic events rather than work as direct mediators in fibrosis<sup>101-103</sup>.

Another chemo-attractant for eosinophils, eotaxin was shown to be up-regulated in human asthma fibroblasts when exposed to both IL-13 and TGF- $\beta$ <sub>1</sub> alone or in combination. Eotaxin would then recruit eosinophils to maintain the remodeling response<sup>104</sup>. Eosinophils have also been suggested to stimulate fibroblast migration and proliferation<sup>105;106</sup>. The hypotheses that eosinophils can regulate TGF- $\beta$  response (in mice) link the fibroblasts to remodeling events. Interestingly, in a report from Shen

*et al* the peptidyl-prolyl- isomerase (PPIase) Pin1 in human eosinophils was suggested to be important since the Pin1 promoted the stability of TGF- $\beta_1$  mRNA. By selective blockade of Pin1 in a rat asthma model, eosinophilic pulmonary inflammation, collagen expression, TGF- $\beta_1$  and airway remodeling was reduced<sup>107</sup>.

Mast cells have for long time been associated with asthma, however their role in COPD has not been clarified. Mastcells interacts with smooth muscle cells and beside the production of mediators such as leukotriene LTD<sub>4</sub>, prostaglandin PGD<sub>2</sub> and histamine they also contribute to fibrogenesis and an increase in smooth muscle as part of the remodeling response<sup>10;108;109</sup>. Both mast cells and eosinophils are important sources of the zinc-dependent matrix metalloproteinases (MMP-3, MMP-9), which through their degradation of matrix proteins and proteoglycans are important in remodeling of the airways<sup>110;111</sup>. It has been suggested that the differentiation and function of myofibroblast is regulated by mast cell mediators such as tryptase. By blocking the proteolytic activity of tryptase with specific inhibitors, a fibrotic response was reduced<sup>112-114</sup>. Interestingly, TGF- $\beta_1$  has shown to be very chemotactic to mastcells which suggest a positive feedback loop in the activation of the fibroblast-mast cell interaction<sup>115</sup>. Mast cells are general divided into tryptase and tryptase/chymase positive cells but emerging data now suggests that subpopulation within each compartment of these cells exists in human lung<sup>116</sup>. This could have impact on remodeling features since it is likely that these subtypes have different properties and therefore could influence the fibroblast differently.

A cell that participate in both Th1 and Th2 responses is the macrophage. Macrophages are involved in both the innate and the adaptive immune responses depending on which cytokines they are exposed to. IFN- $\gamma$  and LPS induces a phenotype that aim to destroy microorganism and exposure of IL-4, IL-13 induces a proliferative and collagen production phenotype. With this function, macrophages are likely to play a role in both asthma and COPD<sup>117</sup>. Macrophages are capable to produce fibroblast activation cytokines such as IL-1 and TGF- $\beta_1$  thus play an important role in the fibroblast crosstalk. The close interaction of Th1 cells and macrophages is required to change the macrophage into an activated macrophage which could fight intracellular and extracellular bacteria and viruses. Importantly, the interferon- $\gamma$  produced by the Th1 locally inhibits fibroblast activation, collagen deposition and proliferation<sup>118;119</sup> (Fig 7)

The chemotactic signals produced by neutrophils make an important contribution to the recruitment, activation and programming if the antigen presenting cells (APCs) such as macrophages and DCs in inflammatory response. The principal contribution of neutrophils to wound healing is microbial sterilization by engulfing the bacteria. However, the capacity of the neutrophils to release proteases and subsequently tissue breakdown is believed to play a role in pathological remodeling in severe asthma and COPD (Fig 7)<sup>1;120</sup>. Interleukin-8 is the major chemotactic substance for neutrophil migration and subsequently accumulation of these cells at site of infection. Importantly, binding of IL-8 to GAGs can be competed with soluble GAGs such as heparin sulphate, chondroitin sulphate and dermatan sulphate. The soluble GAGs inhibit the binding of the chemokine ligands to their receptors which could have impact on the inflammatory



response<sup>121</sup>. In addition, neutrophils also produce tumor necrosis factor and interferon- $\gamma$  which help to drive dendritic, macrophage and T cells activation.

## Smooth muscle cells

Structural cells such as the airway SMC have a pivotal cell in regulating the bronchomotor tone and the acute bronchoconstrictions. However, despite this role, the airway SMC also secrete immunomodulatory mediators such as VEGF, IL-8 and TGF- $\beta_1$  in response to stimuli which affect the connective tissue remodeling and eventually lung function<sup>122;123</sup>. SMCs express cell adhesion molecules that interact with inflammatory cells such as T-cell and mast-cell. Furthermore, SMC also have the capacity to express TLR-2 and TLR-4. This expression could be of importance in regulating hyperresponsiveness since microbacterial products such as lipopolysaccharid (a major component of the external membranes of gram-negative bacteria) have the capacity to modulate airway SMC hyperresponsiveness<sup>124</sup>. It has also been suggested that human airway SMC increase their proinflammatory responses in response to rhinovirus<sup>125</sup>. The close interaction with the tissue matrix illustrates the wide function of the airway SMC<sup>122;123;126</sup>. Furthermore, the ECM may have an important role in persistent lung diseases since components such as fibronectin and collagen I increase human airway SMC mitogenesis in response to platelet derived growth factor-BB and thrombin whereas laminin inhibits proliferation. The increase in cell proliferation was accompanied by a decrease in expression of SMC contractile proteins such as  $\alpha$ -actin, calponin and myosin heavy chain suggesting that ECM could modulate smooth muscle phenotype<sup>127</sup>.

A common histopathological manifestation reported in fatal asthma is an increase in airway SMC mass which was reported in the 60s. The mechanism for the increased airway SMC mass is primarily due to hyperplasia and not hypertrophy and new evidence suggests that increase in airway SMC mass occur even in patients with mild asthma

128-130.

## Epithelial phenotypes and origin

The adult human bronchial tree is covered with a continuous layer of epithelial cells that play a role in maintaining and integrity of the lung<sup>131</sup>. The dominating celltypes are ciliated, columnar, undifferentiated, secretory and basal cells. In normal adult lung, these subpopulations vary as function of airway level with decreased number of cartilage cells, submucosal glands and the apparent of Clara secretory cell as the airway branch from large to small airways<sup>132</sup>. In the large airways (Branch<sup>0</sup> to Branch<sup>5</sup>) the



most abundant cells are the ciliated, undifferentiated columnar, secretory and basal cells. The ciliated epithelial cells are the predominant cell type within the airways accounting for over 50% of all epithelial cell and originate from either basal or secretory cells<sup>133</sup> (Fig 2). Within the epithelium, the basal cells are the only cell that is tightly attached to the basement membrane and it is thought that basal cells are the primary stem cells that give rise to mucous and ciliated epithelial cells. In addition to these properties. In the small airways (B<sup>6</sup> to B<sup>23</sup> branches) there are similar cell types but more Clara cells instead of secretory cells. The Clara cells are believed to be the primary stem cell in the smaller and alveolar compartments. After B<sup>23</sup> branches, the airway epithelium merges with the alveolar epithelium constituted by type I and type II cells.

The epithelial sheet does not function as an independent unit but more as a functional unit with the epithelial cells, mesenchymal cells, endothelial cells and the extracellular matrix in bronchial walls. Repeated environmental and airborne exposure in epithelial cells makes them vulnerable for further pathological change in major lung diseases including COPD, asthma and bronchogenic carcinoma<sup>131;134;135</sup>. Epithelial cells have the capacity to generate a wide variety of different mediators from the epithelial cells (e.g. IL-6, IL-8, Granulocyte-s macrophage colony stimulating factor (G-CSF), IL-1, IL-16, IL-2, CCL5 (RANTES), TNF- $\alpha$  and TGF- $\beta$ ) which influence cells such as T-cell, eosinophils, neutrophils and fibroblasts for subsequently recruitment and activation<sup>136-139</sup> (Fig 2, 7). The function of epithelial/fibroblast interactions has been investigated in co-culture systems where epithelial cells are chemically or physically damaged. 48 hours after insult, the fibroblast release growth factors including endothelin, insulin growth factor and basic fibroblast growth factor which are all involved in proliferation, migration and matrix production<sup>140-142</sup>. This is also manifested by changes of the basement membrane that functions as a base for the epithelium regeneration and as a structural barrier for the subepithelial fibroblasts<sup>131</sup>. Another molecule produced by epithelial cells is nitric oxide (NO) which participates in the host defense and in the pathogenesis in a number of airway diseases. Asthmatics as well as individuals with COPD have increase exhaled NO levels but the exact mechanism and what effect it has on disease development is not known<sup>131;143;144</sup>.

Epithelial cells also respond to various stimuli, such as environmental, viruses, bacteria and mechanical and have the ability to induce host specific defend mechanisms. Viral respiratory infections, especially respiratory syncytial virus are associated with the majority of asthma exacerbations in children and adult<sup>145-147</sup>. A similar association can be seen in COPD where respiratory virus is linked to exarcebations<sup>148</sup> (Fig 7).

# Connective tissue remodeling and inflammation

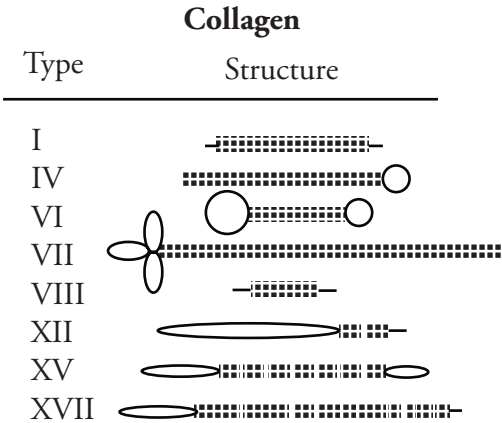
The established description of the extracellular matrix is that it forms a framework and supports the cells by stabilizing the tissue and preserving the architecture. However, the matrix is very active and participates in both normal and pathological processes. The matrix has major influence on structure, viability and function of cells. In diseases such as cancer, fibrosis and atherosclerosis, the matrix changes is now consider to play an important role in combination with inflammatory cells. Fibroblast in various cancer types are the cell types that have been studied and support the changes of matrix composition in pathological situations<sup>131;149</sup>. By definition, the matrix is composed by two classes of the matrix molecules 1) The glycosaminoclycans (GAGs), which are polysaccharide chains that are covalently linked to proteins and 2) the fibrous protein such as collagen, elastin, fibronectin and laminin<sup>150</sup>. Importantly, in chronic diseases and in tissues which are under mechanical tension the connective tissue is continuously changed with a high turnover rate. In this context, the balance of matrix metalloproteinases, which cleaves the extracellular matrix, vs. the ECM production from mesencymal cells could play a critical role when distinguishing between physiological and a pathological response. The next section will briefly describe the major components of the ECM and their biological effect in remodeling and inflammation.

## Collagen

Collagens are trimeric molecules composed of three polypeptide chains which contain the sequence  $(\text{Gly-X-Y})_n$  where X are proline and Y hydroxyproline. Due to its small size, glycine allows narrow turns. Proline can get hydrated and from intra- and inter-chain hydrogen bonds that keep the structure together. The characteristic structural feature of collagen is the repetition of this sequence which allows the formation of a triple helix. Up to 23 different combinations of the  $\alpha$ -chains (collagen type I-XIX) have

been identified. Beside the triple helical domains (CD), collagen contain non triple-helical (NC) domains<sup>151;152</sup>.

Depending on their capability to form organized fibers collagen can be divided into two major classes, the fibril forming collagens (such as I, II, III and XI) and the non-fibrillar collagens (such as IV and IX). There are distinct differences between these groups and one difference is that in the fibril forming collagens almost the entire length of the molecule is constituted by a single CD domain whereas the nonfibrillar collagen molecules contain one or several CD and NC domain of variable size depending on which collagen (Fig 8). The fibril collagen form fibrils which form fibres and finally fibre bundles that are the main providers of mechanical support and resistance to the ECM<sup>153</sup>. In the synthesis of collagens, the enzymes prolyl-4-hydroxylases (P-4-OH) have a central role. The 4-hydroxyproline residues generated by the endoplasmic reticulum (ER) luminal collagen are essential for the stability of the collagen triple helix. The hydroxylation of 4-hydroxyproline residues is catalyzed by collagen P-4-OHs hence could be used as a marker for collagen production in cells such as fibroblasts and fibrocytes (paper II). Prolyl-4-hydroxylases are cytoplasmic and nuclear enzymes that exist in three isoenzymes differing in their catalytic alpha subunit. One of the prolyl-4-hydroxylase families, the HIF P-4-OHs, hydroxylates specific prolines in the alpha unit in hypoxia inducible transcription factor (HIF) that regulating oxygen homeostasis<sup>154</sup>.



**Figure 8** Schematic representation of typical collagen molecules. Dotted area: triple helical domain, circles and ellipses: Non triple helical domain (NC). Adapted from Aumailley *et al*<sup>152</sup>

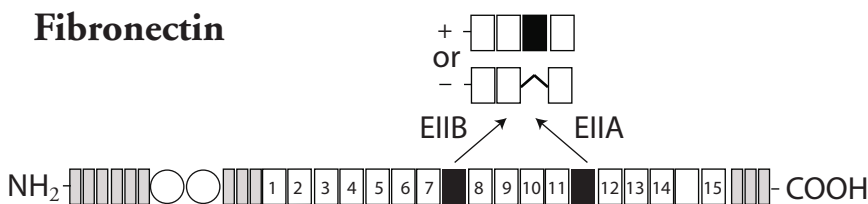
Characteristic of feature in patients with asthma is the remodeling which correlates with disease.<sup>31;155</sup> Histological *in vivo* studies revealed that patients with asthma had an enhanced deposition of collagen I, III compared to non asthmatic subjects whereas another study showed decreased expression of collagen IV<sup>156;157</sup>. Collagen I which is the most abundant protein in humans is produced and deposited by asthma fibroblasts and smooth muscle cells following TGF- $\beta_1$  stimulation<sup>158;159</sup>. Furthermore, collagen fragments could have both pro inflammatory or anti inflammatory properties<sup>160,161</sup>. It has been shown that degraded lung collagen can have the same function as IL-8 and is che-

motactic for lung neutrophils. When mice were long term exposed of this fragment, the mice developed enlargement of the alveoli and increased levels of VEGF. Interestingly, this demonstrates that in the absence of inflammatory cells, the matrix has the capacity to induce chronic inflammation<sup>162</sup>

## Fibronectin

Fibronectin (FN) is a large plasma glycoprotein that is composed of three different domain types: FNI, FNII and FNIII that in combination form one subunit. The functional molecule consists of two identical subunits of 220-250 kDa. Briefly, fibronectin exists in the circulation as plasma fibronectin which is secreted by hepatocytes directly, and cellular fibronectin which is found in the extracellular matrix. The cellular fibronectin have the alternatively spliced EDA and EDB sequences and EDA is believed to play a major role in the differentiation of a fibroblast to a myofibroblast phenotype<sup>26;163</sup> (figure 9). The link between EDA fibronectin and myofibroblast differentiation *in vivo* has been recently demonstrated using the bleomycin model of lung fibrosis. In EDA  $-/-$  mice receiving bleomycin, lung fibrosis was completely prevented<sup>164</sup>.

Today approximately 20 different variant of fibronectin exists in humans<sup>163;165</sup>. Apart from the role in fibroblast differentiation, it has been shown to stimulate the production of cytokines (RANTES, GMSCF and eotaxin) SMCs, mediate migration of fibroblast and small airway epithelial cells<sup>166-172</sup>. Another function of the fibronectin molecule is the ability to bind cytokines that can be rapidly released hence a gradient for the recruitment of inflammatory cells<sup>173;174</sup>. Asthma and COPD individuals have change in their fibronectin levels. For example, fibronectin levels measured by immunohistochemistry is increase in the airways of asthmatics and fibroblasts from COPD patients have reduced migration capacity toward fibronectin and reduced contraction capacity in three dimensional collagen gels compared to controls which propose that fibroblasts have an impaired wound healing capacity<sup>158;175</sup>.



**Figure 9** A schematic representation of the splicing of fibronectin pre-mRNA. The 3 different domains (I, II and III) and the number of each type III domain is shown in the boxes. The extra alternative splice repeat (EIIA and EIIB) are excluded. Adapted from C. ffrench-Constant<sup>165</sup>

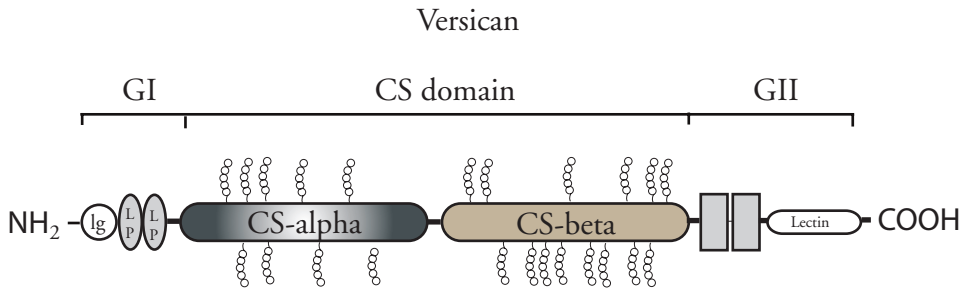
# Proteoglycans

In chronic inflammation a dynamic crosstalk takes place where primarily fibroblasts, but also other mesenchymal cells, serve as a scaffold by secreting proteoglycans and collagens to form a network. The important role of proteoglycans in diseases such as kidney fibrosis, asthma, COPD, cancer and systemic sclerosis (SSc) is well documented at both cellular and physiological levels<sup>176-181</sup>. Proteoglycans have a core protein with one or more covalently attached glycosaminoglycan (GAG) chain(s). These GAG chains are heavily sulphated and influence their behavior and role in the connective tissue. Depending on their structure, proteoglycans could further be subdivided based on the GAG chains that are attached to their protein core and their localization in the tissue<sup>182</sup>. Briefly, depending on the GAG chain structure, the proteoglycans are divided into four groups: chondroitin sulfate (CS)/dermatan sulfate (DS), heparin sulfate (HS)/heparin, keratan sulfate and hyaluronan<sup>182</sup>. The proteoglycans could further be subdivided into: Basement membrane proteoglycan (e.g. perlecan, agrin), the small leucine-rich proteoglycans, SLRPs (e.g. decorin, biglycan, fibromodulin, lumican) and the hyalactans (versican, aggrecan, neurocan). However, by sequence many of the core proteins have revealed that there is a high diversity in the proteoglycan family. This has been shown where members such as biglycan and decorin (see below) are expressed as isoforms either with or without GAG chains<sup>183, 184</sup>.

In this thesis the production of the four main proteoglycans secreted from fibroblasts derived from asthma and COPD patients have been analyzed. These proteoglycans are: Versican, Perlecan, Biglycan and Decorin.

## *Versican*

Versican (265-370kDa) is a member of the large aggregating CS family but also has DS. It is associated with tissue injury, plays a critical role in determining the water content and influence tissue viscoelastic behavior and is also expressed during development. Versican belongs to the hyalactan family that have derived its name due to its ability to bind hyaluronan (by its N terminal domain) and lectin (by versican C-terminal region)<sup>182</sup>. Structurally, the versican molecule has two globular domains, the N-terminal G1 domain and the C-terminal G2 and the attachment sequence for CS. Furthermore, the CS sequence separated by two alternative spliced domains (CS $\alpha$  and CS $\beta$ ) that could generate four different isoforms VO (CS $\alpha$ +CS $\beta$ ), V1 (CS $\alpha$ ), V2 (CS $\beta$ ), V3 (Non of the isoforms) (fig 10)



**Figure 10** Versican which is a CD/DS proteoglycan are schematic presented in this figures. Ig: Immunoglobulin type repeat, LP: Link protein, GAG $\alpha$  and GAG $\beta$  are the glycosaminoglycan binding domains where alpha and beta are the two alternative spliced variants. Lectin = lectin-like module. Adapted from Iozzo<sup>182</sup>

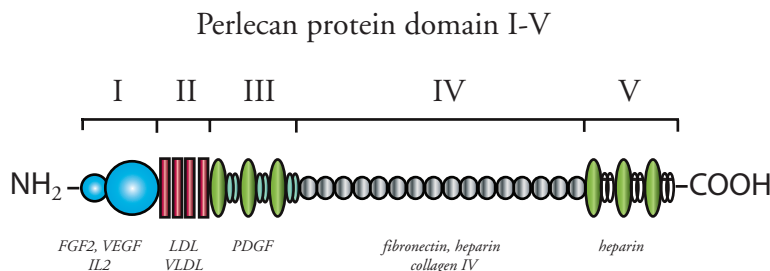
In a study by Huang *et al* where endobronchial biopsies were obtained from patients with mild atopic asthma the expression of versican was significantly increased in the subepithelial zone. Interestingly, the deposition of versican in the smooth muscle in moderate and severe asthmatics were altered which suggest that versican play a role in the function of airway smooth muscle cell<sup>186</sup>. It so also suggested that versican play an important role in maintaining lung elasticity by inhibiting elastic binding protein (EBP) and thus interfere with assembly of the elastic fibers hence direct interfere with lung capacity. The molecular mechanisms for this is not known but it is proposed that the high negative charge in versican (due to the high chondroitin/dermatan sulphate content) is responsible for this phenomena<sup>179</sup>. In response to TGF- $\beta_1$  and platelet derived growth factor fibroblast and SMCs upregulate versican production<sup>187;188</sup>. In SMCs this upregulation can be prevented by treating the cells with the tyrosine inhibitor genistein<sup>188</sup>. The role in cancer has been described in human colon cancer where abnormal versican expression is a feature and stroma of various tumours<sup>189;190</sup>. By RNA interference technology, knockdown of versican expression significantly inhibited tumor growth *in vivo* but not *in vitro*<sup>191</sup>.

### Perlecan

Perlecan is a large HS (400 kDa) multi-domain extracellular matrix proteoglycan and member of the basement membrane family proteoglycans. This proteoglycan is predominantly expressed in the basement membrane, but it also play a role in developmental processes, wound healing, cartilage formation and is produced by variety of cells including fibroblasts, smooth muscle cells, endothelial cells and epithelial cells<sup>192-196</sup>. Perlecan is composed of 5 protein domains and the GAG sites are located in the N-terminal domain. The protein domains individually bind molecules such as FGF2, VEGF, IL-2, laminin-1 and collagen type IV (unit I), VLDL, LDL (unit II), PDGF (unit III), fibronectin, heparin, collagen IV (unit IV) and heparin (unit V)<sup>196</sup> (Figure 11).

These different molecules display the broad functions of perlecan. It is likely that perlecan have an important function in angiogenic mechanisms since perlecan null

mouse have vascular abnormalities<sup>197</sup>. Theoretically, heparanase- mediated release of FGF2 and VEGF would cause persistent activation of these pathways which are important in angiogenesis and restricted release would lead to the opposite effect. In centrally derived fibroblast from COPD patients, perlecan production is decreased compared to controls (Paper IV).

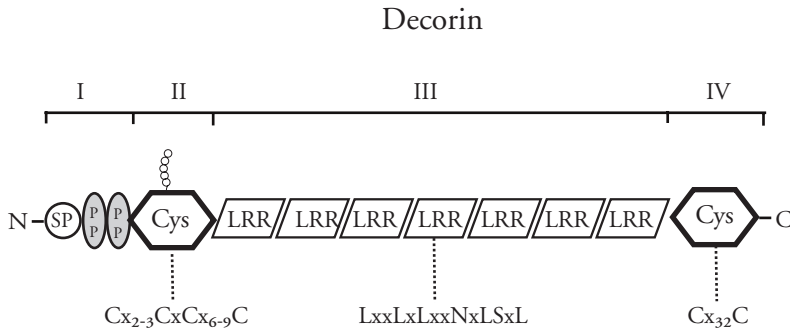


**Figure 11** Schematic model of human protein core. The 5 protein domains and the GAG sites are located in the N-terminal domain. The protein binds molecules important for inflammation and airway remodeling. Adapted from Iozzo<sup>192</sup>

### *Small leucine- rich proteoglycans (SLRPs)*

Two members of the class I SLRPs family are biglycan and decorin. There are additional 4 classes which individual members all fulfill the criteria to be a SLRPs. Briefly, The basis for the classification are: leucine rich, conservation, homology (protein and genomic levels), the presence of N-terminal Cys clusters and chromosomal organisation<sup>198</sup>. Decorin and biglycan are CS/DS proteoglycans with a coreprotein (40kDa) showing an amino acid homolog of 57%<sup>185</sup>. At the N-terminal of biglycan, two CS/DS chains are substituted while decorin only is substituted with only one CS/DS chain (figure 12).

Regardless of their similarity, they have different function *in vivo*. For example, decorin is generally down regulated by TGF- $\beta_1$  it binds and neutralizes TGF- $\beta_1$  activity whereas biglycan is up regulated by TGF- $\beta_1$ . It has been shown that decorin and in a recent study, recombinant human decorin inhibited TGF- $\beta_1$  induced contraction of collagen lattice<sup>199-201</sup>. This have also been confirmed in animal models where induction of fibrosis can be inhibited by decorin treatment<sup>202;203</sup>. Decorin could therefore potentially be used as an antifibrotic treatment in lungfibrosis, cancer and other fibrotic diseases. When human smooth muscle cells were cultured on plates coated with decorin a decrease in proliferation and increase rate of apoptosis was observed. Furthermore, biglycan did not affect smooth muscle proliferation or apoptosis. Interestingly, decorin induced decrease in cell number could be restored when decorin coated matrices were exposed to the enzyme that degrades GAG chains (chondroitinase ABC)<sup>204</sup>. In combination with platelet derived growth factor-BB and epidermal growth factor, TGF- $\beta_1$ , biglycan production from fibroblast was increased.



**Figure 12** Schematic representation of decorin which is a member of the small leucine rich proteoglycans. Its 4 domains are separated by domain I: SP= signal peptide and the propeptide (PP), domain II: Cys= cysteine rich regions, domain III: leucine rich repeats and domain IV: Cys, The small circles represents the CS/DS side chain. Biglycan has the similar size of the protein core but have two CS/DS side chains. Adapted from Iozzo<sup>182</sup>

Different proteoglycans are expressed in various timeframes in a disease. This was studied in bleomycin induced fibrosis where biglycan increase early in the inflammatory response to induce an “inflammatory matrix”. This expression was in contrast to decorin which was increased later in the fibrotic development<sup>177</sup>. Importantly, when adding biglycan to fibroblasts *in vitro* the fibroblasts acquire a more migratory phenotype (inflammatory) supporting a possible role of biglycan in early remodeling events<sup>53</sup>

## Transforming growth factor- $\beta$

A large number of mediators produced by many different cell types are known to promote fibroblast proliferation, collagen synthesis, migration and differentiation (e.g. Fibroblast growth factors, Tumor necrosis factor- $\alpha$ , endothelin-1, platelet- derived growth factor, epidermal growth factor, insulin growth factor, IL-1 and IL-13). Among these, TGF- $\beta_1$  is the most potent pro-fibrotic player and is a key player in many diseases such as asthma, fibrosis and cancer<sup>1;205-209</sup>. Five TGF- $\beta_1$  isotypes are present in mammals, but only 1-3 are present in humans and these share 60-80% homology but are encoded by different genes. These 3 isotypes bind to the same receptor. The biological activities of the different isotypes are the same but the link to fibrosis and aberrant wound healing processes is primarily due to activity of TGF- $\beta_1$ . In the tissue, the primary source of TGF- $\beta_1$  is probably macrophages and eosinophils, but also fibroblasts and epithelial cells have the capacity to produce TGF- $\beta$ . It is produced in a latent form consisting of TGF- $\beta$  and the non-covalently bound latency-associated protein (LAP) that must be released for activation (LAP is derived from the N-terminal of the TGF- $\beta$  precursor). Binding of TGF- $\beta$  to its receptor requires the dissociation from LAP and



this process is catalyzed by agents such as plasmin, integrin  $\alpha v\beta 6$ , metalloproteinases and heparin<sup>210-213</sup>. LAP-TGF- $\beta_1$  is usually secreted as a large latent complex consisting of LAP-TGF- $\beta_1$  that is covalently bound to a latent TGF- $\beta$ -binding protein (LTBP)<sup>214</sup>. It has been suggested that these protein modulate the TGF- $\beta$  response. Deficiency of LTBP-4 in mice results in defective elastin structure, developmental abnormalities, emphysema and colorectal cancer<sup>214</sup>. In addition to the cellular TGF- $\beta$ , it has also been shown that inactive TGF- $\beta$  could be bound in the connective tissue molecules such as trombospondin-1, decorin, fibronectin, elastin, and some collagens<sup>214</sup>.

Following the binding of active TGF- $\beta$  to the receptor, signals are transduced through Smad pathways and a number of non-Smad pathways. Briefly, ligand binding to the serine/threonine kinase TGF- $\beta$  receptor II (TGF- $\beta$ RII) leads to phosphorylation of TGF- $\beta$ RI<sup>215</sup>. There are a number of TGF- $\beta$ RI (also known as ALK (activin-receptor-like kinase)). ALK is not necessary for the binding but is important for the initial signal event. One of the ALKs, ALK5 is known to be involved in TGF- $\beta_1$  signaling. Smad2 and Smad3, then gets phosphorylated and form a complex with Smad4. This complex translocates into the nucleus and bind to DNA which will activate or repress gene expression. In contrast, Smad7 inhibit ALK5 signaling. Target gene expression and cellular responses are also modulated via activation of PI3K and various MAPK pathways that are Smad independent<sup>210;216</sup>. However, a continuously crosstalk take place and the true nature of the signaling pathways remains to be elucidated.

TGF- $\beta$  has a unique and essential function in regulating immune responses and differentiation of cellular subtypes. In general TGF- $\beta$  inhibit proliferation in most of the immune cells including Th1 cells, Th2 cells, macrophages, NK cells, B cells, prevents dendritic cell maturation, but induce both differentiation of Th17 cells and T regulatory cells (Treg)<sup>209;217;218</sup>. However, it has bipolar properties e.g. TGF- $\beta$  can either increase or decrease apoptosis of lymphocytes depending on its phenotype and stage of differentiation<sup>217</sup>. Simultaneously, TGF- $\beta$  is a very chemotactic and induces migration and accumulation of leucocytes such as macrophages, granulocytes, eosinophils and mast cells. The local environment, dose, the specific cell phenotypes and stage of disease are all factors that determine the final role of TGF- $\beta$ . Modification of the TGF- $\beta$  system could be critical in disease development.

# The inflammatory response in asthma and COPD

Inflammation in asthma and COPD is a hallmark of the diseases, but the cellular and molecular response is different in the diseases. However this view is challenged when patients with severe asthma and asthmatic subject who smoke during exacerbation have similar responses as in COPD (Fig 1). Classically, asthma has been regarded to be Th2 (CD4<sup>+</sup>) skewed disease where the link to allergy, recruitment of eosinophils and release of IgE from mast cells play a major role in disease development. On the other hand, COPD has been considered to be a Th1 (CD8<sup>+</sup>) type disease where the repetitive exposure to cigarette smoke induce trafficking of neutrophils and macrophages to inflammatory sites<sup>1</sup>. Both diseases then give rise to distinct pattern of chronic inflammation.

## The inflammatory response

That Th2 driven inflammation is important in asthma is well established. The first line of defense is the innate immune system where cells such as macrophages, immature dendritic cells (DCs) and natural killer (NK) cells respond in selective ways.

The innate immune response is not that unspecific as originally thought and it recognizes motifs that are commonly found on foreign antigen or altered self antigen from bacteria, fungus, parasites and viruses<sup>219</sup>. A group of receptors, pattern recognition receptors (PRRs) that are important for these missions are the Toll like receptors (TLRs). Based on their primary sequences, TLRs can be subdivided into subfamilies which recognize related pathogen associated molecular patterns (PAMPs). One family recognizes lipids (TLR1, -2, -6) and another family recognizes nucleic acids (TLR7, -8, -9)<sup>220</sup>. TLRs are also localized in the cell membrane (TLR1, -2, -4, -5) and in intracellular localization (TLR2, -7, -9). The primarily role is to as pro inflammatory activators

which produce cytokines such as interferon (IFN), CXCL10 and CCL3 which attract immature DCs<sup>221</sup>.

At site of inflammation, the dendritic cells engulf the antigen and undergo maturation. Downregulation of some receptors (CCR1, CCR5, and CCR6) and upregulation of CCR7 allows the DCs to migrate to the local lymph node via the interaction of CCR7 and its corresponding cytokine CCL21<sup>222;223</sup>. Upon arrival to the lymph node, the DC present their antigen to T cells in the T-cell area. The non matured t-cells are recruited to the lymph nodes via high expression of L- selectin (CD62L) which is present on specialized post-capillary venules, high endothelial venules (HEV) <sup>224</sup>. Importantly, matured T-cells (memory cells, activated lymphocytes) do not express L-selectin. In the lymph follicles, B-cells search for antigen and after antigen binding, they interact with T-helper cells to get activated, form germinal centers and subsequently production of specific B-cells.

Simultaneously, monocytes migrate into the wounded area, differentiate into macrophage further enhance the local response by engulfing the antigen and increase the cytokine production. The cytokines produced activates the structural cells such as the fibroblasts in the tissue remodeling process. The trafficking of lymphocytes to the inflamed tissue involves the emigration from the bloodstream via tethering, rolling and escape from the blood vessel (diapedesis). It is believed that the homing pattern of the lymphocytes is uniquely dependent upon the anatomical location of the lymph node where the T cell was activated<sup>219</sup>.

Activated T-cells can be polarized to either T helper Th1 or Th2 cells. These different subtypes produce Th1 and Th2 specific cytokines. Th2 cells are typical IL-4, -5 and 13 producers while IFN- $\gamma$ , IL-10 and IL-22 are typical for Th1 cells. Moreover, they also have separate chemotactic properties due to their different expression of receptors (Th1: CCR5, CCR3, P-selectin, E-selectin and Th2: CCR4, CCR8 and CXCR4).

## Asthma and COPD immunology

### Asthma

With the allergen exposure and allergic (IgE mediated) sensitization of Th2 cell CD4+ cells it was believed that the mechanism behind asthma was elucidated. This mechanism is still very important, however recent studies of asthma have demonstrated that <50% of asthma cases have feature of eosinophilic airway inflammation which suggest that a non-eosinophilic asthma phenotype exists which is suggested to be TLR related<sup>225;226</sup>.

In atopic asthmatics, the primary cells involved in the Th2 response are eosinophils, mastcells, dendritic cells and the B-cells. Importantly, as the disease becomes more severe and chronic, the presence of Th1 cells is also a feature<sup>227</sup>. Other subtypes of CD4 cells that may participate in asthma regulation are the T regulatory cells (Treg) and the

Th17 cells which are believed to regulate Th2 cell function and linked to neutrophilic inflammation, respectively<sup>228;229</sup>. Mast cells are also associated with asthma and following an inhaled allergen provocation the mast cells release preformed granules that host mediators such as histamine, tryptase, heparin, cytokines, newly formed eicosanoids (PDG<sub>2</sub>), leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>). These mediators contract smooth muscle cells and increase microvascular permeability thus being important in asthmatic reactions<sup>230</sup>. Another cell that is suggested to participate in asthma pathology is the eosinophil as previously described (*chapter: Asthma and asthma phenotypes*). In non-allergic asthma, endotoxins, particulates pollutants and viruses are believed to activate epithelial cells and macrophages which will produce IL-8 and subsequently recruitment of neutrophils. This condition is thought to play a major role in the persistence of severe asthma phenotypes<sup>231</sup>.

## COPD

The cigarette smoke will mainly activate the epithelium and macrophages will produce/release factors that attract inflammatory cells in the lung. The primary cellular composition in COPD patients are Th1 cells, macrophages, neutrophils, Th1/Tc1 (Tc = CD8<sup>+</sup>) and fibroblasts<sup>1</sup>. The presence of the macrophages and neutrophils are believed cause degradation of the elastin when these cells release proteases and matrix metalloproteinases in close proximity to the epithelium. IFN- $\gamma$  produced by the subsets of T cells macrophages and epithelial cells resulting in a persistent inflammatory activation<sup>232</sup>. Cigarette smoke activates the epithelium which produces cytokines and molecules contributing to the pathogenesis. The cigarette smoke also enhances production of MMPs by macrophages where MMP-2, MMP-9 and MMP-12 are involved in the pathogenesis of emphysema. The balance of MMP and its inhibitor TIMP has also been suggested to be impaired in favor of MMP which would promote emphysema<sup>233;234</sup>. Furthermore, Tc-cells have been shown to contribute to COPD pathogenesis by participate in the emphysema development<sup>235;236</sup>. However, today no clear mechanism has been provided which could explain the full pathogenesis of COPD.



# Present investigations

## Aims

The overall aim of this thesis was to explore the origin and different fibroblast phenotypes in the human lung and if these have altered properties in disease. To receive further insight into these questions, the aims of the thesis were as follows:

- 1) Are the fibrocytes, which are believed to be a fibroblast progenitor cell present in BALF and tissue section from asthmatics and COPD individuals, compared to controls and could their presence be correlated to structural changes?
- 2) Are there any chemotactic molecules or mechanisms that could explain the presence of activated fibroblast phenotypes in the BALF which could explain why resident fibroblasts migrate into the lumen during pathological conditions?
- 3) Have fibroblasts from different localizations (central and distal) within the human lung from rhinitis-, asthma- and COPD subjects adapted disease- and localization related phenotypes as measured by proteoglycan profiling and proliferative properties?



# Methods

The major methods used in this thesis were as follow:

## Characterization of subjects

### *Control subjects (paper I-IV)*

The control subjects (n=5 paper I and n=3 paper II, respectively) were 24-51 years of age, non-asthmatic, healthy individuals, no allergic symptoms and who did not respond to metacholine lower than 2 mg/ml. In these studies, the bronchial biopsies were collected from the central right lung.

From healthy controls in paper III and IV, fibroblasts were obtained in 12 cases. Seven of the controls were non-atopic and 5 were atopic, defined as confirmed sensitization to one or more allergens (birch, timothy, and/or mugwort, dog, cat, host dust mite). Atopic subjects without respiratory symptoms but with confirmed hyper- responsiveness to methacholine was excluded. Central control fibroblasts were sampled from central airways (third generation in central airways) and distal fibroblasts were sampled from parenchymal airway (transbronchial biopsies  $\approx$  1 inch from the pleura).

### *Characterization of patients with asthma (paper I-III)*

The disease group in study I and II groups consisted of patient suffering from mild asthma and define bronchial hyperresponsiveness which fulfilled the criteria of the American Thoracic Society (ATS guidelines). The subjects were rather young, 25-40 years of age,  $PC_{20} < 2$  mg/ml of metacholine stimulation and displayed asthma symptoms. The subjects had no glucocorticosteroid treatment 6 months prior the study and all of the subjects were atopic and sensitive to pets. In addition, five of the patients with asthma had perennial allergy and one patient had seasonal allergy (birch pollen). The asthma group was further subdivided into two groups whether or not they displayed fibroblasts derived from their BALF (BALF fibroblast). In study I, 9 patients were include (5 with fibroblasts and 4 without fibroblast) and compared to 5 healthy controls. In study II, 6 asthma subjects were included (3 with fibroblasts and 3 without fibroblast and their results were compared to controls (n=3).



Of 13 asthma subjects enrolled in study III, fibroblasts were established from 11 of them (7 centrally derived fibroblasts and 11 distally derived fibroblasts). These individuals were asthma patients with defined bronchial hyper-responsiveness defined as  $PD_{20} < 2000\mu\text{g}$  methacholine. All individuals were free from infection, had stable asthma symptoms and did not use any corticoid treatment 3 months prior the start of the study. Central and transbronchial biopsies were obtained from all subjects.

BAL was performed by flushing the airways with up to 140 ml of 0.9 M NaCl, and the resulting fluid was used for analysis.

#### *Characterization of patients with COPD (paper IV)*

In study IV, patients ( $n = 8$ ) suffering from severe smoke-induced COPD (GOLD stage 4) who were undergoing lung transplantation at Lund University Hospital were included in the study.

## Cell culture

Fibroblasts were isolated from controls and patients with asthma. Bronchial (central) and transbronchial (distal) biopsies were cut and cultured in 25 cm<sup>2</sup> tissue culture flasks, with DMEM supplemented with 10% Fetal Clone III, 1% L-glutamine, 0.5% gentamicin, and 5  $\mu\text{g}/\text{ml}$  amphotericin. The cell cultures were kept in a humidified 37°C cell incubator with an atmosphere of 5% CO<sub>2</sub> and were trypsinized when they reached confluence. All cells were used passage 3-5.

Fibroblasts from COPD patients were obtained as follows: Tissues from central airways (bronchus) and from lung parenchyma were dissected from COPD lungs and were kept in DMEM supplemented with 10% FBS, Gentamicin, PEST, and Amphotericin B on ice until further processing. Bronchial pieces were removed from the luminal side of bronchi and were cut into small pieces. Vessels and small airways were removed from the peripheral lung tissues and the remaining tissues were chopped into small pieces. After rinsing, bronchial and parenchymal pieces were allowed to adhere to the plastic of cell culture flasks for 4 h and were then kept in DMEM with the supplements mentioned above in 37°C cell incubators until outgrowth of fibroblasts were observed. Distal fibroblast cultures were achieved from all patients, while central fibroblasts were achieved from 6 of 8 COPD patients. Bronchial and parenchymal fibroblasts were then referred to as central and distal fibroblasts, respectively. Experiments were performed in passage 3-6. In some cases cells were frozen before use but this did not result in changes in phenotypes defined as proteoglycan production, proliferation or expression of mesenchymal cell markers.

Fibroblast cells from BALF were isolated by separating the cells from the lavage fluid at 500 g and then immediately culturing them in 25 cm<sup>2</sup> tissue culture flasks containing supplemented DMEM. After 5-6 days, the outgrowth of fibroblast-like cells was

observed. To yield a pure fibroblast population from the separated BALF cells, the cells were trypsinized and used in passage 5-7 for the studies.

## Fibrocyte and fibroblast identification

### *Paper I and II*

Fibrocytes in tissue and BALF were stained for hematopoietic markers such as CD34, CD45, and CXCR4 in combination with mesenchymal markers such as Prolyl-4-hydroxylase, pro-collagen, vimentin and  $\alpha$ -SMA. For cultured BALF fibroblasts, cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% Triton x-100 prior intracellular staining such as  $\alpha$ -SMA. Followed by the incubation with the primary antibody appropriate secondary antibodies were applied on relevant chamber or tissue section for 1h incubation and wash prior nuclear staining. Control experiments were performed with and without primary antibody, and with and without secondary antibody. Control sections were included in all experiments to correct for background fluorescence. Isotype controls were used in combination and for each fluorochrome and adequate blocking steps were performed to avoid unspecific staining.

### **Immunofluorescence, confocal**

In the two first papers, multiple probes were analyzed by the use of confocal microscopy. By using laser with different wavelength and appropriate optical filters, multiple emission wavelengths could be analyzed in parallel and co-localization could be recorded. This approach was specifically adapted when analyzing triple stained tissue section and to avoid so-called “spectral overlap” where the emitted signal from one probe overlaps with an emitted signal from a secondary probe hence creating “false positive” results. Classical fluorescent workstation was also used where mercury lamp emits light which after reflected at the specimen pass through a filter that separate the different wavelength. The major difference between these systems is the ability of the confocal microscopy to create 3D images by changing its specific focus on a plane. In addition to this, the confocal microscopy limits the exposure of the specimen source by specific control its focal plane. However, both systems are fully appropriate to use in the studies.

## 2D gels and Mass spectrometry

### Sample preparation and two-dimensional electrophoresis

Cells were washed in PBS and scraped in solubilization solution containing 7 M urea, 2 M thiourea and 2% CHAPS, and stored at -70°C until further use. Immobiline DryStrips (180mm, pH 4-7) were rehydrated in 350 ml of the solubilization solution with sample. Prior to the rehydration, 10 mM DTT and 0.33% IPG pH 4-7 buffer was added to the samplesolution. The isoelectric focusing (IEF) step was performed at 20°C in a Multiphore II according to the following procedure: 300 V 1 minute, 3500 V 25 hours until approximately 85 kVh were reached. The strips were equilibrated for 10 minutes in 65 mM DTT, 6 M urea, 30% w/v glycerol, 2% w/v SDS and 50 mM tris-HCl pH 8.8 (equilibration buffer). A second equilibration step was performed for 10 minutes in 259 mM iodacetamide inequilibration buffer. The strips were soaked in electrophoresis buffer (24 mM Tris, 0.2 M glycine and 0.1% SDS) and run on 14% homogeneous duracryl slab gels. Gel electrophoresis was performed using a Hoefer DALT gel apparatus at 20°C and 100 V for 18 hours. Gels were stained by silver nitrate and scanned using a Bio-Rad GS-710 gelscanner (Spot analysis was performed using the PDQuest 7.01 2-D gel analysis system. Each spot on the gel was given an integrated optical density (IOD) value by the software that was compared to the total amounts of spots. Each spot is therefore referred to as ppm of the total IOD of all valid spots.

### MALDI-TOF mass spectrometry

Gel pieces were washed with 50 mM ammonium bicarbonate buffer followed by three rounds of acetonitrile. The excised spots were treated overnight with 70 ng/ml trypsin, acidified with 0.5% TFA and the peptides were applied through Zip-Tip columns to an Anchor-chip™ plate with 2 mg/ml of DHB (2,5-dihydroxybenzoic acid) matrix. The MALDI-TOF instrument used was a Bruker REFLEX and operated in reflector mode at an accelerating voltage of 20 kV.

Spectra were analyzed and gels were compared with an online BALF protein database hosted by expasy.org as well as with proteins identified in a previous study (*Paper II*).

### Western Blot

Equal amounts of protein samples were loaded for all western blotting which was performed. Primary polyclonal sheep anti-human haptoglobin antibody was added follow-

ing addition of the secondary antibody (polyclonal rabbit anti-sheep antibody conjugated with horseradish peroxidase). The intensity of the optical density of the bands on the membrane was measured using Gel-Pro Analyzer 3.0 (*Paper II*)

## Proteoglycan measurement

### *Versican, Perlecan, Biglycan and Decorin (Paper III and IV)*

Fibroblast cells from non asthmatics (controls, atotics), asthmatics and COPD subjects were cultured. Cells were cultured in 6- well plastic plates and allowed to grow until confluence. COPD fibroblasts had a very contractile phenotype and experiments were therefore performed on cell culture plastics coated with 1% collagen-1. Prior the experiments, the serum level were lowered to 1% for 2h to adjust cells to a low serum milieu. The cells we then incubated with [<sup>35</sup>S]-supplemented (50 µCi/ml) sulfate-poor DMEM for 24 hours supplemented with 0.4% FBS w/wo 10ng/ml TGF-β<sub>1</sub>. Unincorporated [<sup>35</sup>S] was washed out with buffer. Labeled [<sup>35</sup>S]-proteoglycans were separated on a DEAE-52 cellulose column equilibrated with 6M urea, 50 mM sodium acetate, 5 mM N-ethylmalimide, 1 mM EDTA, and 0.5% ovalbumin, pH 5.8. Hyaluronan was then removed from the gel using a higher acetate concentration (0.5M) and the proteoglycan was further eluted with 4 M guanidinium hydrogen chloride, 50 mM acetate, and 0.5% ovalbumin, pH 5.8. Total [<sup>35</sup>S]-labeled proteoglycan was quantified using a scintillation counter. Aliquots of the PG elucidates that equaled a volume to 10.000 dpm were precipitated for at least 24 h in 95% ethanol supplemented with 0.4% Sodium Acetate and 100µg dextran and 25g CS6, Dried PG pellets were solubilized in TRIS- buffer. Individual proteoglycans (versican, biglycan, perlecan, and decorin) were separated on a 3–12% polyacrylamide gel, analyzed and adjusted for total cell protein (BCA protein assay kit). The proteoglycans isolated had been identified previously both by western blot and mass spectrometry MALDI-TOF- MS

## Proliferation assay

### *Paper III and IV*

Cells were plated on 96-well plates (5,000 cells/well) and cultured in 10% serum for 6, 24, and 48 hours. They were fixed in 1% glutaraldehyde for 30 min and stained thereafter for 30 min in 0.1% crystal violet. The excess crystal violet was then gently removed by washing with water. The cells were then lysed overnight with 1% Triton-

X100 and absorbance was measured at 595 nm. Data are presented as ratio to the expression of the crystal violet after 6h.

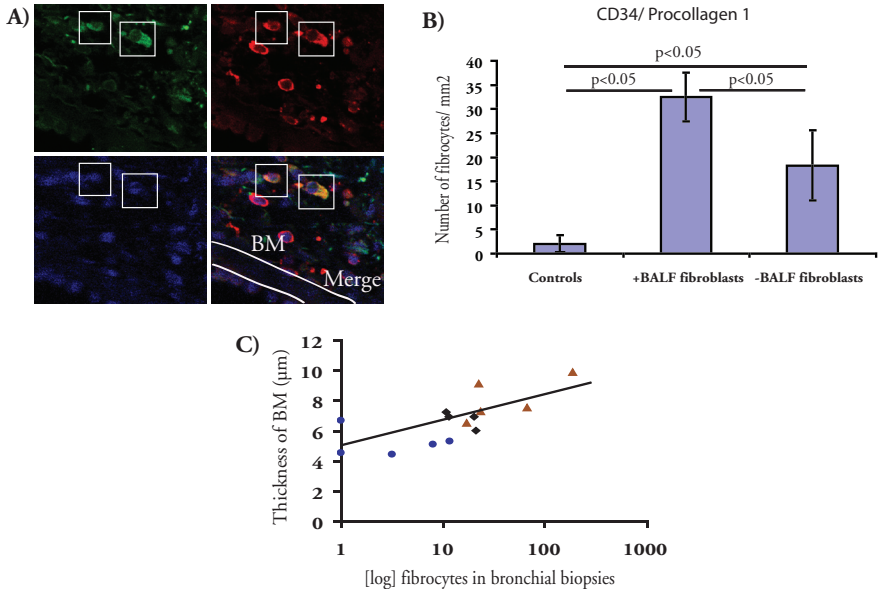
# Results and comments

## Paper I

*Tissue fibroblasts in patients with mild asthma: A possible link to thickness of the reticular basement membrane?*

Despite the capacity of fibroblasts to produce matrix molecules thus affecting cellular response and lung mechanisms the origin of these cells are still not established. A possible origin is from blood derived fibrocytes that have the potential to differentiate into matured mesenchymal cells. In this paper the presence of fibrocytes in patients with mild asthmatic was investigated. By immunofluorescence fibrocytes were identified in bronchial biopsies with antibodies directed to the hematopoietic markers CD34 and CD45RO in combination with the mesenchymal markers  $\alpha$ -SMA, procollagen I and prolyl-4-hydroxylase (figure 13a). Mild asthma patients were divided into two categories based on whether or not fibroblast like cells could be established from BALF. Based on these different subtypes we hypothesized that patients with BALF fibroblast (these patients also had increased eosinophils in BALF) would have increased numbers of the fibroblast progenitor cells, fibrocytes, beneath the basement membrane. Furthermore we hypothesized that presence of these cells could correlate to structural changes such as thickness of the basement membrane. Three different antibody combinations (to detect the fibrocytes) were used and in all case the presence of fibrocytes were significantly higher in patients with BALF fibroblasts compared to controls and patients without BALF fibroblasts. Patients with BALF fibroblasts displayed a 17-fold increase of CD45RO/prolyl-4-hydroxylase positive fibrocytes beneath the basement membrane compared to controls (figure 13b). Importantly, the increase of this antibody combination was also noticed in asthmatics without BALF fibroblasts, although the numbers were still significantly lower compared to asthmatics with BALF fibroblasts. This suggests that fibrocytes are present in all mild asthma individuals and that their presence can be enhanced upon ongoing inflammation. Furthermore, basement membrane thickness could be correlated to the number of fibrocytes. This can be viewed as an indication of pathological remodeling in the tissue and demonstrates that presence of

fibrocytes could influence the composition of the basement membrane (figure 13c). The findings in this paper suggest that even mild asthmatics have signs of remodeling and that this possibly could be estimated by the presence of fibrocytes.



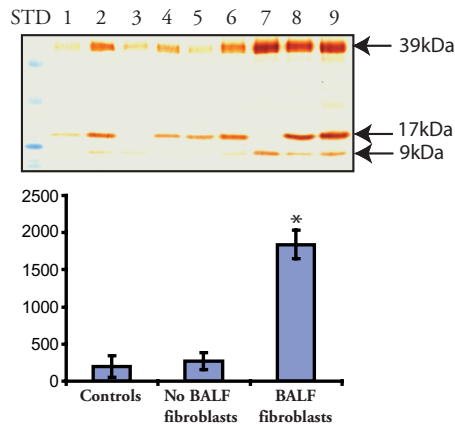
**Figure 13** A) Example of staining combination used in the present study to identify fibrocytes. Biopsies were stained against CD34 (green), pro-collagen (red) and nuclei (blue) and positive cells are positioned (in white boxes) close to the basement membrane. B) Quantification of CD34/pro-collagen I positive cells in asthmatic patients and controls. C) Basement membrane thickness correlates to thickness of the basement membrane in the individuals. *Definition av abbreviations:* BM = Basement membrane, +BALF fibroblasts = asthma patients with fibroblasts in their BALF, -BALF = asthma patients without fibroblasts in their BALF, BALF = Bronchoalveolar lavage fluid, Filled brown triangles = patient with BALF fibroblasts, Filled black squares = patients without BALF fibroblasts, Filled blue circles = controls.

## Paper II

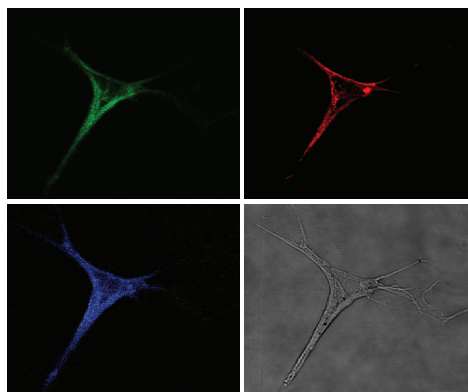
### *Specific haptoglobin expression in bronchoalveolar lavage during differentiation of circulating fibroblast progenitor cells in mild asthma*

In this study we hypothesized that the fibroblasts present in BALF in mild asthmatics are fibrocytes and that these cells have changed their phenotype as judged by analysis of the BALF proteome. The discovery that fibroblasts could emerge in the BALF in combination with the presence of fibrocytes beneath the basement membrane in mild asthmatics started this study<sup>38;41</sup>. By the gel-based proteomic approach regulation of proteins in the BALF as measured by optical density were calculated. Among the

expressed spots the four haptoglobin- $\beta$  domains were the most highly up-regulated peaks from the haptoglobin spectrum showed peptide fragments covering 33% of the haptoglobin- $\beta$  domain including regions near both the N- and C-termini. This up-regulation was further validated with Western blot protein expression and a 2.5 fold increase of the haptoglobin- $\beta$  domain was observed in BALF from asthmatic patients with BALF fibroblast compared to controls ( $p < 0.05$ ) (Fig 14). Importantly, the BALF fibroblasts were positive for the fibrocytes markers CD34, CD45 and  $\alpha$ -SMA (17%) confirm the hypothesis of recruitment of progenitor cells (figure 15). Importantly, asthmatics without fibroblast in their BALF and controls did not express the haptoglobin-domains suggesting that the fibroblast in these patients participates in disease specific mechanisms.



**Figure 14** Protein expression after Western Blotting for haptoglobin in BALF from controls (lane 1-3), asthmatic subjects without BALF fibroblasts (lane 4-6) and asthmatics with BALF fibroblasts (lane 7-9). The haptoglobin domains are marked as follows: 39 kDa  $\beta$ - domain, 14 kDa and 9 kDa are the  $\alpha$ 1 and  $\alpha$ 2 domain respectively. Values are presented as means  $\pm$  SEM for  $n=3$ /group.



**Figure 15** Fibrocytes in BALF. The fibroblasts were stained with antibodies against the fibrocyte markers CD34 (green), CD45RO (red) and  $\alpha$ -SMA (blue) and subjected to confocal microscopy. Transmission image are shown to visualize the cell.



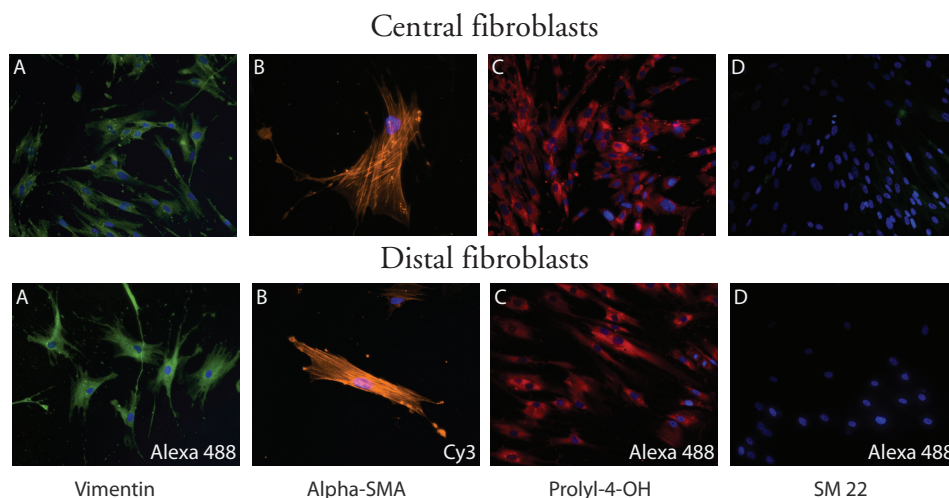
## Paper III and IV

### *III Altered matrix production in the distal airway of asthmatic and atopic individuals*

### *IV Altered fibroblast proteoglycan production in COPD and possible effects on pathogenesis*

The major role of the fibroblast is to produce matrix and maintain the tissue homeostasis. With the novel findings that fibroblast could originate from localizations such as the bone marrow (fibrocytes) we wanted to investigate whether we could obtain fibroblast different phenotypes based on their localization (central and distal) within the human lung and if these cells are altered in disease situations. In these two studies, proliferation from healthy controls, atopic individuals, patients with asthma and COPD patients were compared in proliferation capacity, proteoglycan production. Furthermore, the effect of TGF- $\beta$  stimulation on the proteoglycan production from the fibroblasts was also investigated.

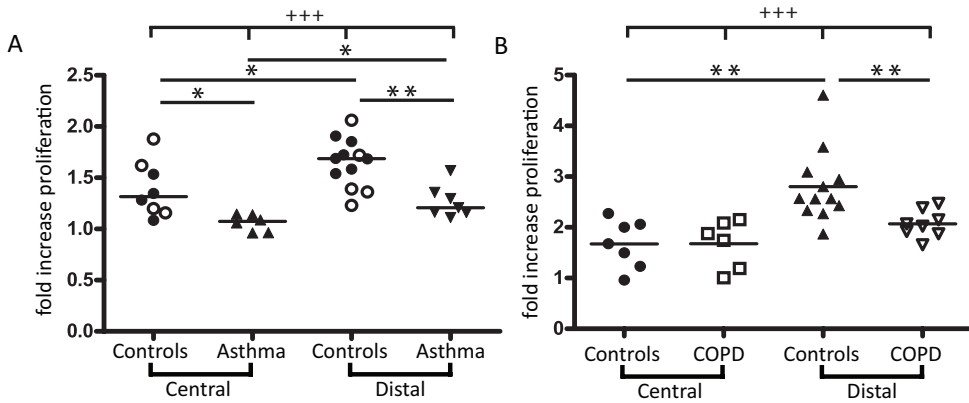
The absence of smooth muscle cell marker SM22 in the cultured fibroblast from controls, patients with asthma and COPD patients suggests that we obtained a homogenous fibroblast population and no contamination of smooth muscle cells in the culture (figure 16)



**Figure 16** Characterization of distal fibroblasts. The figure illustrates asthma distal fibroblasts expressing fibroblast (vimentin, prolyl-4-hydroxylase) and myofibroblast ( $\alpha$ -SMA) proteins, but absence of SM22 which is a sensitive marker of smooth muscle cells. Fibroblasts from COPD patients displayed the same expression

In this study, proliferation rate from asthmatics was reduced compared to controls which suggest that the fibroblast have acquired a “resident, spindle shaped” fibroblast phenotype which produce matrix. This reduction in proliferation was not observed in atopic individuals or in COPD subjects. By compare fibroblast proliferation from central and distal localization, distal control fibroblasts have significantly increased their

proliferation capacity compared to central fibroblast. This was apparent in controls and asthma. Importantly, distal fibroblast from both asthma and COPD had reduced capacity compared to distal control fibroblast suggesting a disease related phenotype shift in proliferation capacity (figure 17a and b)

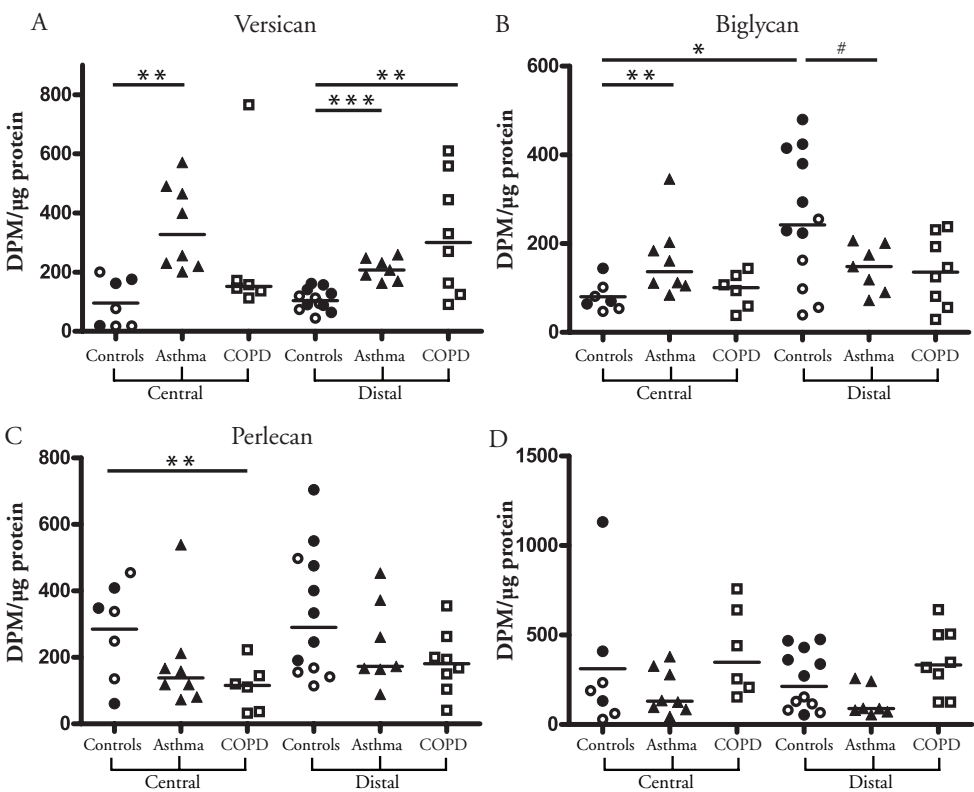


**Figure 17** Proliferation capacity of fibroblasts from central and distal localization from controls, atopics, patients with asthma and COPD were investigated. 24hours proliferation was calculated in A and 48hour in B respectively.

Increasing interest has been directed to the fibroblast ability to produce extracellular matrix molecules such as proteoglycans that could modulate the local environment. To test if the fibroblast from the subjects had changed their proteoglycan production, versican, perlecan, biglycan and decorin was measured. Versican production from central asthma and COPD fibroblasts was increased compared to controls (figure 18a). In addition, the versican production from distal asthma fibroblasts was significantly elevated in compared to controls. Versican and its spliced variants have been suggested to influence cellular responses such as proliferation and tissue elasticity by affecting the elastin binding protein. Production of biglycan from centrally derived asthma fibroblasts, and this was in contrast to atopic individuals and COPD patients where the levels were not changed compared to controls (figure 18b). Production of the basement membrane proteoglycan, perlecan, was significantly decreased in COPD patients. This phenomenon could not be seen in the asthma or atopic subjects. This suggests that the proteoglycan profiling from fibroblasts in various diseases are different and need to be considered when interpreting remodeling of ECM (figure 18c). In addition to this, lung function parameters such as  $PD_{20}$  and bronchial flux were correlated to production of individual proteoglycans in centrally- and distally fibroblast. This suggests that individual proteoglycan production can be linked to hyperreactivity and NO production in distal airways and not only in central airways as previously described<sup>237</sup>

To elucidate the role of TGF- $\beta_1$  on the proteoglycan production, TGF- $\beta_1$  was administered to the cells. Although no significance increase in any of the individual proteoglycans could be measured from atopic or asthma subjects, the result demonstrate that

fibroblast from these patient groups response to TGF- $\beta_1$  and the differences from basal level persist. This suggests that despite absence of TGF- $\beta_1$ , fibroblasts from in these patients have the capacity to alter the ECM. Importantly, centrally derived COPD fibroblasts did not respond to TGF- $\beta_1$  stimulation as distal COPD which propose that these cells are not that sensitive to growth factor thus partly lost their reparative potential.



**Figure 18** Proteoglycan expression from non asthmatic controls (atopics- open circles, controls- closed circles), patients with asthma and COPD patients. The significances in the figure described difference when comparing asthma and COPD versus non-asthmatics (controls).

# General discussion

This thesis demonstrates the complexity of the human lung when consider the ECM and its contribution to chronic pathological airway remodeling. Moreover it establishes that different fibroblast phenotypes are present in various geographical localizations which could have a major impact on disease development in chronic diseases.

Matrix has classically been regarded to be a quiescent element. The major function has been to construct the scaffold for the inflammatory cells and to maintain the integrity. With this view, the fibroblast was often forgotten when experiments and inflammatory responses were presented and discussed. However, the lack of treatments that fully restore lung function has forced the scientists to re-think and the ECM is now regarded to play a significant role in chronic lung diseases. As the major ECM producing cells, fibroblasts are now considered to be key cells in pathological remodeling processes. The possibility to use fibroblast phenotypes and their proteoglycan expression as read-out of the fibrotic status would give a tremendous insight into the fibrotic status of the individual.

In the first two papers the hypothesis of fibroblast progenitor cells in mild asthma patients was investigated. At first, the presence of fibrocytes was investigated. Fibrocytes express several inflammatory surface molecules that are important in the recruitment of fibrocytes to sites where they are needed but also have the ability to produce matrix proteins<sup>42;75</sup>. These dual functions make these cells interesting as possible target to control remodeling. The presence of fibrocytes close to the basement membrane suggests that they have been recruited and differentiate into a matrix producing cell<sup>41</sup>. By addition TGF- $\beta_1$  to purified peripheral blood fibrocytes these cells lose expression of their hematopoietic surface molecules and increase production of mesenchymal proteins. However, it is not known if these cells have unlimited proliferation and differentiation capacity. But does it really matters? The patients used in the first two papers had increased numbers of eosinophils and neutrophils in the BALF fluid. This suggests that an ongoing inflammation recruit these cells to specific environment. In theses situation, on ongoing recruitment of fibrocytes potentially could harm the host in same way as a fully differentiated fibroblast. Fibrocytes also express several types of the TLR receptors (TLR-2, TLR-4 and TLR-7) and perhaps some of these individuals have an asthma phenotype that by be linked to an innate immune response, as judged by the neutrophils.

The presence of fibrocytes in the BALF in study I and II suggests that the cells are recruited into the lumen by some chemotactic gradient. Candidates for this recruit-

ment are chemokines such as SDF-1 produced by epithelial cells and newly discovered lysophosphatidic acid and its corresponding receptor LPA1. This could explain the large increase of fibrocytes beneath the basement membrane compared to controls<sup>82,238</sup>. It is tempting to speculate the role of these cells in the airway lumen but their presence is likely to be pathologic since these cells cannot be found in BALF from healthy individuals. Furthermore, mesenchymal like cells in the BALF have also been found in more fibrotic diseases such as systemic sclerosis (SSc) and IPF<sup>239,240</sup>. In addition to this the observation, the increased presence of fibrocytes in asthma patients with BALF fibroblast compared to asthma patients without BALF fibroblast suggest different asthma endotypes. Presence of these cells could theoretically therefore be used as a predictor for future remodeling events.

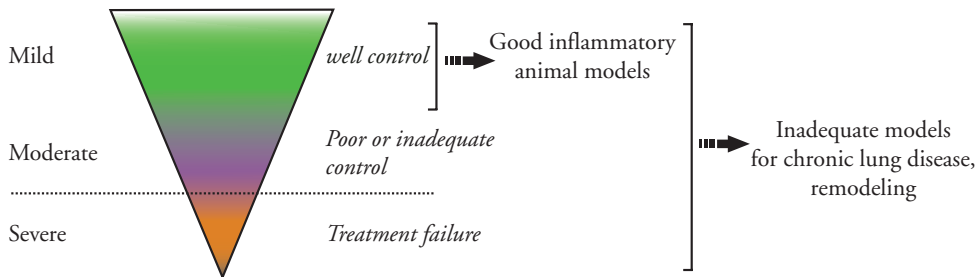
The hypothesis that fibrocytes may contribute to pathological remodeling received attention since investigations where bone marrow MSCs were administered in various animal models was partly disappointing. Even though labeled MSCs are found in the bleomycin exposed lung, the cells cannot be found in peripheral blood when searching for MSC surface antigen. This suggests that the administered mesenchymal stem cells do not express bone marrow MSC surface antigens. A possible explanation might be that labeled cell are a subtype of MSC, e.g. fibrocytes<sup>66,241,242</sup>. Furthermore, presences of the MSC in human lung are very few. It is therefore reasonable to question current animal models and if they truly represent the clinical situations.

The most used animal model to study fibrotic disease is bleomycin induced fibrosis. However this model has many disadvantages to clinical settings. The major weakness is that fibrosis in this model is self-limiting and the exposed mice/rats will recover after administration (if not a lethally dose will be administered). Basically, bleomycin, this fully reversible model, is used as a model for clinical non-reversible lung diseases such as IPF. It is therefore more likely that this model correspond to acute inflammation. In addition, the fact that not a single compound has been accepted for use in human (in 20 years), but all have failed the clinical trials also suggests that the use of this model as a representative fibrosis model needs to be re-evaluated<sup>243</sup>. However, if the protocols could be modified thus include repetitive administration and lower concentration the bleomycin this model might be representative to clinical situation. Not to forget, bleomycin is frequently used in treat cancer such as squamous cell carcinomas and has historically been an important molecule in the inflammatory research.

Study remodeling events in asthma situations have mainly been focused on OVA-albumin inflammation or LPS induced lung injury. These models have similar disadvantages as bleomycin but not that extensive. Their strength is the importance in acute and immediate situations where allergen or bacterial exposure induces an inflammatory reaction. This has highly clinical relevance and the roles of these models are and have been important in characterizing inflammatory events. Nevertheless, the protocols used have primarily not been design to study chronic events and it will be necessary to design new protocols. This chronic exposure has been considered in host-dust mite models which have described chronic remodeling events<sup>244</sup>. And even though patients with

moderate and severe asthma symptoms are under poorly or inadequate controls no animal model exists to shed light on the mechanisms in these diseases (Fig 19).

As a consequence, it is importance to encourage scientists to start translational research which would give a new dimension to the results. In this thesis, only human primary fibroblasts and tissue has been used. Despite low n-numbers and large heterogeneity of the samples it is necessary to continue research on primary samples to achieve more knowledge of the clinical situations. One commonly used argument in obtaining fibroblast from biopsies is that the cells do not represent the fully heterogeneity of structural cells in the lung. The possibility that the cells have changed their phenotype has also been stated. However, despite these arguments, differences can be measured in the fibroblasts cultured from controls as well as patient with asthma and COPD.



**Figure 19** Overview of asthma phenotypes, how they are controlled and how relevant the current animal models are to represent true disease, modified from<sup>12</sup>

Why are remodeling necessary to study in COPD? Scientists have for many years studied the inflammation (influence from inflammatory cells) as a major target for treatment of patients with COPD. However, reduce/diminish the traditionally inflammatory response may not have the same effect on the tissue repair and mechanisms driven by the matrix producing cells such as the fibroblasts. The matrix changes are also a part of the inflammation and needs to be considered in all inflammatory events, acute or chronic. Patients with COPD have predominantly areas of emphysema where the loss of lung structure will have an enormous impact on lung function and quality of life. However, areas of fibrosis are also present making this disease less homogenous as initialized thought. Thus it is highly motivated to study and compare the matrix production from the fibroblast derived from different localizations and from diseases such as asthma and COPD.

Collagen is the most abundant molecules in the human lung and therefore also the most studied molecule when investigation fibrosis and emphysema. However, presence of collagen should only be used as a guideline to further go deeper into the connective tissue composition and role in lung. We therefore studied the proteoglycan production from fibroblasts derived from individuals with asthma and COPD. Proteoglycans are molecules that consist of a core protein and substituted with GAG side chains. Proteoglycans can be very abundant and at cell surface of epithelial cells, syndacan-1 is highly expressed and cartilage have a high density of proteoglycan, mainly aggrecan.

Despite their wide expression throughout the human body, very little is known about their role in adult connective tissue homeostasis. The developmental biology and the cancer research have contributed with importance messages that these molecules have essential roles during normal and pathological conditions. It is therefore reasonable and important to describe presence and function of these molecules in chronic lung diseases as well. GAGs are highly sulphated which enabled the GAGs to selectively interact with chemokines thereby forming gradients for recruitment of cells. In study III and IV, the fibroblast from asthma and COPD patients produce different levels of the versican, perlecan, biglycan and decorin compare to controls. These differences suggest that the cells are producing a “disease” specific proteoglycan pattern which potentially could be used as a fingerprint.

Asthma, has for many years been considered to be bronchial disease and the increase of the versican and biglycan production from central derived asthma fibroblasts are confirming the immunohistochemical investigation presented by others<sup>180,245</sup>. The role of the increased versican production is not known, but it is suggested to give stability in the tissue. It is a large and very charged molecule which could bind water. In this way, the increased versican production would be a secondary effect to compensate for something else such as repetitive epithelial injury which eventually ends up in breaking the epithelial barrier. Indeed, increased thickness of the basement membrane suggests that the EMTU compartment is altered.

Biglycan production is also increased in central asthma fibroblast and this is in contrast to the centrally derived COPD fibroblasts that not are changed compared to controls. On the other hand, the decreased perlecan production from centrally derived COPD fibroblasts suggests that basement membrane composition in central airways is effected in COPD patients. Perlecan, which is a heparan sulphate proteoglycan, is expressed in the lamina densa in the basement membrane. Perlecan has been shown to bind basement membrane protein including laminin and collagen IV. Alterations in the basement membrane composition may therefore be important for the basement membrane integrity in adults and subsequently activation of the EMTU. Even though COPD is regarded to be a small airway and distal airway disease the united airway hypothesis suggests that pathological events in central airway may have an effect on other localization in the lung.

By investigating the proteoglycan production in distal airway from asthma and COPD subjects interesting comparisons can be accomplished. The increase of distal versican production in both asthma and COPD may have important relevance for the clinical situations. In COPD it has been proposed that versican due to its high chondroitin sulphate/dermatan sulphate content could be negative for the lung elasticity by inhibiting elastin binding protein (EBP) and thus interfere with assembly of the elastic fibers. This suggests that enhanced versican production can be regarded to be pathological in these clinical situations. Importantly, the COPD patients in the study are GOLD IV and these cells are under pressure to re-create lost tissue architecture whether in mild asthmatic, the patients are not believe to have the same effects. This comparison postulates that despite different diseases (asthma and COPD) the fibroblasts try to restore



the altered connective tissue back to normal composition by mechanisms that could be quite similar. It is tempting to speculate that the sulphating levels of the proteoglycan could have an impact on the properties of the proteoglycans and subsequently on their mechanisms.

One molecule that is known to have an effect on proteoglycan production is TGF- $\beta_1$ . Its effect on cultured fibroblast was therefore investigated. In the controls, TGF- $\beta_1$  induced an increase in proteoglycan production in both central and distal fibroblasts. However, fibroblasts from patients with asthma did not further increase their proteoglycan production compared to controls. This suggests that TGF- $\beta_1$  is not required to induce an altered proteoglycan production. This was in contrast to fibroblast from COPD patients and indicates that COPD fibroblasts might have been exposed to TGF- $\beta_1$  in the patients thus are primed to TGF- $\beta_1$  stimulation.

By studying the proliferation capacity from the central and distal fibroblasts, important information of the fibroblast phenotype can be obtained. The decrease in proliferation rate in central asthma fibroblasts compared to controls suggests that the fibroblast have changed its phenotype, adapting a more resident, contractile and matrix producing cell. The increased versican expression described earlier strengthens this hypothesis. On the other hand, the increased biglycan production suggests that the connective tissue have adapted a more inflammatory composition. Previously published data have shown that the SLRPs such as biglycan can induce morphological and cytoskeletal changes in fibroblast. This suggests that biglycan may have a role in the conversion of the resident fibroblast to an inflammatory fibroblast phenotype<sup>53</sup>. The migratory capacity of the cells in the study was not evaluated, however, ongoing work try to elucidate this. Furthermore, control subjects have increased proliferation capacity of the distal lung fibroblasts compared to central control fibroblasts which suggests that the role of distal fibroblast might be different compared to central.

Structural cells present in the upper airway are surrounded by a variety of structural elements such as cartilage and smooth muscle cells. In the lower airways such as the alveolus the fibroblast are present in close proximity where the gas exchange takes place. In alveolus, fibroblasts are continuously exposed to mechanical forces and it is tempting to speculate that fibroblasts have to respond directly to reparative signals. The increased distal proliferation therefore represents a more active fibroblasts compared to central airways. As a consequence, a decrease in proliferation capacity observed in distal fibroblasts from patients with asthma and COPD compared to controls might have an important role in the reparative potential in these subjects. The proliferation capacities of central fibroblasts from patients with asthma further strengthen the hypothesis of asthma as a unified airway disease. It is also tempting to speculate that the increased proliferation capacity potentially could be a risk factor for develop cancer in the distal airways.

In conclusion, considering the complex mechanism behind asthma and COPD this thesis demonstrates that different fibroblast phenotypes are present in human lung. These fibroblasts can be separated by their proliferation and matrix production. Presence of fibroblast progenitor cells close to the basement membrane in suggests that



ongoing remodeling processes take place. Further studies are needed to clarify the role of different fibroblast phenotypes in human adult lung diseases.

# Future Perspectives

In this thesis the presence of different fibroblast phenotypes and their possible origin has been studied. The concept that central fibroblasts are different compared to distal in control as well as disease fibroblast is novel and must be taken into account in future studies. The establishment of fibroblast in the BALF in patient with mild asthma has led into speculation regarding the origin of these cells and their role in the lumen.

As a main goal, future studies should therefore continue to include research on the different fibroblast phenotypes including the progenitor cells such as fibrocytes and tissue resident fibroblast stem cells. Differentiation of these phenotypes to fibroblasts/myofibroblasts and how the ECM such as proteoglycans potentially regulate this differentiation will shed light into the role of fibrocytes in lung diseases. The ability to migrate to site of ongoing remodeling has to be further investigated. Chemokines such as SDF-1 and lysophosphatidic acid (LPA) are interesting molecules in the recruitment process and the influence from angiogenic factors such as VEGF in differentiation process.

Since the proteoglycans are composed by variety of GAGs it would be interesting to study the composition and detailed structures of these GAGs in patients. This could be achieved by study proteoglycan production in fibroblasts from patients with asthma and COPD patients. In addition, it is not known if expression or functions of the enzymes that are involved in the GAG synthesis are different in controls vs. patients with asthma and COPD. This would provide understanding of how the connective tissue is composed in chronic lung diseases.

To explore the density of mesenchymal cells in human lung tissue, mesenchymal markers can be used in various combinations such as prolyl-4-hydroxylase, VEGF, SM22, vimentin in combination with markers that visualize proliferating cells (ki67) and apoptotic cells (e.g. caspase 3). This will give important information of the localization and expression of the mesenchymal cells.

The different fibroblast phenotypes presented in this thesis suggest that the cells have different roles in central and distal airways. To get more insight into these roles migration capacities of these cell populations will be explored. This will give us insight into the responsiveness of the cell. To explore if distal fibroblast exhibit a more inflammatory phenotype the production of prostaglandin such as  $\text{PGI}_2$ ,  $\text{PGE}_2$  and  $\text{PGD}_2$  would be interesting to study. In addition, production from distal fibroblasts of other inflammatory molecules such interleukins as IL-8, IL-10 and IL-17 would shed light on the reparative crosstalk in the distal lung. These have been suggested to play a role

neutrophilic, T-regulatory and Th17 responses which are newly discovered cells that may participate in disease progression.

It would also be interesting to study a possible role of nitric oxide in the production of proteoglycans. This is particular interest since both patients with asthma and COPD patients have increased levels of nitric oxide in exhaled air and the increased levels have not been explained. A future study could thus be to examine if nitric oxide could be used as a marker for remodeling in combination with inflammation.

# Populärvetenskaplig sammanfattning

Astma och kronisk obstruktiv lungsjukdom (KOL) är två folkhälsosjukdomar som är mycket kostsamma för samhället. Idag är vi framgångsrika i att behandla de flesta astmatiker så att dessa individer inte behöver förändra sin livsstil. Däremot finns det inte någon bra behandling för de som utvecklat svårare symtom. Behandling av astma är livslång. Den främsta orsaken till att utveckla KOL är rökning. Om man slutar röka kan lungornas försämring saktas ner, men det går aldrig att återställa lungans fullständiga funktion.

En viktig gemensam nämnare i båda sjukdomarna är att det pågår processer som kan liknas vid en konstant sårsläkning som aldrig tar slut, det läks aldrig. Idag behandlar man främst den inflammatoriska delen med anti- inflammatoriska läkemedel. Trots denna behandling finns det nu bevis för att andra celler, s.k. fibroblaster är förändrade i båda sjukdomarna. Fibroblasternas roll är att tillverka ämnen som är viktiga för lungornas uppbyggnad och struktur. I denna avhandling har fibroblaster från olika delar av lungan studerats samt hur dessa fibroblaster tillverkar komponenter som är viktiga i sårsläkningen. Dessa fibroblaster kommer från astmatiker och KOL patienter. Det har även undersökts om cellerna redan finns i lungvävnaden eller om de kanske kommer från blodet och rekryteras till astma och KOL lungorna vid den konstanta sårsläkningen. Resultatet från denna avhandling är att fibroblaster är olika beroende på var de finns i lungan. Celler uppe i bronkerna är annorlunda än cellerna långt nere i lungan. Ännu viktigare är att astma och KOL fibroblasterna har förändrats och tillverkar komponenter som kan vara skadliga för respektive sjukdom. Vi upptäckte även att stamceller till fibroblaster, sk fibrocyter finns hos astmatiker i områden där tydlig sårsläkning sker. Närvaron av dessa stamceller överensstämde även med förändringar av lungornas struktur. Mina fynd i avhandlingen ger en ny syn och ökad förståelse på hur sårsläkningsprocesser kan bidra till sjukdomar som astma och KOL för att kunna utveckla nya läkemedel.



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