INTRACELLULAR SIGNALING IN THE LUNG:
A role for C/EBP transcription factors in chronic obstructive pulmonary disease, glucocorticoid signaling and lung development

Lukas Didon
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ABSTRACT

Over the last decade, a previously unknown role has been established for members of the C/EBP transcription factor family in lung gene expression. In other organs, C/EBPs are well known regulators of cell differentiation and linked processes such as proliferation, apoptosis, gene expression as well as central regulators of inflammatory responses and infectious defenses. The scope of this thesis is to investigate the role of C/EBP transcription factors in the human and mouse lung as well as a putative role in the pathogenesis of chronic obstructive pulmonary disease (COPD). As a first approach, DNA-binding activity of C/EBPs was studied in the airway epithelium of humans and in adult mice. We found that C/EBPβ is the dominant DNA-binding C/EBP transcription factor. Interestingly, we also found that C/EBPβ-activity is increased in the airways of asymptomatic smokers, whereas smokers that develop COPD lack this increase. We hypothesize that altered activity of C/EBPβ in airway epithelial cells has a previously unknown role in the pathogenesis of COPD. Here it could affect the production of inflammatory mediators and genes involved in anti-oxidative and infectious defenses in addition to affecting epithelial cell proliferation, thereby making the lungs more susceptible to destruction and inflammation which augment the progression of the disease.

Inflammation in COPD typically exhibits partial resistance to the anti-inflammatory action of glucocorticoids. When studying glucocorticoid signaling in the lung epithelium, we found that the glucocorticoid receptor, at least partially, mediates the effects of glucocorticoids in lung epithelium by inducing phosphorylation of C/EBPβ, thereby augmenting its DNA-binding activity. This raises the possibility that the decrease in C/EBP-binding activity in the airway epithelium of patients with COPD may have a causative role for the relative resistance to glucocorticoids seen in this disease.

As a means to deepen our understanding of the C/EBP family’s role in the lung, as well as to critically address whether C/EBPs have a role in COPD pathogenesis and related pathological processes, an animal model was used due to the limitations in sampling the human lung. As a first approach to evaluate whether the mouse is a suitable model to study C/EBP functions in the lung, we investigated the expression of C/EBPs in the human and mouse lung epithelium in addition to the lung epithelium of COPD patients. By using immunohistochemistry we found that the adult expression pattern of C/EBPs in the mouse lung is highly similar to the expression pattern of C/EBPα and C/EBPβ in the human lung, suggesting the mouse as a suitable model to study the C/EBP family’s role in lung. In addition, we found that C/EBPα displays dynamic expression during lung development that together with the respiratory distress of neonatal C/EBPα knockout mice, suggest a crucial role for C/EBPα in the development of the lung. We generated a gain-of-function mouse model ectopically expressing C/EBPα in the lung epithelium (SFTPC-Cebpa mice), and a loss-of-function mouse model using the Cre-LoxP technique, with lung epithelial disruption of the C/EBPα gene (CebpaΔLE mice) to address this hypothesis. Both CebpaΔLE mice and SFTPC-Cebpa mice display strikingly similar impaired lung phenotypes during development characterized by a decreased number of growing epithelial tubules which are larger in size as well as a thickened interstitium, indicating that the tempo-spatial expression of C/EBPα is important for correct lung development. Further, adult CebpaΔLE mice, that survive the perinatal lethality, demonstrate a severe pathological picture with 1) goblet cell hyperplasia, bronchiolar metaplasia, fibrosis and mucus plugging, together pathologically defined as bronchiolitis, 2) emphysema and 3) extensive macrophage and lymphocyte infiltrations. C/EBPα has a vital role in lung development and lung epithelial differentiation. Repair processes generally descend from mechanisms and signaling pathways used during organ or tissue development. Therefore, C/EBPα could have a potential role also in remodeling processes, which in COPD patients either is impaired or inadequate. The diagnosis of COPD is based on clinical, radiological and functional features but there are well-recognized histopathological correlates including all the histopathological findings in the CebpaΔLE mice. In line with this, it is tempting to speculate that the pathological processes in COPD and CebpaΔLE mice share at least some underlying mechanisms, with a linkage between the epithelial differentiation-repair process inherent in COPD and the epithelial differentiation during lung development. In summary, the findings presented in this thesis suggest that investigations of the role of C/EBPs in the pathogenesis of COPD could provide important knowledge, that may potentially serve as a base for the development of new treatments for this devastating disease.
LIST OF PUBLICATIONS

I. **Didon, L.,** Qvarfordt, I., Andersson, O., Nord, M. and Rüse, GC.
Decreased CCAAT/Enhancer Binding Protein Transcription Factor Activity in Chronic

II. Berg, T.*, **Didon, L.*,** Barton, J., Andersson, O. and Nord, M.
Glucocorticoids increase C/EBPbeta activity in the lung epithelium via phosphorylation.
*Biochemical and Biophysical Research Communications* 334(2): 638-45. 2005
(*these authors contributed equally)

III. Berg, T., **Didon, L.,** and Nord, M.
Ectopic transgenic expression of C/EBPalpha in the lung epithelium disrupts late lung
development. *American Journal of Physiology (Lung Cell and Molecular Physiology).*

IV. **Didon, L.,** Elmberger, G., Gonzalez, F.J. & Nord, M.
Adult mice with a lung-specific inactivation of the transcription factor C/EBPalpha develop
an emphysematic lung phenotype with impaired differentiation of the respiratory
epithelium. Submitted.

Publications not included in the thesis:

Barton, J. L., Berg, T., **Didon, L.** and Nord, M.
The pattern recognition receptor Nod1 activates CCAAT/enhancer binding protein beta
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<td>Full Form</td>
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<tr>
<td>α-SMA</td>
<td>α-Smooth muscle actin</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>ATCC</td>
<td>The American Type Culture Collection</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>CC16</td>
<td>Clara cell 16 kDa protein (also known as CCSP)</td>
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<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
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<tr>
<td>CHOP</td>
<td>C/EBP homologous protein</td>
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<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
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<tr>
<td>CK-8</td>
<td>Cytokeratin 8</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>E-cadherin</td>
<td>Epithelial cadherin</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<tr>
<td>Floxed</td>
<td>Flanked by lox P sites</td>
</tr>
<tr>
<td>FOX</td>
<td>Forkhead box</td>
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<td>FOXJ1</td>
<td>Forkhead box transcription factor J1</td>
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<td>GC</td>
<td>Glucocorticoids</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HFH</td>
<td>HNF-3/Forkhead homologue</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte nuclear factor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
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<tr>
<td>NF-kB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NKX2-1</td>
<td>NK2 homeobox 1 (also known as TTF-1)</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal day</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>RA</td>
<td>Retinoic acid</td>
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<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
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<tr>
<td>RSK</td>
<td>Ribosomal S6 kinase</td>
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<td>SFTPC</td>
<td>Surfactant protein-C gene</td>
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<td>SHH</td>
<td>Sonic hedgehog</td>
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<tr>
<td>SP</td>
<td>Surfactant protein</td>
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<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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INTRODUCTION

The lung

Structure

The respiratory system consists of conducting airways that lead air into the alveoli, where gas exchange occurs. The epithelium of the proximal conducting airways in humans contain several cell types including a large proportion of ciliated epithelial cells, interspersed mucous cells (goblet cells) without cilia that most importantly produce mucus secreted into the airway lumen, basal cells that constitute the stem cells of the proximal respiratory epithelium and serous secretory cells, mostly located in the submucosal tracheobronchial glands together with mucous cells. Other cell populations are also sparsely present in the respiratory epithelium, such as the columnar, microvilli-bearing brush cells, and the small granule cells. Beneath the epithelium lies the basement membrane, composed of densely packed collagen fibers; the loose connective lamina propria with numerous cells, including lymphocytes, plasma cells, mast cells, eosinophils and fibroblasts; and the more deeply-located submucosa. Bronchi are encircled by cartilage plates which decrease in number in a distal direction. Bronchioles (in the human lung often said to be less than 1 mm in diameter, though this value can actually range from 5 mm to 0.3 mm in humans) have neither cartilage nor glands in their mucosa but a relatively thick layer of smooth muscle cells. The pulmonary acini, with a terminal bronchiole as its central and most proximal structure, branches into respiratory bronchioles, alveolar ducts, sacules and alveoli, and is defined as the lung parenchyma (figure 1).

The bronchiolar epithelium initially has a ciliated pseudostratified columnar epithelium with scattered goblet cells that gradually, in a distal direction, transforms into a simple ciliated columnar epithelium with interspersed non-ciliated secretory serous cells and Clara cells that dominate in number in the terminal bronchioles (Ross and Pawlina, 2005; Junqueira et al., 1986). Clara cells are secretory epithelial cells responsible for the production of bronchiolar surfactant, and they also retain a stem cell potential in the lower airways, where basal cells are scarce (Hong et al., 2001). The most distal airways are the respiratory bronchioles lined with scattered alveoli extending directly from the lumen of the respiratory bronchiole, which branch into alveolar ducts at the most distal portion. Alveolar ducts have almost no walls but are instead lined by alveolar openings and usually terminate at an alveolar sac, which is a space surrounded by clusters of alveoli. The alveolar ducts, alveolar sacs and the alveolus, the distal-most part of the respiratory tree approximately 0.2 mm in diameter, are lined by squamous thin-walled type I cells (pneumocytes) and the cuboidal surfactant-producing type II pneumocytes.
The delicate alveolar structures are the functional unit of the lung where gas exchange occurs over the thin air-blood barrier. The alveolar septum that comprises the air-blood barrier contains a surfactant layer at its thinnest part, and the thin cytoplasm of type I pneumocytes with the epithelial basal lamina fused with the basal lamina of the adjacent capillary endothelial cell. The very thin but rich matrix of elastic fibrils in the interstitium provides the support for the ducts and alveoli. In its thicker parts, fibroblasts, lymph vessels, type II pneumocytes and alveolar macrophages can be present. The interalveolar septa is perforated by alveolar pores, allowing air circulation from adjacent alveoli or acini if the bronchiole is obstructed and ventilation is poor. Alveolar macrophages are located both in the alveolar septum and in the alveolar lumen where they phagocytose inhaled particles and microbes, and in concert with the alveolar epithelium, constitute the first line of defense in the alveoli against inhaled pathogens. The capillaries of the interalveolar wall originate from the pulmonary arteries of the respiratory circulation. Pulmonary arteries carry oxygen-depleted blood from the right ventricle of the heart along the dividing respiratory tree from the hilus down to the alveoli. Cells of the conducting airways are however supplied by oxygenated blood from the bronchial circulation originating from the aorta. Although the respiratory circulation

Figure 1: Overview of the human lung.
and the bronchial circulation are functionally divided, they are both drained largely by pulmonary veins from the lung periphery to the left atrium of the heart (Ross and Pawlina, 2005; Junqueira et al., 1986).

Grossly, human and mouse lungs differ obviously by size, but also by the number of left and right lobes. The mouse lung has four right lobes and one left lobe, whereas humans have three right lobes and two left lobes. The difference in size is also apparent when comparing the size of the airways, since a human bronchirole (5 - 0.3 mm in diameter) would be referred to as a large airway in a mouse lung. Histologically, the major differences between human and mouse lungs is the lack of respiratory bronchioles in mice, the ratio of ciliated cells to secretory Clara cells, as well as the spatial distribution of secretory Clara cells in the mouse airways. The respiratory bronchioles in humans constitute a relatively large transitional zone between conducting airways and the gas-exchanging alveoli, whereas in mouse this transitional zone is minimal (Plopper and Hyde, 1992). This is an aspect to bear in mind when studying emphysema induced by tobacco smoke, which in humans primarily affects the respiratory bronchioles, a structure that does not exist in mice. The epithelium lining the distal bronchioles has species-specific differences in cellular composition, as in humans Clara cells are found interspersed between the ciliated cells in the distal airways. In mice on the other hand, Clara cells are the predominant cell type in both proximal as well as distal airways, and basal cells are restricted only to the most proximal airways (Evans et al., 1990; Evans et al., 1989). Despite the differences in cellular distribution between human and mouse airway epithelium, ciliated cells are more frequent in the proximal airways of mice than in the distal airways, as seen in humans. Also the general pattern of cytodifferentiation in the respiratory epithelium is similar (Plopper and Hyde, 1992; Plopper et al., 1992).

Function

The respiratory system performs three main functions: air conduction, air filtration and, most importantly, gas exchange in the alveoli where oxygen from the air is transferred to the blood and carbon dioxide is removed. The vascular system that carries the blood through the respiratory circulation is discussed in the previous section. Conducting airways condition and filter inhaled air by warming and moistening the air, in addition to removing particulate materials including inhaled microorganisms. Mucous and serous secretions produced by goblet and serous cells as well as mucus-secreting glands plays a central role in this process, and also provide a protective coat against dehydration of the epithelium. A mucus coat covers the major part of the epithelial surface of the conducting airways, and is constantly moved upwards in coordinated sweeping movements by the ciliated epithelium. This mucociliary escalator serves as an important protective mechanism by removing small inhaled particles and pathogens from the lung.

Type II pneumocytes, or alveolar type 2 cells, produce and secrete surfactant, a protein-lipid mixture that serves to decrease surface tension in the alveoli, thereby preventing their collapse upon expiration. Surfactant is comprised of about 90% lipids, 10% proteins and a lesser amount of carbohydrates, which are all synthesized, stored, secreted and recycled or catabolized by alveolar type 2 cells (Roth-Kleiner and Post, 2003). Of the surfactant lipids, DPPC or dipalmitoylphosphatidylcholine is by far the most important for reducing surface tension. However, surfactant also includes proteins that not only aid in surfactant distribution and homeostasis (as surfactant protein B (SP-B) and SP-C), but also proteins that have a role in host defense against inhaled pathogens such as SP-D and SP-A (Ross and Pawlina, 2005). Bronchiolar Clara cells are also known to secrete surfactant phospholipids, in addition to the abundantly-secreted Clara cell
protein (CC16) that is used as a pulmonary marker for respiratory injury reflecting damage of the distally located Clara cells. Following respiratory damage, CC16 levels decrease in bronchoalveolar lavage (BAL) fluid and increase in serum as a result of leakage across the air-blood barrier (Ross and Pawlina, 2005).

**Chronic obstructive pulmonary disease**

Chronic obstructive pulmonary disease (COPD) remains a major public health problem and is the fourth leading cause of mortality worldwide. COPD is characterized by airflow limitation that is not fully reversible accompanied by a chronic inflammatory response of the lungs to noxious particles and gases, most commonly cigarette smoke (Calverley and Walker, 2003; Rabe et al., 2007). The characteristic symptoms of COPD is chronic and progressive dyspnea, cough, and sputum production. Since 2007, COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) consortium as: “a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases” (Rabe et al., 2007). The progressive loss of lung function in COPD patients associated with continued smoking is caused by a mixture of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of the two components vary from person to person (James and Wenzel, 2007; Rabe et al., 2007). The airway inflammation in COPD persists also in ex-smoking COPD patients (Turato et al., 1995). A COPD exacerbation is defined as “an event in the natural course of the disease characterized by a change in the patient’s baseline dyspnea, cough, and/or sputum that is beyond normal day-to-day variations, is acute in onset, and may warrant a change in regular medication in a patient with underlying COPD” (Rabe et al., 2007). Persistently increased airway inflammation as a likely consequence of reduced viral and bacterial clearance, is a known complication following COPD exacerbations and often result in a consistent decrease in baseline lung function compared to before the exacerbation as well as promoted airway remodeling (Aaron et al., 2001; Donaldson et al., 2002; Seemungal et al., 2000).

**Classification**

Early classification attempts relied on epidemiological definitions of chronic cough and sputum production lasting for 3 months over a period of at least 2 years (chronic bronchitis) or on the presence of emphysema in pathological specimens, although neither approach was of much help in clinical management (Calverley and Walker, 2003). Later on, Fletcher, Peto and colleagues showed in epidemiological studies that death and disability were related to progressive deterioration in the FEV1 (forced expiratory volume in second) rather than persistent symptoms of cough and sputum production so, as a result spirometric measurements were suggested as the most reliable and widely available clinical diagnostic tool (Fletcher and Peto, 1977; Peto et al., 1983). The spirometric GOLD classification system of COPD (Rabe et al., 2007) includes four stages: stage I, mild; stage II, moderate; stage III, severe; stage IV, very severe (see table 1). The very simple spirometric classification of disease severity is based on specific spirometric cut points (e.g., post-bronchodilator FEV1/FVC ratio, 0.70 or FEV1, 80, 50, or 30% predicted), but are used only for purposes of simplicity, and these cut points have not been clinically validated. The pathophysiology will be discussed below.
Table 1. Spirometric classification of COPD severity based on post-bronchodilator FEV1.

<table>
<thead>
<tr>
<th>Stage</th>
<th>FEV1/FVC &lt; 0.70</th>
<th>FEV1 &gt; 80% predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I: mild</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II: moderate</td>
<td>FEV1/FVC &lt; 0.70</td>
<td>50% &gt; FEV1 &lt; 80% predicted</td>
</tr>
<tr>
<td>Stage III: severe</td>
<td>FEV1/FVC &lt; 0.70</td>
<td>30% &gt; FEV1 &lt; 50% predicted</td>
</tr>
<tr>
<td>Stage IV: very severe</td>
<td>FEV1/FVC &lt; 0.70</td>
<td>FEV1 &lt; 30% predicted or FEV1 &lt; 50% predicted</td>
</tr>
</tbody>
</table>

* Respiratory failure: arterial partial pressure of oxygen (PaO2) < 8.0 kPa (60 mm Hg) with or without arterial partial pressure of CO2 (PaCO2) > 6.7 kPa (50 mm Hg) while breathing air at sea level (Rabe et al., 2007).

Chronic bronchitis

Chronic bronchitis primarily affects smokers and is characterized by daily coughing and increased mucus production during at least three consecutive months over a period of more than two years. The diagnostic criterion of chronic bronchitis is based solely on the case history, and no clinically useful objective method to diagnose chronic bronchitis has been described. Bronchial epithelium from patients with chronic bronchitis has an increased number of goblet cells in the bronchi and in the bronchioles as well as an increased number of infiltrating leucocytes (Riise GC, 1992). Chronic cough and sputum production may precede the development of airflow limitation and COPD by many years, however, as significant airflow limitation may develop without chronic cough and sputum production, it cannot be used to identify people “at risk” of developing COPD (Rabe et al., 2007).

Asthma and COPD

Asthma is characterized by periodic reversible airway obstruction, airway inflammation, and airway wall remodeling. The similarity between asthma and COPD led to the ‘Dutch hypothesis’ where asthma and COPD were suggested to be different forms of the same disease, chronic obstructive lung disease, and this is still a topic of debate. (Barnes, 2006a; Kraft, 2006). COPD shares many similarities with asthma, but one of the clinical definitions that differentiates between the two diseases is whether or not the airway obstruction is reversed by β2-agonists and / or a longer period of glucocorticoid treatment. In contrast to COPD, in which pathology involves both the large and small conducting airways in addition to the lung parenchyma (Sin and Man, 2006), the pathology of asthma is more dominated by the larger conducting airways. Involvement of the small conducting airways and the lung parenchyma has however been suggested by Kraft and colleagues (Kraft, 1999; Kraft et al., 2006). Severe asthma, in contrast to mild asthma, displays more similarity to COPD, with increased numbers of neutrophils, IL-8 and TNFα (Barnes, 2008), increased oxidative stress, and a poor response to glucocorticoids. Moreover, there is a mixture of Th1 and Th2 cells and also more CD8+ T-cells in severe asthma, which more closely resembles the immune-cell population seen in COPD than the characteristic Th2-inflammation in mild asthma (Barnes, 2008). The etiology of COPD is typically related to tobacco smoking, although, both smoking and non-smoking asthma patients may develop a fixed (irreversible by treatment) airflow
obstruction, which is by definition COPD. Smokers with features of asthma have been suggested to be more likely to develop COPD (James and Wenzel, 2007).

**Histopathology**

COPD is associated with two main pathologic lesions: 1) Inflammation of the small airways with airway wall thickening, infiltration of inflammatory cells, mucus plugging and peribronchiolar fibrosis and 2) Destruction of lung parenchyma with enlargement of air spaces and loss of lung elasticity, i.e. emphysema (Barnes, 2000; Barnes, 2004b; Rabe et al., 2007; Travis et al., 2002). Chronic bronchitis is a clinical diagnosis often, but not always, concurrent with COPD (Hargreave and Parameswaran, 2006; Travis et al., 2002). Most patients with COPD have both histopathologic conditions, but their relative extent within individual patients can vary widely.

**Emphysema.** Emphysema is pathologically defined as an ‘abnormal, permanent enlargement of the airspaces distal to the terminal bronchiole accompanied by destruction of their walls’. There are different types of emphysema and they are classified by the localization of the disease (Wright and Churg, 2006). Centrilobular (or centriacinar) emphysema is the type commonly found in COPD and is usually more severe in the upper than in the lower zones of the lung. In milder forms, single alveoli entering directly into the walls of terminal and respiratory bronchioles are affected, whereas the distal alveolar bundles connected to the alveolar ducts of the acinus remain more intact. The respiratory bronchioles are centrally located in the acini, hence the name centriacinar or -lobular. Although, in more severe lesions, destruction will advance toward the periphery of the lobule. A subset of COPD patients have a concurrent α1-antitrypsin deficiency, and they generally display a more widespread unison enlargement of alveoli within the acini, called panlobular (or panacinar) emphysema. Alveoli can be difficult to distinguish from the alveolar ducts and respiratory bronchioles in panlobular emphysema, and the lung architecture appears simplified with formation of small box-like structures (figure 2; Wright and Churg, 2006).

![Image](image.png)

*Figure 2.* Hemmatotoxylin-eosin staining of sections from tissue obtained from volume-reduction surgery for emphysema (B), and in tissue sections with normal histology from patients that underwent lung surgery of other causes (A).
Small airway disease. The major contributor to airway obstruction in COPD is the “small airway disease”, which refers to the narrowing of small airways (airways < 2 mm in diameter, including both membranous and respiratory bronchioles) due to 1) increased mucus production and malfunction of the mucociliary clearance apparatus, resulting in accumulation of inflammatory exudates (usually referred to as mucus plugging); 2) thickening of the airway wall by remodeling processes related to tissue repair and infiltration of inflammatory cells; 3) shortening of smooth muscle surrounding the airway; 4) collapse of airways due to loss of alveolar attachments to the airway wall and loss of elastic recoil of the lung parenchyma (figure 3; Finucane and Colebatch, 1969; Hogg et al., 2004) (Saetta et al., 1985; Wright and Churg, 2006).

Mucociliary clearance is an important part of the respiratory innate host defense, and impairment of the clearance apparatus result in the presence of intraluminal mucus and increased airway obstruction (Aikawa et al., 1989; Hogg et al., 2004). Metaplastic transdifferentiation of Clara cells into goblet cells under inflammatory settings contribute to the goblet cell hyperplasia and mucus hyper production in COPD (Kouznetsova et al., 2007). Intraluminal macrophages can be found in the accumulating mucus and represent the earliest pathological change in smokers (Wright and Churg, 2006). Thickening of the airway walls and deposition of extracellular matrix proteins (fibrosis) in the subepithelial compartment contributes considerably to the persistent narrowing of the small airways in COPD and is associated with declining lung function (Hogg et al., 2004; Kranenburg et al., 2006). Deposition of extracellular matrix proteins in the airways is suggested to be a consequence of remodeling process related to tissue repair (Cosio et al., 1978; James and Wenzel, 2007; Kranenburg et al., 2006; Wright and Churg, 2006). Airway obstruction can be further enhanced by increased mass and tension of airway smooth muscle bundles (James and Wenzel, 2007). Another factor that contribute significantly to the obstruction caused by small airway disease is collapse of the small airways (Hyatt, 1983). This is primarily a result of emphysematous destruction, which disrupts the peribronchial alveolar attachments that normally prevent airway obstruction.
closure through recoil of the lung parenchyma (Lamb et al., 1993; Willems et al., 1990; Wright et al., 1984), but also a result of the thickening of the adventitium mostly due to peribronchiolar inflammation (Petty et al., 1986; Saetta et al., 1985; Wright and Churg, 2006).

The lung epithelium and pulmonary hypertension

The airway epithelium has several defense mechanisms that respond to the stress imposed by cigarette smoke, in some individuals these defenses are exaggerated, resulting in chronic inflammation, and eventually, chronic bronchitis and bronchiolitis (Hogg, 2004b; Maestrelli et al., 2001; Mills et al., 1999; van der Vaart et al., 2004). One of the earliest tobacco smoke-induced consequences in the airway epithelium is disruption of the protective barrier that is normally mediated by tight junctions between adjacent epithelial cells, resulting in increased permeability across the epithelium (Jones et al., 1980; Simani et al., 1974). The mechanism for increased permeability is unclear, but prior studies have shown that damage to the epithelial cell itself, and to a lesser extent, disruption of the tight junctions between epithelial cells are partly responsible (Burns et al., 1989; Walker et al., 1984). The disruption of the epithelial barrier is associated with an innate immune response, typified by the migration of inflammatory cells into the epithelial layer (Hogg, 2004a) and, if the inflammatory response becomes chronic results in an airway pathology and dysfunction associated with chronic bronchitis (Hogg et al., 2004). Shedding of the columnar epithelium, which is constituted of more than 50% ciliated cells in the proximal airways, cells that play a key role in innate immune host defense by carrying out mucociliary clearance, has been reported in the larger airways of COPD patients (Di Stefano et al., 1998; Jeffery, 2001; Stellato, 2007; Tiddens et al., 1995). Since the airway epithelial morphology in cigarette smokers not typically demonstrate epithelial necrosis, the most likely mechanism for epithelial cell death and shedding in response to cigarette smoke is apoptosis (Jeffery and Reid, 1981a).

20 - 30% of all COPD patients suffer from concurrent pulmonary hypertension, which contribute to the deteriorated lung function. The pulmonary hypertension is associated with thickening of the musculature around pulmonary arteries and intimal vascular fibrosis and is suggested to be a direct response to cigarette smoke, and not a secondary response to emphysema or hypoxia as previously assumed (Kranenburg et al., 2006; Wright and Churg, 2006; Chatila et al., 2008; Falk et al., 2008).

Immunology and inflammatory cells of COPD

Macrophages are believed to have a major regulatory role on the inflammation in COPD through the release of proteases and chemokines that attract neutrophils, monocytes and T cells (Barnes, 2004a). Alveolar macrophage numbers are significantly increased in COPD, mainly thorough chemokine (C-C motif) ligand 2 (CCL2; also known as MCP1) and chemokine (C-X-C motif) ligand 1 (CXCL1; also known as GROα) mediated recruitment of circulating monocytes expressing the CCL2 receptor (CCR2; also known as MCP1R) and the CXC-receptor 2 (CXCR2; also known as IL8R) (Traves et al., 2004). Whereas the alveolar inflammation in COPD is dominated by increased numbers of macrophages, the airway inflammation shows a striking neutrophil dominance. Neutrophils are recruited by CCL2 and CXCL8 (also known as IL-8) secreted mainly by airway epithelial cells through the CXCR2 expressed on neutrophils (figure. 4). Reviewed in (Barnes, 2008).
The increased levels of INFγ in the COPD lung induce the expression of chemoattractants such as CXCL9 (also known as MIG), CXCL10 and CXCL11 from both the epithelium and macrophages. These chemoattractants are all ligands to CXCR3 (also known as GPR9 and MigR), a receptor expressed on Th1 cells, hence the predominance of Th1 cells in COPD patients. CD8+ T cells exceed the number of CD4+ T cells in the airways and lung parenchyma of patients with COPD (Saetta, 1998), but their role in disease pathogenesis is not yet certain. Cytotoxic T-cells have been suggested to enhance COPD pathogenesis by releasing granzyme B and perforins which may induce apoptosis of type 1 pneumocytes, thereby contributing to the development of emphysema (Majo et al., 2001).

Destruction of the parenchymal elastin framework has been a leading potential mechanism for alveolar destruction in emphysema and neutrophil elastase and MMPs are the most studied candidates that account for the increased protease activity that is a factor contributing to the protease/antiprotease imbalance in COPD (reviewed in Yoshida and Tuder, 2007). The evidence in support of a significant role of neutrophil elastase in the pathogenesis of emphysema has relied on the finding of early emphysema in patients deficient in α1-antitrypsin, the major inhibitor of neutrophil elastase (Ranes and Stoller, 2005). MMP-12 has also been suggested as major candidate responsible for pulmonary emphysema and increased levels are observed in the sputum, BAL, bronchial biopsies, and peripheral lung tissue of patients with advanced emphysema (Demedts et al., 2006; Molet et al., 2005). MMP-12 also degrades elastin and is predominantly produced by alveolar macrophages. Increased MMP-12 expression is further associated with parenchymal destruction in several experimental emphysema models, and MMP-12 knockout-mice are protected from development of cigarette smoke-induced emphysema (Hautamaki et al., 1997). MMP-9, MMP-2 (which also degrade elastin) and MMP-1 (which degrade collagen) expression have also been shown to be increased in COPD lungs, however, whether they have a central role in emphysema remains unclear (Imai et al., 2001; Segura-Valdez et al., 2000). MMPs are antagonized by the protease specific TIMP inhibitors and a increased ratio in MMP/TIMP favor a proteolytic environment and are believed to cause alveolar destruction. This is supported by experimental models and the finding of decreased TIMP-1 levels in alveolar macrophages from COPD patients (Yoshida and Tuder, 2007).
Whether B cells or dendritic cells play a role in COPD pathogenesis is still unclear. However, in severe forms of the disease, B cells are suggested to be activated by bacterial or viral antigens as a consequence of chronic bacterial colonization or latent viral infection in the airways, thereby inducing the formation of lymphoid follicles. COPD has also been suggested to have an autoimmune component, where new antigenic epitopes are created by tobacco smoke-induced tissue damage, oxidative stress or chronic bacterial infection (Agusti et al., 2003; Barnes, 2008). The autoimmune theory has gained support by the discovery of elastine antibodies in COPD patients (Lee et al., 2007), in addition to the finding of antigen-induced expansion of B cell clones in patients with severe emphysema (Sullivan et al., 2005). Cigarette smoking has been shown to induce an increase in the number of mature dendritic cells in the airways and alveolar walls of smokers (Soler et al., 1989). However, others have demonstrated a decrease of dendritic cells in the airways of smokers with COPD compared to smokers without airway obstruction (Rogers et al., 2008). Reviewed in (Barnes, 2008).

Figure 4. Immunology and inflammatory cells of COPD. Adapted from (Barnes, 2008).
Transcription factors and intracellular signaling pathways

Expression of multiple inflammatory proteins is increased in the respiratory tract of COPD patients, including cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors. Most of these inflammatory proteins are regulated at the level of gene transcription. Changes in gene transcription are achieved through activators and repressors (specific transcription factors; TFs) that can either induce or repress transcription of various target genes by binding to cis-activating/repressing elements located in regulatory regions of target genes (Barnes, 2006c). TFs can be expressed ubiquitously or in specific cell types. The phenotypic characteristics of a cell are largely determined by the specific expression profile of TFs in that particular cell, resulting in a cell-type unique gene expression profile. TFs need to contact intermediary factors (co-transcription factors) which may function as coactivators, corepressors or both, that convey regulatory information to the transcription machinery (including RNA polymerase II and general transcription factors). Co-transcription factors are distinct from TFs in that they, in most cases, do not have the capacity to bind DNA with sequence specificity (Heldin and Purton, 1996; Lodish et al., 2000).

DNA in eukaryotes is packaged into a tight chromatin structure that prevent the transcription machinery from accessing gene promoters (Kornberg, 1974). Gene expression is to a significant extent regulated by a balance between acetylation/deacetylation of core histones by various transcriptional coactivators recruited by TFs. Acetylation of histones, by histone acetyl transferases (HATs), open up the chromatin structure and promotes transcription of the gene(s) in a specific region of the chromatin structure. Histone acetylation is reversed by histone deacetylases (HDAC) thereby tightening the interaction between histones and DNA, leading to repression of transcription (Rundlett et al., 1996). In recent years, several different types of modifications have been shown to take place on histones, in addition to on the DNA itself, to regulate transcription including phosphorylation, methylation and ubiquitination (Fischle et al., 2003).

Signals mediated by cytokines, for example, are commonly transmitted to the nucleus through a series of cytoplasmic mediators, a so-called signal transduction pathway. The use of several mediators allow both a fine-tuned and tightly regulated pathway, in addition to efficient crosstalk between different signaling pathways. An initial trigger, typically a signaling molecule binding to its receptor, activates the intracellular signaling pathway. The cytoplasmic mediators are often kinases or phosphatases that mediate the signal by phosphorylating or de-phosphorylating their specific target protein, and thereby activating the next mediator in the signaling pathway. Each step is amplified in a kinase pathway, since a phosphorylated and thereby activated kinase can in turn phosphorylate and activate a vast number of its substrates, in this case the next subunit of the signaling pathway, which enables a single signal transmitter (e.g. a cytokine) to induce a fast and robust response. In addition, each mediator of the pathway is regulated by the equilibrium of activated upstream kinases and phosphatases, providing a mechanism to fine tune the signaling pathway as well as synchronize with other signaling pathways. Many signal transduction pathways culminate in the activation or inactivation of TFs, most commonly through post-transcriptional modifications such as phosphorylations. Hence, transcription factors act as ‘nuclear messengers’, as they convert transient signals from the external or internal environment through cytoplasmic mediators and nuclear co-activators into long-term changes of gene expression (figure 5; Lodish et al., 2000).
CCAAT/enhancer binding proteins (C/EBPs) are key-regulators of cell differentiation and linked processes such as proliferation, apoptosis and gene expression in several organs. C/EBPs are also central for inflammatory responses and infectious defenses. There are six C/EBP-genes in mammals: C/EBP-\(\alpha\), -\(\beta\), -\(\gamma\), -\(\delta\), -\(\epsilon\), and -\(\zeta\) and they all share a strong sequence homology. Due to alternative translation start sites and so called leaky ribosomal scanning, C/EBP\(\alpha\) exists in two different isoforms (42 and 30 kDa) and C/EBP\(\beta\) in at least three different isoforms (38, 35 and 20 kDa)(Ramji and Foka, 2002). C/EBP\(\alpha\) is expressed at high levels in the adipose tissue, liver, intestine, lung, adrenal gland, peripheral-blood mononuclear cells and placenta. C/EBP\(\beta\) is present in the liver, intestine, lung, adipose tissue, spleen, kidney and myelomonocytic cells at significant levels. The expression of C/EBP\(\delta\) is low in liver, kidney, spleen and heart as well as relatively high in the lung and intestine. C/EBP\(\epsilon\) expression is restricted primarily to myeloid cells, and both C/EBP\(\gamma\) and C/EBP\(\zeta\) are expressed ubiquitously (Alam et al., 1992; Ramji and Foka, 2002). C/EBPs are modular proteins, consisting of an activation domain, a basic DNA-binding region, and a leucine-rich dimerization domain (Ramji and Foka, 2002). The basic region and the dimerization domain of C/EBPs display a high sequence similarity and consequently they can interact with virtually identical DNA sequences, and also form both homo- and heterodimers with each other (Cassel and Nord, 2003). Furthermore, C/EBPs are auto-regulatory and cross-regulate each other’s expression (Niehof et al., 2001). However, each protein exhibits unique functional properties in vivo through differences in their transactivating ability, tissue- and differential stage-specific expression, changes in

**Figure 5.** General mechanism of intracellular signaling pathways and transcriptional regulation.
C/EBP-isoform-ratio through leaky ribosomal scanning, post-transcriptional modifications, and minor variable DNA binding specificities. Their unique properties are highlighted by the diverse phenotypes of the different C/EBP-deficient mice generated (Flodby et al., 1996; Fuchs, 2007; Martis et al., 2006; Screpanti et al., 1995; Tanaka et al., 1997; Wang et al., 1995; Zhang et al., 1997). Several extracellular stimuli have been shown to post-transcriptionally activate different C/EBP factors, most thoroughly studied are phosphorylations of different different amino acid-sites on C/EBPβ (Boruk et al., 1998; Buck et al., 2001a; Buck et al., 2001b; Cho and Kim, 2003; Harris et al., 2001; Hu et al., 2001; Kang et al., 2003; Trautwein et al., 1993). C/EBPs can also be regulated through cytoplasmic-to-nuclear shuttling as well as intranuclear shuttling. Shutting is often regulated through direct phosphorylation of C/EBPs and is dependent on a kinase signaling pathway (Billiard et al., 2001; Buck et al., 2001b; Cho and Kim, 2003; Kang et al., 2003; Schaufele et al., 2003). Furthermore, C/EBP-specific inhibitors like GC-Induced Leucine-Zipper (GILZ) and C/EBPζ can influence the activity of C/EBP factors (Ramji and Foka, 2002).

C/EBPs in regulation of inflammatory responses and infectious defenses

C/EBPβ has a specific role in the control of inflammatory and defense responses and its activity and/or expression level is, together with C/EBPα and C/EBPδ, regulated by a number of inflammatory agents, including lipopolysaccharide (LPS) and a range of cytokines (Ramji and Foka, 2002). C/EBPβ was first identified on the basis of its ability to regulate gene transcription in response to IL-1 and IL-6 (Akira et al., 1990; Poli et al., 1990) but is now known to regulate many additional inflammatory genes, including tumor necrosis factor α (TNF-α), Intercellular adhesion molecules 1 (ICAM-1), Cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS), as well as genes involved in the innate immune response, both receptors such as some of the toll-like receptors (TLRs), and effectors such as antibacterial peptides of the β-defensin and cathelicidin families, also lysozyme and collectins (Cassel and Nord, 2003; Diamond and Eichler, 2002; Gudmundsson et al., 1996; He and Crouch, 2002; Musikacharoen et al., 2001; Poli, 1998; Ramji and Foka, 2002; Rosenberg et al., 2002; Takeshita et al., 2004).

Nuclear factor κ B (NFκB) is a key inflammatory transcriptional regulator that regulate the expression of pro- and anti-inflammatory genes, as well as acute phase genes including host defense genes in a tightly regulated, cell type and promoter specific cross-talk with C/EBPβ (Poli, 1998). This cross-talk is typically mastered by protein-protein interaction and/or by altering the phosphorylation status of the several regulatory phosphorylation sites on C/EBPβ and NFκB (Basak et al., 2005; Faour et al., 2006; Gorrill and Khalili, 2005; Plevy et al., 1997; Poli, 1998; Stein et al., 1993; Zwergal et al., 2006). C/EBPβ and NFκB are known to synergistically activate the transcription of acute phase proteins such as serum amyloid A1, A2, A3 and α1-acid glyco protein, in addition to the cytokines IL-6, IL-8, IL-12 and granulocyte colony-stimulating factor (G-CSF) (Betts et al., 1993; Dunn et al., 2003; Lee et al., 1996; Li and Liao, 1992; Matsusaka et al., 1993; Plevy et al., 1997; Poli, 1998; Ray and Hannink, 1995). However, their interactions have also been shown to lead to antagonistic effects where C/EBPβ can repress NFκB activity on the IL8 and the HIV-1 long terminal repeat promoter (Stein and Baldwin, 1993; Stein et al., 1993; Zwergal et al., 2006). The crucial role of C/EBPβ in regulation of inflammatory and host defense responses becomes evident in C/EBPβ deficient knockout-mice that are highly susceptible to bacterial and fungal infections, and display impaired expression of several inflammatory genes. Macrophage responses when challenged with pathogens are also impaired, a consequence of C/EBPβ’s vital role in macrophage activation and differentiation (Poli, 1998; Screpanti et al., 1995; Tanaka et al., 1995).
C/EBPs in regulation of cellular differentiation and proliferation

In lung, as well as in other organs including liver, adipose tissue and myeloid blood cells, C/EBPs are key regulators of differentiation. In adipocyte differentiation, C/EBPβ and C/EBPδ are expressed during the early clonal expansion phase of adipogenesis. Subsequent to this period of extensive mitotic cell growth, C/EBPβ and C/EBPδ expression levels diminish markedly and come to be replaced by C/EBPα (Cao et al., 1991). Changes in the ratio between different C/EBP members have been suggested to be part of the tight regulation that determines whether a cell proliferates or differentiates (Borger et al., 2002). C/EBPα generally stimulates expression of genes characteristic of the mature differentiated organ, and is associated with terminal differentiation (Ramji and Foka, 2002). C/EBPα, β, δ and ε are all expressed in myeloid cells and display a unique expression profile during myeloid differentiation (Ramji and Foka, 2002). C/EBPβ is up-regulated during macrophage differentiation and C/EBPβ-deficient mice have impaired macrophage functions (Natsuka et al., 1992). Also C/EBPα-deficient mice have impaired myeloid differentiation, but in contrast to C/EBPβ-deficient mice they lack mature neutrophils (Zhang et al., 1997). Furthermore, C/EBPα knockout-mice demonstrate deficient development and function of the liver, adipose tissue and lung (Fuchs, 2007; Linhart et al., 2001; Ramji and Foka, 2002; Sugahara et al., 2001; Yang et al., 2005). C/EBPα is a strong inhibitor of cell proliferation and is likely to promote growth arrest by cell-specific mechanisms (Fuchs, 2007). Several human tumor types, including non-small cell lung cancers, display reduction in the levels of C/EBPα, suggesting that C/EBPα is a tumor suppressor gene (Halmos et al., 2002; Tada et al., 2006; Takai et al., 2005). The different models suggested for C/EBPα-mediated cell-cycle arrest include direct interaction with cyclin-dependent kinase 2 (cdk2) and cdk4 that arrests cell proliferation by inhibition of these kinases (McKnight, 2001; Ramji and Foka, 2002; Wang et al., 2001) or by indirect inhibition of Cdk2 by stabilization of the Cdk2 inhibitor, p21WAF1/CIP1 (Timchenko et al., 1996). C/EBPα also enhances the formation of cdk4-ubiquitin conjugates and induces degradation of cdk4 through a proteasome-dependent pathway (McKnight, 2001). Several additional models for C/EBPα mediated growth arrest are known and discussed in e.g. (Fuchs, 2007).

C/EBPs in the lung

Of the tissue-specific C/EBP family members, three have been demonstrated to be expressed in lung, namely C/EBPα, β and δ. C/EBPα and C/EBPβ are expressed in alveolar type II cells as well as bronchiolar Clara cells. In addition, C/EBPβ is also expressed in alveolar macrophages and interstitial cells. C/EBPδ is expressed at higher levels in the lung than in any other tissue, and expression has been found in alveolar type II cells and in all Clara cells (discussed in paper III and preliminary results; Cassel and Nord, 2003). C/EBPα is, together with the two transcription factors FOXA2 (also known as HNF-3β) and NKX2-1 (also known as TTF-1), a central player in the transcriptional regulation of lung epithelial differentiation, and a major regulator of other processes in the early developing lung (Cassel and Nord, 2003). Several genes specifically expressed in the lung epithelium such as CC16 (also known as CCSP or CC10), surfactant protein-A (SP-A) and SP-D, have been shown to be regulated by the C/EBP transcription factor family (Li et al., 1995; Matlapudi et al., 2002; Nord et al., 1998; Park and Kemper, 1996; Rosenberg et al., 2002; Skarin et al., 1999). CC16 is a small secretory protein abundantly expressed in the Clara cells of the bronchiolar epithelium and in similar cells in the larger airways that was the first lung-specific gene demonstrated to be regulated by C/EBP transcription factors (Nord et al., 1998; Nord et al., 2000). CC16 has been suggested to play a role in the protection against oxidative stress and to have anti-inflammatory functions (Zhang et al., 2000; Stripp et al., 2000). Both SP-A and SP-D are hydrophilic innate host defense proteins of the collectin family that aid in clearing the lungs of...
invading microbes (Kingma and Whitsett, 2006). SP-A and SP-D are expressed in alveolar type II cells in addition to bronchiolar Clara cells and SP-D is additionally expressed in the tracheobronchial glands of the lung (Kuroki and Voelker, 1994). Expression of C/EBPβ and C/EBPδ increase dramatically in the lung after lipopolysaccharide (LPS) and bleomycin treatment, which both induce inflammatory reactions (Sugahara et al., 1999). Furthermore, recent findings in bleomycin-treated C/EBPβ deficient mice suggest that C/EBPβ possesses an essential and multifactorial role in pulmonary fibrosis, including regulation of cytokine expression in addition to inducing myofibroblast differentiation and proliferation (Hu et al., 2007). Cytochrome P450 enzymes (CYPs) are key enzymes in metabolic reactions, and the CYP2B as well as CYP2A family metabolize a number of inhaled drugs and toxic xenobiotics. (Schoedel and Tyndale, 2003). The C/EBP-regulated CYP2B1 and CYP2A13 enzymes are expressed in the lung epithelium and are believed to have a role in the metabolic activation of inhaled toxins from tobacco smoke, including tobacco-specific nitrosamines and nicotine (paper II; Berg et al., 2002; Ling et al., 2007a). The role of C/EBPα in asthma airway smooth muscle hyperplasia has been thoroughly investigated by Black and colleagues (Borger et al., 2002; Borger et al., 2007; Roth et al., 2004; Roth et al., 2002). They suggest that the proliferation of airway smooth-muscle cells and the differentiation of fibroblasts into myofibroblasts, in patients with asthma may result from an absence of C/EBPα (Roth and Black, 2006). Further, they show that the antiproliferative action of C/EBPα requires complex formation with an activated glucocorticoid receptor, which in turn induces the expression and activation of the cell-cycle regulator p21 (Waf1/Cip1) (Roth et al., 2004).

Glucocorticoid signaling

Glucocorticoids have been used as an effective treatment for inflammatory diseases for over 60 years (Hench PS, 1949), and asthma was one of the first disorders where positive results were reported. Administration of exogenous GCs in doses that far exceed physiologically released levels result in adverse effects in multiple organ systems, consequently topical administration by inhaled corticosteroids is preferred rather than oral corticosteroids in the treatment of diseases characterized by airway inflammation, such as asthma and COPD (Bazzy-Asaad, 2001). However, current pharmacological treatment of COPD is unsatisfactory, even though GCs have positive effects on exacerbations, they do not significantly influence the severity of the disease or its natural course (Soriano et al., 2007). The specific characteristics of the inflammatory response in COPD and asthma, as well as the site of inflammation differ, but they both involve recruitment and activation of inflammatory cells and changes in the structural cells of the lung. It is widely accepted that the success of GCs in treating airway inflammation directly reflects their anti-inflammatory effects, such as decreased expression of cytokines and chemokines that activate endothelial cells and leukocytes, but the precise mechanisms by which steroids improve lung function remain unclear (Sha et al., 2004). Recent findings have shown that GCs induce relaxing effects on the smooth muscles of the bronchioles, and also initiate the protective actions of innate immune responses, an effect that can diminish the occurrence of exacerbations brought by respiratory infections in both COPD and asthma (Homma et al., 2004; Schleimer, 2004; Zhang et al., 2007).

Endogenous GCs are produced by the adrenal cortex from cholesterol and are essential for organ development and cell differentiation in addition to their role in regulating inflammation and immune responses (Bamberger et al., 1996). The cellular response to GCs is mediated by the glucocorticoid receptor (GR), which functions as a transcription factor and regulates transcription of several steroid-responsive target genes (Barnes, 2006b). The GR is in its unliganded state bound to
other proteins in the cytoplasm such as heat shock proteins (hsp90, hsp56, hsp70) (Bazzy-Asaad, 2001). But once activated by a GC, GR homodimerize and translocate to the nucleus, where they can bind to specific positive or negative GC responsive elements (GREs or nGREs) on gene promoters and/or interact with other regulatory proteins. Anti-inflammatory GC-signaling can be mediated by several different mechanisms such as 1) Direct DNA binding of GR to the target gene promoter to induce (through binding to GREs) the expression of anti-inflammatory target genes or to repress (through binding to nGREs) expression of pro-inflammatory target genes; 2) Interaction with and inhibition of pro-inflammatory cytosolic signaling pathway mediators such as some MAPKs; 3) Inducing apoptosis in inflammatory cells; and 4) Directly inhibit pro-inflammatory transcription factors (e.g. AP-1 and NFkB) either by repressing their activity at the promoter level, or by inhibiting their nuclear translocation. GR exercises its regulation at the target gene promoter by different mechanisms including protein-protein interaction with other transcription factors (e.g. AP-1, NFkB, C/EBPs and STATs), with the basal transcriptional machinery and co-activators/repressors (e.g. HATs and HDACs), by binding to competitive binding sites in the promoter region to block overlapping binding sites for other TFs, or by competing for co-activators/repressors with other TFs (Barnes, 2006b; Heldin and Purton, 1996; Stellato, 2007).

C/EBPs and glucocorticoid signaling

All three C/EBP factors specifically expressed in the lung can interact with GR in the presence of an unknown intermediary factor (Boruk et al., 1998). Borger et al demonstrated that the response from GC treatment of the expression and regulation of C/EBPs differed in bronchial smooth muscle cells depending on whether the cells were from asthmatic patients or from patients with COPD (Borger et al., 2007). The response in these cells also differed from the response in the airway epithelium (paper II; Berg et al., 2002), suggesting that GC-signaling is both tissue and disease specific. A number of the GC-target genes in the lung epithelium, which GCs are known to stimulate transcription from, lack GREs. Our group and others have shown that GCs regulate transcription of at least some of these genes via the action of C/EBP transcription factors (paper II; Berg et al., 2002; Rudiger et al., 2002; Zhang et al., 2007). That GR depend on other regulatory proteins in the lung to transmit the glucocorticoid signal was emphasized by the findings that even though knock-out mice lacking GR succumb at birth due to respiratory immaturity (Cole et al., 1995), mice that have been genetically modified so that their glucocorticoid receptors cannot bind DNA, but still can interact with other proteins, do not exhibit respiratory problems (Reichardt et al., 1998). This provides a clue to the mechanism of up-regulation of several lung-specific, GC-stimulated genes (such as the surfactant proteins and CC16), as their promoters do not include any functional GREs (Karim, 1998). Interestingly, C/EBPα knockout-mice (Flodby et al., 1996; Wang et al., 1995) also display severe respiratory problems and a very similar lung histopathology as the GR knockout-mice (Cole et al., 1995), supporting the possibility that these pathways may share common elements. Zhang and co-workers have studied the effects of GCs on local innate immunity and inflammation in the airway epithelium after TNF-α and TLR activation. They report that respiratory epithelial cells manifested an “acute phase response” upon TNF-α and TLR activation and that GCs spared, or enhanced, the epithelial expression of molecules involved in host defense, including complement, collectins and other antimicrobial proteins, but as expected, GCs inhibited expression of inflammatory cytokines and chemokines. Further, the selective effect on gene expression was shown to be mediated by activation of C/EBPβ. This brings forth an additional explanation for the ability of GCs to reduce exacerbations in COPD (Zhang et al., 2007).
Lung development

The section lung development is divided into two parts, one focusing on the structural development, and the other on cellular differentiation, primarily on differentiation of the lung epithelium.

Structural development

Lung development is traditionally divided into four stages that reflect the histological appearance of the lung during embryonic development (table 2). In general, growth and maturation of pulmonary structures proceed from the proximal to the peripheral portions of the lung, therefore, the various morphological–anatomical stages of development may overlap at various sites. The first stage, the pseudoglandular period, starts with the formation of the lung bud as a simple budding of the epithelium from the foregut into the surrounding mesenchyme at embryonic day (E) 9.5 in mouse (week 5 in humans). By interacting with the surrounding mesenchyme, the main parts of the bronchial and respiratory tree are formed during the rest of the pseudoglandular period that ends at E16.5 (week 17 in humans) by a tightly regulated process of epithelial branching, called branching morphogenesis (Ten Have-Opbroek, 1981; Warburton et al., 2000). During the next period, the canalicular (E16.5-17.5 in mouse and weeks 16-26 in human), the distal epithelium continues to branch through a process morphologically different from the preceding branching morphogenesis where the tubular branches increase in size and width (reviewed in Prodhan and Kinane, 2002). As the interstitial mesenchyme thins out, the pulmonary vasculature develops, tightly synchronized to the development of airways, resulting in the start of what will become the future air-blood barrier. This process initiates differentiation of the cuboidal epithelium lining the distal airway region into alveolar type I and type II cells and maturation of the primitive capillary network. Together this serves to form the prealveolar saccules and constitutes the start of the next stage of lung development, the saccular stage. The saccular stage spans through E17.5 to postnatal day (P) 5 in mice and weeks 24-38 in human embryonic development. The epithelial cells of the distal lung differentiate during the saccular period and now resemble cells from the fully developed lung. In parallel, the capillary network continues to mature and elastic fibers are deposited in the parenchyma, serving to prepare for alveolarization and respiration after birth (Roth-Kleiner and Post, 2005; Ten Have-Opbroek, 1981). Alveolarization is the final step in the development into a mature lung. During this stage, alveoli are formed by a process different from previous branching processes. Here, protein rich septa invaginate in to the prealveolar saccules which drastically increases the surface area of the lung. In humans, this period starts just prior to birth and continues approximately until the age of three, while in mice alveolarization occurs entirely postnatally (figure 6; reviewed in Groenman et al., 2005; Hislop, 2005; Roth-Kleiner and Post, 2005; Ten Have-Opbroek, 1981).

<table>
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<th>Table 2. Histological stages of murine lung development.</th>
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<td><strong>Stage</strong></td>
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<td>Pseudoglandular</td>
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<td>Canalicular</td>
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<td>Saccular</td>
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<td>Alveolar</td>
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Lung development follows a genetically controlled program. Reciprocal interactions between the endodermal epithelium and the surrounding mesodermal mesenchyme play a key role in inducing lung growth and branching in a temporal- and spatial-specific manner. Several central factors control this complex process. Those that are known to regulate early lung development include 1) the mesoderm-expressed fibroblast growth factor 10 (FGF10) that initiates lung bud formation; 2) the transcription factors GLI, NKX2-1 and endodermally expressed members of the Forkhead family (FOXs), and 3), and the epithelium-secreted molecules sonic hedgehog (SHH) and bone morphogenetic protein 4 (BMP4). SHH is upstream of the GLI and FOX transcription factors, and the FOX family in turn regulates the expression of FGF10, BMP4 and GLI3. Several other major signaling pathways have also been shown to take part in branching morphogenesis including additional members of the transforming growth factor-$\beta$ (TGF-$\beta$) superfamily, the WNT family and glucocorticoid signaling. Reviewed in (Cardoso, 2000; Groenman et al., 2005; Minoo, 2000; Warburton et al., 2000).

**Lung epithelial differentiation**

As branching of the respiratory tubules proceeds, numerous epithelial cell types are formed through cellular differentiation. Growth factor-mediated signaling between the endoderm and mesenchyme (as described above) as well as signals from the extracellular matrix collaborate with several transcription regulatory pathways in this process, together promoting tissue and cell specific gene expression (reviewed in Cardoso, 2000; Warburton et al., 2000). During embryogenesis, pulmonary structures generally mature in a wavelike fashion starting in the trachea and progressing towards the distal airways. During a substantial period of time before epithelial maturation is complete, there is a mixture of mature and immature cell types found within the same epithelium, and in most species, ciliated cells differentiate first and nonciliated cells with secretory granules appear next (Plopper et al., 1992). The earliest signs of cytodifferentiation of ciliated and nonciliated cells in mice appear in the proximal epithelium at E15 (pseudoglandular period) and E17 (canalicular period) respectively. The distal respiratory epithelium differentiate predominantly in the saccular–alveolar stages (from E17.5). However, the first sign of differentiation is a flattening of the epithelium, which is already seen in the canalicular stage around E16.5 (Kawamata and Fujita, 1983; Ten Have-Opbroek, 1981; Toskala et al., 2005).
Secreted molecules. Disruption of BMP4 and WNT-signaling involved in the growth factor-mediated signaling between the future respiratory epithelium and mesenchyme repress differentiation of the distal airway epithelium and cause expansion of the proximal epithelium into the distal region (Bellusc i et al., 1996; Mucenski et al., 2003; Weaver et al., 1999). TGF-β1, on the other hand, performs the opposite function, and instead promotes differentiation of the proximal epithelium (Masui et al., 1986) whilst inhibiting differentiation of alveolar type II cells in the distal region (Beers et al., 1998; Bragg et al., 2001). BMP-4 knockout mice die early in development before formation of the lung bud, and overexpression of BMP-4 in the respiratory epithelium of the developing lung results in a decreased number of mature type II cells (Bellusc i et al., 1996). The SHH signaling pathway, crucial for lung morphogenesis, is also essential for lung mesenchymal differentiation, but does not directly control epithelial cell differentiation. Abnormal expression of FGF18 has also been described to lead to proximalization of the lung (Whitsett et al., 2002) and FGF7 has been shown to be important for maintenance of the alveolar epithelium (Shannon et al., 2001; Sugahara, 1995). Glucocorticoid (GC) signaling is crucial for lung development and GR knockout-mice die at birth, due to respiratory dysfunction with severe atelectasis, in similarity with the Cebpa deficient mice, and they have higher proportions of type-II cells and undifferentiated alveolar epithelial cells as well as peri-saccular hypercellularity (Bird et al., 2007; Brewer et al., 2002; Cole et al., 1995; Flodby et al., 1996; Wang et al., 1995). GCs are used to enhance surfactant production and to promote distal alveolar development as means to reduce respiratory distress of preterm infants. However, GCs may diminish intrauterine growth, at the same time as they enhance maturation of type II cells (Bird et al., 2007; Grummer and Zachman, 1998; Nakahara et al., 2000).

Transcription factors. The initial step in lung development, the formation of the lung bud from the foregut, where endodermal cells becomes committed to respiratory epithelial cell lineages, is associated with the co-expression of members of three distinct families of transcription factors. These include NKX2-1, FOXA2, and GATA-6. FOXA2 is expressed in the pulmonary epithelium from day E10 and is subsequently expressed in both ciliated and non-ciliated cells of the bronchial epithelium, in addition to type II alveolar cells (Ikeda et al., 1996). Later in development, FOXA2 is restricted to a subset of bronchiolar and alveolar cells, inducing transcription of lung epithelial restricted genes, e.g., NKX2-1, SP-B and CC16 (Bohinski et al., 1994; Clevi dence et al., 1994; Ikeda et al., 1996; Perl and Whitsett, 1999). Deletion of FOXA2 disrupts formation of the foregut endoderm and all of its developmental derivatives, including the lung (Ang and Rossant, 1994). GATA-6 displays a similar, but not identical, expression pattern as NKX2-1 and FOXA2 during early lung development, and is believed to regulate expression of NKX2-1 (Shaw-White et al., 1999). GATA-6 is needed for the formation of respiratory bronchioles as well as for the regulation of genes, such as SP-A and SP-C, associated with differentiated respiratory epithelium. Disruption of the GATA-6 gene results in early embryonic lethality, occurring prior to lung bud formation (Morrissey et al., 1998). Also, NKX2-1 is detected in the epithelium of the primordial lung bud at E10 (Zhou et al., 1996), and is present in the nuclei of non-ciliated columnar epithelial cells early in lung development (Stahlman et al., 1996). As development progresses, expression of NKX2-1 becomes restricted to alveolar type II cells and proximal Clara cells (Lazzaro et al., 1991). NKX2-1 stimulates expression of SP-A, SP-B, SP-C, SP-D and CC16 in both mouse and human pulmonary epithelial cells (Groenman et al., 2005; Perl and Whitsett, 1999; Warburton et al., 2000). NKX2-1 also regulates the expression of WNT7B, important for lung development and cellular differentiation (Weidenfeld et al., 2002), and is further suggested to be an upstream regulator of BMP4 (Minoo et al., 1999). Disruption of the gene for NKX2-1 results in tracheoesophageal fistula and markedly hypoplastic lungs lined by a poorly differentiated respiratory epithelium lacking gas-
exchange areas and impaired lung branching morphogenesis (Kimura et al., 1996). Reviewed in (Perl and Whitsett, 1999; Roth-Kleiner and Post, 2005; Warburton et al., 2000).

C/EBPα plays a crucial role in the maturation of the respiratory epithelium and is regulated by NKX2-1 and FOXA2 during lung development (Cassel and Nord, 2003; Martis et al., 2006). One aim of the investigations included in this thesis is to study the role of C/EBPα in lung development and lung epithelial differentiation and will be discussed further in the results and discussion section. C/EBPα knockout-mice mice die from neo-natal hypoglycemia (Flodby et al., 1996; Wang et al., 1995; Sugahara et al., 2001) and deletion of C/EBPα in the respiratory epithelium also cause extensive neo-natal lethality, although due to respiratory failure at birth. The respiratory failure is associated with disrupted structural and biochemical maturation of the lung (paper III and IV; Flodby et al., 1996; Wang et al., 1995; Basseres et al., 2006; Martis et al., 2006). Many other transcription factors have been shown to be involved in lung cellular differentiation, and some are even determinants for cell type specific differentiation. FOXJ1 (also known as HFH-4) is exclusively expressed in ciliated cells (Blatt et al., 1999) and gain-of-function and loss-of-function studies have shown that FOXJ1 is essential for differentiation of columnar and ciliated airway epithelial cells (Chen et al., 1998; Tichelaar et al., 1999). FOXA1 (also known as HNF3-α) is expressed in respiratory epithelial cells of the conducting airways during development (Bingle et al., 1995). Its role is however not clear, and no abnormal lung phenotype is seen when the gene is disrupted (Kaestner et al., 1994).

Retinoic acids (RAs) are major regulators of lung development, and exert their signaling through the retinoic acid receptors (RARα, -β, and -γ) and the retinoic X receptors (RXRα, -β, and -γ). RA signaling is suggested to be of great importance in the very beginning of organogenesis and in the late phase of lung development. Maternal overdoses of retinoic acid and vitamin A deprivation, is associated with pulmonary agenesis and other defects in lung growth and lobulation. While gene targeting of individual RARs only provoke minor or no abnormalities of lung morphogenesis in mice (likely as a result of overlapping functions of the different receptor isotypes. 39,41), double knockouts of RARα and RARβ cause severe impairment in early pulmonary morphogenesis (Mendelsohn et al., 1994). RA signaling is crucial for the septation process, where particular fibroblasts that produce RA in the alveolar walls play a key role by producing elastin at the sites of outgrowth of new secondary septa (Shannon et al., 1998; Wessells, 1970). During lung development RARγ localizes preferentially to the epithelium of the distal endbuds and lungs of mice bearing deletions for RARγ produce less alveolar elastin and display a reduced number of alveoli (Hogan and Yingling, 1998). Also, RARα deletion and overexpression impaires alveolarization, while RARβ signaling in the early postnatal period is an inhibitor for alveolar formation. Reviewed in (Roth-Kleiner and Post, 2005).

Platelet derived growth factor-A (PDGF-A) signaling is an other of the central signaling pathways regulating alveolarization, and mice lacking PDGF-A die either at early gestation or within the first weeks of life due to emphysematous lungs without any signs of alveolarization (Boström et al., 2002; Boström et al., 1996). NMYC displays lung epithelial-specific expression in the lung and is believed to be crucial for lung epithelial differentiation and proliferation (Okubo et al., 2005a). Consequently, mice with a deleted Nmyc gene also die of respiratory problems (Moens et al., 1993). In addition to GATA-6, GATA-5 is also expressed in the developing lungs but its role is still not clear, however it appears not to be as essential as GATA-6 (Perl and Whitsett, 1999). The nuclear factor I family (NFI) is involved in the regulation of the gene for SP-C that is associated with type II cell differentiation (Bachurski et al., 1997). Activation protein 1 (AP-1) and retinoic acid receptors (RARs) co-regulate several of the respiratory epithelial differential dependent genes with
other transcription factors more fundamental for lung epithelial differentiation, such as NKX2-1, FOXA2 and GATA-6 (Perl and Whitsett, 1999). Components of the EGF signaling pathway, including EGF, TGF-α, and epithelial growth factor receptor (EGFR), are expressed in lung. EGF signaling has been suggested to promote respiratory epithelial cell differentiation and induce surfactant lipid and protein expression. However, the role of EGF in lung development and epithelial differentiation is not generally accepted. (Kaartinen et al., 1995; Miettinen et al., 1997; Whitsett et al., 1987). Insulin-like growth factor (IGF) receptors are expressed in a stage- and cell-specific manner in the developing and postnatal lung, and one of the receptors, IGFR-2, has been suggested as a marker of mature alveolar type 1 cells (Maitre et al., 1995). Understanding the complex interactions among the signaling pathways directing pulmonary morphogenesis and epithelial differentiation will likely provide knowledge important in understanding the pathogenesis of inherited and acquired lung diseases, which will hopefully lead to advances in diagnosis and therapy of pulmonary disorders in the future.
SCOPE OF THE PRESENT INVESTIGATION

To establish the expression and activation of C/EBPs in the respiratory epithelium of patients with chronic obstructive pulmonary disease (COPD).

To investigate the role of C/EBPβ as a mediator of glucocorticoid signaling in the respiratory epithelium.

The investigate the murine developmental expression of C/EBPα in the lung, and its role in cellular differentiation in the lung epithelium.
COMMENTS ON METHODOLOGY

**Bronchial brush biopsies**

Biopsies can be taken at different depths of the airways, from bronchial mucosal biopsies of the large airways to transbronchial biopsies of the peripheral airways and lung parenchyma. Compared with these, bronchial brush biopsies are much safer for the patient and sample a larger area of the airway and gives information specific to the superficial airway epithelium, since principally cells above the basement membrane are collected. Bronchial brush biopsies are dominated by >90% epithelial cells, but inflammatory cells have also been documented (Riise et al., 1996; Riise et al., 1992). Comparisons between endobronchial and transbronchial biopsies show evidence of differences in inflammation between central airways, peripheral airways and the alveolar region (Holz et al., 2000), although there are marked similarities in epithelial inflammation in all compartments of the lung, suggesting that the lung epithelium responds in some aspects as one entity. In order to sample the upper airways of patients and control subjects included in paper I and II, we used the clinical protocol established by Riise and colleagues (Riise et al., 1994). Approximately one million cells were obtained by this method which enabled preparation of nuclear extracts for further analysis by electrophoretic mobility shift assay (EMSA). In comparison to endobronchial biopsies, this method provides the advantage of studying not only the presence of C/EBPs, as can be done with immunohistochemistry, but also the DNA-binding activity. This is due to the fact that high quality nuclear extracts are harder to prepare from whole tissue biopsies due to a higher fraction of extracellular matrix proteins that is avoided in the brush biopsies that yields free epithelial cells, with only a minor contamination of other cell types. An alternative approach is to obtain primary human airway epithelial cells from surgically resected specimens, but obviously this limits the possible study population.

**Lung epithelial cell lines**

Human primary epithelial cells can be retrieved by various methods (including surgical dependent and independent methods) and are also commercially available. However, it is difficult to keep primary lung epithelial cells in a differentiated and proliferative state in cell culture. Consequently cell lines is often used for mechanistical studies since they are well characterized, not contaminated with other cell types and easy to work with. Human derived cell lines frequently used to model the lung epithelium include A549, Calu-1, NCI-H441 and BEAS-2B. These cell lines are derived from different cell types. A549 and NCI-H441 cells are both derived from human lung adenocarcinomas but A549 cells are classified as being of alveolar type II cell origin, whereas NCI-H441 are classified as being of Clara cell origin (Giard et al., 1973; American Type Culture Collection (ATCC)). The Calu-1 cells are derived from a squamous cell carcinoma (Fough, 1975) and BEAS-2B cells were isolated from normal human bronchial epithelium and immortalized with adenovirus 12-SV40 (Reddel et al., 1989). The NCI-H441 cells show characteristics of a natural differentiated Clara cell and do express SP-A and SP-B (Kalina et al., 1992). However, NCI-H441 cells do not express CC16 but do support transcription of the CC16 promoter and express C/EBPβ and C/EBPδ (Nord et al., 1998). As this cell line has been previously used for studying transcription and does respond to glucocorticoid treatment, the NCI-H441 cells were used in paper II to study the involvement of C/EBPs in glucocorticoid regulation of gene transcription.
Generation of genetically modified mice

As a means to deepen our understanding of the role of the C/EBP-family in the lung, and to critically address whether C/EBPs have a role in COPD pathogenesis and related pathological processes, we wanted an animal model in light of the limitations in sampling the human lung. A number of different species are used as animal models for the human respiratory system, including monkey, horse, pig, dog, cat, sheep, rabbit, ferret, guinea pig, rat and mouse (Kurucz and Szelenyi, 2006). However, the large number of genetic and molecular tools available for the mouse, in addition to the possibility of using inbred mice that are genetically identical, is a considerable advantage that outweighs the value of other animal species. In order to study the function of C/EBPα in the lung epithelium in vivo, we generated mice that over-expressed C/EBPα specifically in the lung epithelium \((SFTPC-Cebpα\) mice), and mice with a lung epithelial specific disruption of the \(Cebpα\) gene \((Cebpα^{ΔLE}\) mice; figure 7). Mice with disruption of the \(Cebpα\) gene in the whole organism exist, however, they display neonatal lethality due to hypoglycemia (Flodby et al., 1996; Wang et al., 1995), and the impact of lung epithelial deletion of \(Cebpα\) in these mice is not easy to assess since the phenotype of these mice reflects the combined impairment of \(Cebpα\) in all cells that normally express C/EBPα. Thus, in order to address the role of C/EBPα specifically in the lung epithelium, we generated a conditional knockout-mouse using the Cre-LoxP system (reviewed in Kos, 2004; Kwan, 2002; Kühn and Torres, 2002).

Briefly, the gene of interest, here \(Cebpα\), is flanked on each side by a short base pair sequence, LoxP sites, without affecting the expression or function of the gene. This is done by homologous recombination in murine embryonic stem (ES) cells from the 129 strain. Clones of ES-cells whose genome had undergone recombination is then selected and introduced into an early recipient mouse embryo (usually at the blastocyst stage) from the C57/B6 strain. In some of the resulting off-spring, the recombined ES-cells will have contributed to the germline and these mice are used for breeding with C57/B6 mice to generate mice that carry the recombined ‘floxed’ (flanked by LoxP sites) allele \(Cebpα^{fl/+}\) mice; this strain was generated by Gonzalez and colleagues, NCI, NIH, USA). To generate a tissue specific knockout-mouse, a “floxed” mouse-strain is intercrossed with a mouse strain expressing the Cre-recombinase in the specific tissue or cell type of interest, commonly generated by the procedure described above. Cre recognizes the LoxP-sequences and excise the gene in between the LoxP-sites, generating a conditional knockout. We use a mouse strain in which the Cre recombinase was specifically expressed in the lung epithelium under the control of the 3.7 kb human Surfactant protein C (SP-C) promoter \((SFTPC-Cre\) mice), which is active in all lung epithelial cells in mice. The \(SFTPC\)-promoter is active in all epithelial cells of the primitive lung and commences expression from at least E10 during development (Okubo and Hogan, 2004; Okubo et al., 2005b).

The use of inbred strains (e.g. the 129 and C57/B6 strain) have the strong advantage over outbred strains, that mice from different litters will be genetically identical. However, if different inbred strains are intercrossed this homology will be abruptly lost. This is a common problem when genetically modified mice are generated due to the common procedure where available efficient ES-cell lines are derived from the 129 strain, that are then injected into blastocysts from C57BL/6 mice and the use of C57BL/6 mice as the breeding strain to efficiently screen for transmission of the ES-cell, and thus 129 derived genome. Both the \(Cebpα^{fl/+}\) mice and the \(SFTPC-Cre\) mice used in paper IV were generated in this way, although, the \(SFTPC-Cre\) mice were backcrossed (consecutive crossings with mice from a specific genetic background) on the outbred ICR strain that is well known to generate large litters. Hence, the offspring generated when \(SFTPC-Cre\) mice were intercrossed with \(Cebpα^{fl/fl}\) mice were a mix of two different inbred strains and an outbred strain.
Offspring from outbred strains and mixed inbred strains do not have a pure genetic background and should preferably only be compared within the litter to keep variation due to genetic background as small as possible. A genetic modification can however be transferred to an inbred genetic background again, by at least 9 consecutive backcrosses, which generates a congenic strain with <99.5% genetic homology with the inbred strain used for backcrossing (Armstrong et al., 2006).

Cre-recombinase only needs to be expressed at one occasion in a progenitor cell to permanently excise the gene of interest in all cells derived from that progenitor cell. Rosa26 reporter mice (ROSA26Sortm1Sor/tm1Sor) that harbor the β-galactosidase gene is commonly used to monitor Cre-induced recombination. When Rosa26 reporter mice is intercrossed with a Cre-expressing strain, Cre will excise a stop-codon that has been inserted within the promoter controlling the β-galactosidase gene in Rosa26 reporter mice, which results in β-galactosidase expression in all cells were Cre is expressed or cells originating from precursor cells were Cre has been expressed (Soriano, 1999). In contrast to the CebpaΔLE mice with disruption of the Cebpa gene in all lung epithelial cells, in the SFTPC-Cebpa mice, the ectopic Cebpa-expression is restricted to the cells where the SFTPC-promoter is currently active. Expression from the SFTPC-promoter has been shown to be restricted to the lung epithelium, with higher expression in the distal region during lung development (Okubo and Hogan, 2004; Okubo et al., 2005b; Wert et al., 1993).

Figure 7. Generation of a lung epithelial specific Cebpa knockout-mouse (CebpaΔLE)
RESULTS AND DISCUSSION

Over the last decade, a previously unknown role for a group of transcription factors called CCAAT/enhancer binding proteins (C/EBPs) has been established in the regulation of lung gene expression (Cassel et al., 2000a; Cassel and Nord, 2003; Cassel et al., 2000b; Cassel et al., 2000c; Nord et al., 1998). C/EBPα is an important regulator of differentiation in the lung and other organs such as liver and adipose tissue. It stimulates the expression of genes characteristic of the mature differentiated organ and inhibits proliferation (paper III; McKnight, 2001; Ramji and Foka, 2002). In addition to C/EBPα, two other C/EBPs are expressed in the lung: C/EBPβ and C/EBPδ (paper III; Cassel and Nord, 2003). Although these three C/EBPs exhibit overlapping target gene specificities, C/EBPβ has a specific role in inflammatory and defense responses, most well-studied in myeloid cells. Here, C/EBPβ regulates inflammatory genes and genes involved in the innate immune response (Poli, 1998; Ramji and Foka, 2002). Until our publication in 2005 (paper I), there were no studies investigating whether or not there is an association between the C/EBP transcription factor family and COPD. At the time, only two publications linked C/EBPs to specific lung diseases. These publications investigated the loss of expression of C/EBPα in non-small cell lung cancers that was suggested to contribute to the dysregulated cell growth (Halmos et al., 2002), as well as decreased C/EBPα expression in bronchial smooth muscle cells of asthmatics, associated with the smooth muscle hypertrophy occurring in asthma (Roth et al., 2004).

C/EBP activity in the airways of patients with COPD and chronic bronchitis

The airway epithelium has several defense mechanisms in response to the stress imposed by cigarette smoke, and in some individuals these defenses are exaggerated, resulting in chronic inflammation, and eventually, chronic bronchitis (Hogg, 2004b; Maestrelli et al., 2001; Mills et al., 1999; van der Vaart et al., 2004). The C/EBP transcription factor family has a well-established role in other tissues as regulators of inflammatory processes and immunological defenses, in addition to a key role in differentiation. C/EBPs are known to activate many genes involved in defense mechanisms implicated in the pathogenesis of COPD, including anti-oxidative genes (such as heme oxygenase-1 and superoxide dismutases (Alam, 1994; Jones et al., 1997), and genes within the innate immune system, both receptors such as some of the TLRs, and effectors such as several antibacterial peptides and collectins (Cassel and Nord, 2003; Diamond et al., 2000; He and Crouch, 2002; Musikacharoen et al., 2001; Poli, 1998; Ramji and Foka, 2002). Thus, it is not unlikely that the C/EBP family could have a role in COPD-pathogenesis, as it is a complex disorder with many processes that have links to C/EBPs. As a first approach to investigate whether the C/EBP transcription factor family could be involved in COPD pathogenesis, DNA binding activity of C/EBPs were analyzed in the airway epithelium of patients with chronic bronchitis and COPD (paper I). To do this, we developed a methodology that combines bronchial brush biopsies as mean to sample the airway epithelium, with electrophoretic mobility shift assays (EMSAs) to study transcription factor DNA-binding activity. The airways were brushed very gently to avoid sub-epithelial cell contamination and to avoid contamination by blood leukocytes (Riise et al., 1994). Interestingly, C/EBPβ-activity in the airway epithelium of healthy smokers was four-fold increased compared to healthy never-smokers, suggesting that tobacco smoke induces increased DNA-binding activity of C/EBPβ. However, smokers that have developed chronic bronchitis and/or COPD lack this activation of C/EBPβ in their lung epithelium. C/EBP-mediated regulation is highly cell type
specific, which also seems to be the case in COPD, since Borger et al recently found that C/EBPβ activity was decreased in smooth muscle cells from COPD patients compared to healthy non-smokers, even though healthy smokers would have been a more appropriate control group for COPD patients that most often have a significant smoking history (Borger et al., 2007).

Tobacco smoke induce disruption of the bronchial epithelium, but the epithelium does not typically demonstrate epithelial necrosis, instead the disruption is suggested, at least partially, to be a result of smoke-induced apoptosis, a process associated with C/EBPβ (Buck et al., 2001a; Gelbman et al., 2007; Hoshino et al., 2001; Jeffery and Reid, 1981b; Liu et al., 2003). An important aspect of tobacco smoke-induced injury is the activation of toxic substances in the inhaled smoke following metabolism by cytochrome P450 (CYP) enzymes. Nitrosamines are compounds found in tobacco smoke that become highly active and toxic during metabolism by the selectively expressed and C/EBP-regulated CYP2A13 in the lung epithelium (Ling et al., 2007b). Also, the C/EBP-regulated CYP2B1 (Berg et al., 2002) is expressed in the lung epithelium and metabolizes both certain nitrosamines and other toxic compounds from tobacco smoke, including nicotine. The airway epithelium serves as the first barrier between the inhaled air, that can carry hazardous substances such as tobacco smoke or pathogens, and the body. The airway epithelium is also a major regulator and contributor to the inflammatory and defensive processes in the lung, and is suggested to play a major role in COPD pathogenesis (Barnes, 2008; Mills et al., 1999). We hypothesize that altered activity of C/EBPβ in airway epithelial cells has a previously unknown role in the pathogenesis of COPD, where it could affect the production of inflammatory mediators, genes involved in the antioxidative and infectious defenses, in addition to affecting cell proliferation, thereby making the lungs more susceptible to inflammation, which potentially leads to tissue destruction and increased obstruction of the airways. As there is clearly a gap in our knowledge about the role of this group of transcription factors in COPD, these data suggest that investigations on the role of C/EBPs in the pathogenesis of COPD could provide important insights for the development of new treatments for this devastating disease.

C/EBP expression in the COPD lung and in the mouse lung

To further investigate whether the C/EBP transcription factor family could have a role in COPD pathogenesis, we looked at the expression of C/EBPs in sections obtained from volume-reduction surgery for emphysema, as well as in tissue sections with normal histology from patients that underwent lung surgery of other causes (preliminary results). Here, we found that both C/EBPβ and C/EBPα were expressed in all types of lung epithelium represented in the specimens, ranging from pseudo-stratified airway epithelium to the alveoli. C/EBPβ was also expressed in alveolar and airway macrophages. This is in agreement with previous publications of the murine lung that have reported C/EBPα expression in alveolar type II and lower levels in the bronchiolar Clara cells (Cassell et al., 2000b; Li et al., 1995; Nord et al., 1998; Sugahara et al., 1999), C/EBPβ expression in type II cells (Rosenberg et al., 2002; Sugahara et al., 1999), and high-levels of C/EBPδ expression in the bronchiolar epithelium and lower levels in alveolar type II cells (Cassell et al., 2000b; Lag et al., 2000; Rosenberg et al., 2002; Sugahara et al., 1999). Further, the number and proportion of C/EBPβ-expressing alveolar macrophages in sections from patients with emphysema were increased compared to controls. As with the airway epithelium, alveolar macrophages have been suggested to play a major role in the pathogenesis of COPD, and C/EBPβ is a key regulator of macrophage differentiation and activation. Thus, the increased expression of C/EBPβ could represent a dysfunctional activation of macrophages, that keeps the alveolar macrophages in an
activated state long after the initial inflammatory trigger (tobacco smoke and pathogens) is removed, thereby continuing to promote destruction of the lung parenchyma.

As a means to deepen our understanding of the role of the C/EBP-family in the lung, and to critically address whether C/EBPs have a role in COPD pathogenesis and related pathological processes, we wanted an animal model in light of the limitations in sampling the human lung. A number of different species are used as animal models for the human respiratory system, including monkey, horse, pig, dog, cat, sheep, rabbit, ferret, guinea pig, rat and mouse (Kurucz and Szelenyi, 2006). However, the large number of genetic and molecular tools available for the mouse is a considerable advantage that outweighs the value of other animal species. In paper III, we therefore investigated the respective expression patterns of the different C/EBPs in the mouse lung, both in the adult lung and during development. In the adult mouse lung, C/EBPα is expressed at low levels in most epithelial cells lining the distal conducting airways, and at higher levels in type II cells of the alveolar region in adult mice (Paper III and IV). But C/EBPβ and C/EBPδ were expressed in cells of the conducting airway epithelium, and in the alveolar region in adult mice. Our results are in agreement with the few existing publications on adult murine expression of C/EBPs (reviewed in (Cassel and Nord, 2003)). The adult expression pattern of C/EBPs in mouse (paper III) is highly similar to the human lung expression pattern (preliminary results) of C/EBPs, suggesting the mouse as a suitable model to study the role of the C/EBP transcription factor family in the lung.

**Adult lung epithelial C/EBPα knockout-mice develop a histopathology resembling COPD**

To critically address the role of the C/EBP-family in the lung epithelium and in the COPD lung, lung epithelial specific C/EBPα knockout-mice (Cebpa^ALE^ mice, paper IV) and C/EBPβ knockout-mice (Cebpa^ALE^ mice; see future perspectives), were generated using the Cre-Lox P technique. About 56% of the Cebpa^ALE^ mice died within hours after birth due to respiratory distress in accordance with previous publications (Basseres et al., 2006; Flodby et al., 1996; Martis et al., 2006; Wang et al., 1995). But when followed up to 3 months of age, none of the Cebpa^ALE^ mice surviving the perinatal period died. In contrast to Cebpa^fl/fl^ littermates, adult Cebpa^ALE^ mice surviving the perinatal lethality demonstrate a severe pathological picture with 1) goblet cell hyperplasia, bronchiolar metaplasia, fibrosis and mucus plugging, together pathologically defined as bronchiolitis, 2) emphysema and 3) extensive macrophage and lymphocyte infiltrations. As outlined above (see background), the diagnosis of COPD is based on clinical and functional features, but there are well-recognized histopathological correlates, including all the histopathological findings in the adult Cebpa^ALE^ mice (Barnes, 2000; Barnes, 2004b; Rabe et al., 2007; Travis et al., 2002). Although, the etiology of COPD is in more than 90% of patients associated with longtime exposure to tobacco smoke (Barnes, 2004b). An animal model covering all aspects of COPD is most likely never going to be discovered, yet Cebpa^ALE^ mice develop an adult lung phenotype with a histopathology similar to the histopathology in COPD, and it is tempting to speculate that these two pathological processes share at least some underlying mechanisms (see below).
C/EBPβ as mediator of glucocorticoid signaling in the lung epithelium

In most countries inflammatory lung diseases such as asthma are treated with inhaled glucocorticoids. Even though it is widely accepted that the success of glucocorticoids directly reflects its anti-inflammatory effects, such as inhibition of the expression of cytokines and chemokines that activate endothelial cells, leukocytes, and lymphocytes, the precise mechanisms by which steroids improve lung function remain unclear (Zhang et al., 2007). COPD has many similarities to asthma but one of the clinical definitions that differentiate between the two diseases is whether or not airway obstruction is reversed by glucocorticoids and β2-agonists. In paper II, we set out to study glucocorticoid signaling in the lung epithelium in an attempt to understand the underlying mechanisms better. We found that C/EBPβ is the most active C/EBP factor in both human and mouse lung epithelium, and that glucocorticoids induce DNA binding of C/EBPβ in cultured primary mouse lung epithelial cells. Mechanistic studies in the human epithelial cell line NCI-H441 revealed that glucocorticoids, acting via the glucocorticoid receptor, increase C/EBP-binding as soon as 10 minutes after stimulation. The mechanism is independent of de novo protein synthesis and involves phosphorylation of C/EBPβ at Thr^235. Together, these data indicate that glucocorticoids increase the DNA binding activity of C/EBPβ via post-translational mechanism(s) involving phosphorylation.

The two transcription factors C/EBPβ and NFκB master the expression of pro- and anti-inflammatory genes in addition to acute phase genes, including host defense genes, in a highly complex and cell type specific manner. This regulation is typically commanded by protein-protein interactions and/or by altering the phosphorylation status of the several regulatory phosphorylation sites on C/EBPβ and NFκB (Basak et al., 2005; Faour et al., 2006; Gorrill and Khalili, 2005; Plevy et al., 1997; Poli, 1998; Stein and Baldwin, 1993; Stein et al., 1993; Zwergal et al., 2006). These interactions can both result in positive synergistic effects on gene expression of target genes, but C/EBPβ can also repress NFκB activity (Betts et al., 1993; Dunn et al., 1994; Lee et al., 1996; Li and Liao, 1992; Matsusaka et al., 1993; Plevy et al., 1997; Poli, 1998; Ray and Hannink, 1995; Stein and Baldwin, 1993; Zwergal et al., 2006). Recent finding by Zhang et al show that GCs spare or enhance local innate host defense responses in addition to exerting anti-inflammatory actions in the lung epithelium through a C/EBP-dependent mechanism, which suggest that C/EBPβ can mediate an increased protection against exacerbations in COPD by inducing an innate immune response (Zhang et al., 2007). Our findings in paper I and II suggests, together with the findings by Zhang et al that altered C/EBPβ levels could directly affect the responsiveness to glucocorticoid treatment of inflammatory lung disorders such as COPD and asthma by various mechanisms, which implicates C/EBPβ as a potential novel target for treatment of these devastating diseases.

C/EBPs in COPD - a summary

We have studied the role of C/EBP transcription factors in the human and mouse lung as well as in lungs from patients with COPD. In both the adult mouse (paper III) and human lung (preliminary results), C/EBPα and C/EBPβ are expressed in all types of airway epithelium from cuboidal to pseudo-stratified epithelium and also in alveolar pneumocytes. In addition, C/EBPβ is expressed in alveolar macrophages, and both the number and proportion of C/EBPβ-expressing alveolar and airway macrophages are increased in subjects with emphysema compared to controls. Furthermore, when studying the DNA-binding activity of C/EBPs in the airway epithelium of both humans and mice, we found that C/EBPβ is the dominant DNA-binding C/EBP transcription
factor, and that less activity was seen from C/EBPδ and almost no activity from C/EBPα (paper I and II). The lack of C/EBPα DNA binding activity, despite its presence in the airway epithelium, could suggest that the most important function of C/EBPα in the adult lung is to inhibit proliferation by p21waf1- and cdk2/4-mediated pathways (Timchenko et al., 1996; Wang et al., 2001), a notion further supported by its specific expression at the non-proliferating branch points during lung development (paper III). When we studied C/EBP DNA-binding activity in the airway epithelium from patients with COPD and healthy volunteers, we found that C/EBPβ activity was 4-fold increased in healthy smokers compared to smokers with COPD (paper I). Patients with COPD had the same level of DNA-binding activity as healthy never-smokers, which raises the possibility that injury to the epithelium caused by tobacco-smoke induces C/EBP-activity in healthy smokers. We hypothesize that this serves as a protective mechanism that could stimulate regeneration of the lung epithelium through promotion of cell proliferation. It would also inhibit apoptotic signals induced by the tobacco-smoke and activate early inflammatory genes and defense mechanisms against infection. Thus, activation of C/EBPβ would be of importance to maintain a functional epithelium and adequate defenses against bacterial colonization. It also implies that the low C/EBPβ-binding activity we observed in smokers with chronic bronchitis and COPD reflect an inability to activate C/EBPβ. This could have pathogenic implications, as it renders the epithelium incompetent of efficient regeneration and increases sensitivity to infection, accentuating the vicious circle in the development of COPD.

The inflammation in COPD typically exhibits partial resistance to the anti-inflammatory action of glucocorticoids (Barnes, 2003; Jeffery, 1998). When studying glucocorticoid signaling in the lung epithelium we found that C/EBP, at least partially, mediates the effects of glucocorticoids in the lung epithelium by inducing phosphorylation of Thr235 on C/EBP, hence augmenting its DNA-binding activity (paper II). This raises the possibility that the decrease in C/EBP binding activity in the airway epithelium of patients with COPD could be a contributing factor to the relative resistance to glucocorticoids seen in this disease.

**C/EBP expression during murine lung development**

As a means to deepen the understanding of the role of the C/EBP family in lung biology and provide further insights into the potential role of the C/EBP family in COPD and other lung diseases, we investigated their function in lung development and lung epithelial differentiation. As a first approach, we looked at the developmental expression of C/EBPs in the mouse lung using immunohistochemistry (paper III). C/EBPα displayed a dynamic expression pattern with the first evidence of expression seen at embryonic day (E) 15.5 (the pseudoglandular period), with expression mainly in a subset of cells restricted to the epithelium in the distal parts of the developing tubules. The expression was (at E17.5) more widespread in the future alveolar region and was also seen in the epithelium lining the future distal conducting airways. During the saccular stage (at E18.5), the expression of C/EBPα increased and started to resemble the adult expression-pattern, with low-level C/EBPα expression in most epithelial cells lining the distal conducting airways and higher levels of expression in the type II cells of the alveolar region (Paper III and IV). C/EBPβ was detected during lung embryogenesis from the late pseudoglandular stage (from E 15.5) in both the epithelium and the mesenchyme. From the saccular stage (at E18.5) to adulthood, both C/EBPβ and C/EBPδ were expressed in cells of the conducting airway epithelium and the alveolar region (paper III), in agreement with the few existing publications on C/EBP expression during murine development (Barlier-Mur et al., 2003; Birkenmeier et al., 1989; Lu et al., 2004). In microarray
analysis of developing mouse lung both C/EBPβ and C/EBPδ have been detected in the branching region whereas C/EBPα could not be detected at E11.5 (Lu et al., 2004). In whole rat lung, mRNA expression of C/EBPα, C/EBPβ and C/EBPδ has been seen to increase just prior to birth, decreasing around the time of birth, after which it increases again (Barlier-Mur et al., 2003). The dynamic expression of C/EBPα during lung development (paper III) and the respiratory distress in a fraction of the neonatal Cebpα−/− mice (Flodby et al., 1996; Wang et al., 1995) inspired us to further investigate the role of C/EBPα in lung development by generating a gain-of-function mouse model with ectopic expression of C/EBPα (SFTPC-Cebpa mice; paper III) as well as a loss-of-function mouse model with lung epithelial specific disruption of the Cebpa gene (CebpaΔLE mice, paper IV) as a complement to the previously published embryonic Cebpα−/− mice.

**Ectopic expression of C/EBPα in mouse affects lung development**

In paper III, we studied the role of C/EBPα during lung development and lung epithelial differentiation by the generation of lung epithelial C/EBPα gain-of-function mice with the Cebpa gene under the control of the human surfactant protein C promoter (SFTPC-Cebpa mice). Expression from the human SFTPC-promoter is induced from at least E10, several days earlier than expression from the endogenous Cebpa gene (E15.5), and is restricted to the lung epithelium with higher expression in the distal region when present in mice (Okubo and Hogan, 2004; Okubo et al., 2005b; Wert et al., 1993). C/EBPα was readily detected in most cells of the distal lung epithelium at E15.5 in SFTPC-Cebpa mice, in comparison to the discrete expression of just a few cells in wild type mice. Further, SFTPC-Cebpa mice display fewer and larger developing epithelial tubules than their wild type littermates, indicating that the tempo-spatial expression of C/EBPα is important for correct development during the late pseudoglandular stage (from E15.5). To investigate whether forced expression of C/EBPα resulted in a premature differentiation of the lung epithelium, the expression of several lung epithelial differentiation markers including NKX2-1, pro-surfactant protein C, cytokeratin 8, SP-A and CC16 were studied. No evidence of altered lung epithelial differentiation could be seen as early as E15.5. We also investigated the effects on the developing mesenchyme using the mesenchymal marker α-smooth muscle actin, in addition to morphometric quantification of the lung mesenchymal area. However, both assays indicated that mesenchymal development was not affected at this early time point. SP-A and CC16 are both known to be regulated by C/EBPα and are not normally expressed at this early stage (E15.5) (Korfhagen et al., 1992; Zhou et al., 1996). Neither of them were expressed at E15.5 in SFTPC-Cebpa mice, indicating that ectopic expression of C/EBPα from an earlier time point than normal is not enough to commence premature maturation of the lung epithelium as could have been expected (Cassel et al., 2002). It is likely that the additional factors needed for a high level of CC16 expression are lacking in the lung epithelium at this particular developmental stage.

**Disruption of the C/EBPα gene impairs lung development and epithelial differentiation**

In parallel to the generation of our gain-of-function model for C/EBPα, we generated a loss-of-function model as a complement to the previously published (Flodby et al., 1996; Wang et al., 1995) whole body Cebpα−/− mice (paper IV). A conditional knockout-mouse with a lung epithelial disruption of the Cebpa gene (CebpaΔLE mice) was generated by intercrossing a transgenic mouse strain with the Cre recombinase regulated by the human SP-C promoter (SFTPC-Cre mice), with a floxed (see
C/EBPα strain (CebpaΔLE mice). Cebpa−/− mice have previously been generated (Flodby et al., 1996; Wang et al., 1995), but have not been studied in detail during the embryonic lung development, so we therefore followed both the Cebpa−/− mice (paper III) and the CebpaΔLE mice (paper IV) during development. The lung phenotype of Cebpa−/− mice starts to diverge from wild type mice around E17.5, and is characterized by a decreased number of growing epithelial tubules that are larger in size, along with a thickened interstitium. A very similar phenotype is seen in the CebpaΔLE mice (E18.5) and surprisingly also in the SFTPC-Cebpa mice (paper III, see above). The unexpected finding that lungs of mice over-expressing C/EBPα in the lung epithelium display a similar phenotype as mice that lack C/EBPα in the lung epithelium suggests the need for C/EBPα to be expressed at the right level, but also at the right time and place, during development.

The expression pattern of the airway epithelial cell markers CK-8, CC16 and E-cadherin, from development (E18.5) to adulthood, in CebpaΔLE mice clearly demonstrate that all levels of the lung respiratory tree, including the alveolus and alveolar parenchyma, are affected to different extents by the lung epithelial specific disruption of C/EBPα (paper IV). CC16-expressing cells in the proximal conducting airways of adult CebpaΔLE mice are reduced in number and display an enlarged morphology. Still, both the conducting airway markers CC16 and CK-8 exhibit normal expression in the disrupted distal conducting airway epithelium of CebpaΔLE mice. However, CK-8 also display a notable expression in groups of cells in the alveolar wall and in cells within the interstitium, suggesting an impaired differentiation of the alveolar epithelium. In CebpaΔLE mice, E-cadherin, which normally exhibits an expression pattern specific for the conducting airways, is aberrantly expressed both in cells lining the alveolar/emphysematic airspaces in proximity of bronchioles, and also in cells within the interstitium. Furthermore, we used proSP-C as a marker for type II pneumocytes as a mean to study alveolar epithelial differentiation in both Cebpa−/− (paper III) and CebpaΔLE mice (paper IV). SP-C expressing cells were found in the interstitial septa in addition to lining the alveoli in Cebpa−/− mice (E17.5) (paper III) and CebpaΔLE mice (from E18.5, paper IV). Additionally and the number of SP-C expressing cells is increased in CebpaΔLE mice compared to Cebpafl/fl mice (paper IV), as previously described by Basseres et al in embryonic CebpaΔLE mice.

Together, these findings suggest that deletion of the Cebpa gene leads to an impairment of lung epithelial cell differentiation that induces two parallel processes: 1) Bronchiolar metaplasia, supported by the finding of the conducting airway markers CK-8 and E-cadherin in cells lining the alveolar/emphysematic airspaces, and 2) Transdifferentiation of epithelial cells to mesenchymal cells (a process that can be referred to as epithelial-mesenchymal transition; EMT (Kim et al., 2006) that previously have been associated with C/EBPs (Stahlman et al., 1996), supported by the presence of SP-C and E-cadherin positive cells interspersed within the lung interstitium, which likely contribute to the increased extra cellular matrix deposition and interstitial fibrosis in the CebpaΔLE mice.

C/EBPα and lung epithelial proliferation

C/EBPα is an inhibitor of proliferation and is absent from proliferating cells in other organs such as liver and fat (Birkenmeier et al., 1989). To investigate the relationship between cellular proliferation and C/EBPα expression during lung development, immunohistochemistry against markers for cell proliferation was performed on CebpaΔLE mice (paper IV) and SFTPC-Cebpa mice (paper III). A very limited increase in phospho-histone H3 expression could be seen in CebpaΔLE
mice, indicating that the lung phenotype of $\text{Cebpa}^{\Delta LE}$ mice is foremost a consequence of impaired differentiation of the lung epithelium rather than a consequence of increased proliferation (paper IV). No differences in either phospho-histone H3 or cyclin A expression, nor in the overall lung size could be seen between $\text{SFTP\text{-}Cebpa}$ mice and wild type littermates (paper III). Thus, the phenotype of neither $\text{SFTP\text{-}Cebpa}$ mice, nor $\text{Cebpa}^{\Delta LE}$ mice, can be explained by a general inhibition, or stimulation, of growth that could be expected due to the known anti-proliferative activity of C/EBP$\alpha$ (Harris et al., 2001; Timchenko et al., 1996; Wang et al., 2001).

However, in immunofluorescent double staining of C/EBP$\alpha$ and PCNA on lungs from wild type mice we observed that most of the cells expressing C/EBP$\alpha$ at E17.5 do not proliferate (paper III). This suggests that C/EBP$\alpha$, under normal conditions in the lung, and similarly to what has been seen in other organs, is expressed in non-proliferating cells and could possibly have a role in the regulation of cell growth during development. Epithelial deposition of the extra cellular matrix protein fibronectin is known to be important for lung branching morphogenesis (Sakai et al., 2003), and fibronectin displays an abnormal distribution in the lungs of embryonic $\text{SFTP\text{-}Cebpa}$ mice, suggesting a disturbed branching morphogenesis in the $\text{SFTP\text{-}Cebpa}$ mice. Based on these observations, a model for how C/EBP$\alpha$ could modulate branching morphogenesis during lung development was proposed in paper III. In the proposed model of branching morphogenesis, C/EBP$\alpha$, due to its antiproliferative properties, helps to define cells close to the tips of the growing epithelial tubules that are to stop proliferating. Cells that do not express C/EBP$\alpha$ is then allowed to proliferate and expand into the surrounding mesenchyme, forming new branches.

**Concluding remarks**

Signaling pathways are generally very complex and tightly regulated mechanisms that are highly conserved through evolution. As evolution proceeds and organisms becomes more advanced and sophisticated, new regulatory systems are needed. Rather than reinventing the wheel, nature tends to reuse already existing regulatory systems and modify them slightly to meet the new needs. COPD is a disease with several concurrent destructive processes leading to disruption of the airways and parenchyma. In healthy individuals, repair processes in the lung counteract the destructive processes caused by inflammation, tobacco smoke and respiratory infections. However, in COPD patients repair process is either impaired or inadequate, resulting in the predominance of destructive processes.

Repair processes are generally derived from the same or similar mechanisms and signaling pathways such as those employed during development of the organ or tissue. C/EBP$\alpha$ clearly has a crucial role in lung development and in lung epithelial differentiation, and could therefore also have a role in the remodeling processes that are initiated in order to repair the tissue injuries, which could consequently lead to the development of COPD. In line with such a hypothesis, $\text{Cebpa}^{\Delta LE}$ mice develop an adult lung phenotype with a histopathology similar to that seen in COPD. It is tempting to speculate that the pathological processes in COPD and in $\text{Cebpa}^{\Delta LE}$ mice share at least some underlying mechanisms, and that this connection lies in a linkage between the epithelial differentiation-repair process inherent in COPD, and the epithelial differentiation during lung development.
FUTURE PERSPECTIVES

In order to critically address our hypothesis regarding the role of C/EBP transcription factors in inflammatory lung diseases, we have generated lung specific C/EBPα and C/EBPβ knockout mice that, in a collaborative study, will be challenged with tobacco-smoke and respiratory pathogens. This experimental setup will also provide a model that can help give insights into a potential role of C/EBPβ in glucocorticoid treatment of COPD exacerbations, as well as to study general mechanisms of glucocorticoid signaling in the lung epithelium.

Generation and tobacco-smoke / pathogen challenge of CebpbΔLE mice

In order to be able to generate mice with a lung-specific inactivation of C/EBPβ (CebpbΔLE) we first had to generate a floxed Cebpb mouse strain (Cebpbfl/fl mice). By breeding Cebpbfl/fl mice with SFTPC-Cre mice, CebpbΔLE mice were generated. As with the “whole body” C/EBPβ knockout mice, CebpbΔLE mice are perfectly healthy and do not display an altered lung phenotype when unchallenged. However these mice will be used to investigate the role of C/EBPβ in tobacco smoke-induced lung inflammation, and in the host defense against two common respiratory pathogens, influenza A and Hemophilus influenzae.

The role of C/EBPβ in mediating glucocorticoid signaling in vivo

The inflammation in COPD typically exhibits partial resistance to the anti-inflammatory action of glucocorticoids (Barnes, 2003; Jeffery, 1998). When studying glucocorticoid signaling in the lung epithelium we found that C/EBPβ, at least partially, mediates the effects of glucocorticoids in lung epithelium by inducing phosphorylation of Thr235 of C/EBPβ, hence augmenting its DNA binding activity (paper II). This raises the possibility that the decrease in C/EBP-binding activity in airway epithelium of patients with COPD may have a causative role for the relative resistance to glucocorticoids seen in this disease. Investigations into how C/EBPβ is regulated by GCs and β2-agonists (Roth et al., 2002; Yin et al., 2006) would deepen our understanding of the effects of GC/β2-agonist combinations in COPD and asthma treatment. As an initial step serving to validate the role for C/EBPβ in GC action in the airway epithelium in vivo, CebpbΔLE mice and Cebpbfl/fl mice are treated with GC and/or β2-agonists. Activation of three categories of genes will analyzed using real-time PCR on lung tissue. Firstly, expression of genes that have been shown to be GC-regulated via C/EBPs such as the anti-inflammatory gene CC16 and the P450 CYP2B1 will be evaluated. The second set of genes is involved in non-inflammatory innate immune-defenses such as β-defensins and the collectins SP-A and SP-D, all known to be regulated by C/EBPs and stimulated by GCs, but where the interplay has not been characterized. The third category is C/EBP-regulated genes with a pro-inflammatory role such as IL-6, IL-1β, TNF-α, COX2 and iNOS. These sets of genes will address the interplay between the C/EBPs and GC and β2-agonist-signaling in the regulation of these genes which might provide a first indication whether the in vivo observations (paper II; Zweragal et al., 2006), which suggest that GC activation targets C/EBPβ towards promoters of GC-activated, anti-inflammatory and innate immunity genes, holds true in vivo as well. By critically addressing our hypothesis in vivo, the data generated may thus help uncover a previously
unknown signal transduction mechanism for glucocorticoids in the lung. Together with evidence of decreased levels of C/EBPβ in patients with COPD (paper I), we suggest that mice lacking C/EBPβ specifically in the lung epithelium provide a model that more closely represents the lung epithelium of COPD patients when studying GC and β2-agonist signaling aimed for COPD patients.

**Generation of a lung epithelial specific C/EBPα; C/EBPβ double knockout-mice**

In addition to generating CebpbΔLE mice, we have generated lung epithelial specific C/EBPα; C/EBPβ double knockout mice (CebpaΔLE; CebpbΔLE mice) The primary reason for this is the ability of C/EBP transcriptions factors to replace each other’s functions, and thus the phenotype of both CebpaΔLE and CebpbΔLE mice could be partially rescued by the other C/EBP. We will study CebpaΔLE; CebpbΔLE mice, initially only during development since we expect a severely altered lung phenotype, with the aim of determining if the phenotype diverges from the phenotype of CebpaΔLE mice.
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