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C/EBP transcription factors in lung cellular differentiation and development

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ABSTRACT

During embryonic development the lung is lined with a primitive epithelium. As development proceeds, the epithelium matures and differentiates into several cell types that together with cells of mesenchymal origin constitute the adult lung. From studies investigating the regulation of differentiation-dependent genes in the lung, such as the Clara Cell Secretory Protein (*Ccsp/Scgb1a1*), a role for CCAAT/enhancer binder protein (C/EBP) transcription factors in lung cellular differentiation has been suggested. As C/EBPs have been shown to be important regulators of development, differentiation and proliferation in other organs, their expression was investigated during mouse lung development. C/EBPα, C/EBPβ and C/EBPδ were detected from late pseudoglandular stage and expression increased prior to birth, correlating with the extensive cellular differentiation, and onset of *Ccsp/Scgb1a1* expression, that occurs during this period. When investigating combinatorial actions of C/EBPs and other transcription factors in the regulation of *Ccsp/Scgb1a1*, C/EBPα was found to synergistically transactivate the promoter together with the epithelial-specific transcription factor NKX2.1. Together with the expression-pattern of C/EBPα, this indicates that the synergy could be a major determinant for the high-level, epithelial-specific expression of *Ccsp/Scgb1a1* in adult lung, and for the onset of *Ccsp/Scgb1a1* during development. To further examine the role of C/EBPα during lung development we generated transgenic mice ectopically expressing C/EBPα in the lung epithelium using the human Surfactant protein-C promoter, giving a premature, more widespread C/EBPα expression. Lungs from these mice were characterized by fewer and larger developing epithelial tubules. However no defects in overall proliferation or cellular differentiation were observed. A similar phenotype was observed in *Cebpa*$^{-/-}$ mice and we suggest that these similar phenotypes could possibly stem from dysregulation of airway branching. From our results we conclude that C/EBPα has a role in the later stages of lung development.

Glucocorticoids stimulate cellular differentiation during late lung development. In other organs, a role for C/EBPs in glucocorticoid signaling has been suggested. We investigated the role of C/EBPs in the action of glucocorticoids in the lung epithelium by studying the regulation of the *Ccsp/Scgb1a1* and *CYP2B1* genes. Both these genes are positively regulated by glucocorticoids and C/EBPs. However functional binding sites for the glucorticoid receptor have not been found in their promoters. In transient transfection studies using a lung epithelial cell line, we found that glucocorticoids induced expression from the *Ccsp/Scgb1a1* and *CYP2B1* promoters. In both promoters, induction was lost when the C/EBP-binding sites were mutated. Electrophoretic mobility shift assays revealed that glucocorticoids increased the DNA-binding activity of C/EBPβ and C/EBPδ within 10 minutes. The effect was mediated through the glucocorticoid receptor, independent of protein synthesis and involved phosphorylation of C/EBPβ at residue Thr^{235}. We further demonstrated that C/EBPβ is the predominant C/EBP-factor in both human and mouse lung epithelium and that glucocorticoids increase DNA binding of C/EBPβ in primary cells from mouse lung. These results indicate a previously unknown role for C/EBP-transcription factors in glucocorticoid signaling in the lung epithelium.

LIST OF PUBLICATIONS

I. Cassel TN, **Berg T**, Suske G, Nord M. (2002) Synergistic transactivation of the differentiation-dependent lung gene CCSP (Scgb1a1) by the bZIP factor C/EBPα and the homeodomain factor Nkx2.1/TTF-1. *Journal of Biological Chemistry*. **277**, 36970-7.

II. **Berg T**, Nord, M (2005) Ectopic expression of the transcription factor C/EBPα in the lung epithelium disrupts late lung development. *Manuscript.*

III. **Berg T**, Cassel TN, Schwarze PE, Nord M. (2002) Glucocorticoids regulate the CCSP and CYP2B1 promoters via C/EBPβ and δ in lung cells. *Biochemical and Biophysical Research Communications*. **293**, 907-12.

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

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
ATCC	The American Type Culture Collection
bZIP	Basic region-leucine zipper
bHLH	Basic region-helix-loop-helix
BMP	Bone morphogenetic protein
CaM	Ca^{2+}/Calmodulin-dependent kinase
CBP	CREB binding protein
CDK	Cyclin-dependent kinase
CYP	Cytochrome P450
CCSP	Clara cell secretory protein
CC16	Clara cell 16kDa protein (same as CCSP)
C/EBP	CCAAT/enhancer binding protein
CHOP	C/EBP homologous protein
CHX	Cyclohexamide
Dkk1	Dickkopf-1
E	Embryonic day
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular-signal-regulated kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOX	Forkhead box
GC	Glucocorticoids
GR	Glucocorticoid receptor
GSK	Glycogen synthase kinase
HFH	HNF-3/Forkhead homologue
HNF	Hepatocyte nuclear factor
ID	Inhibitor of DNA binding
LAP	Liver-enriched activator protein
LIP	Liver-enriched inhibitory protein
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MLK	Mixed lineage kinase
NF-κB	Nuclear factor-kappa B
P	Postnatal day
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
RB	Retinoblastoma protein
PKA	Protein kinase A
RSK	Ribosomal S6 kinase
SCGB1A1	Secretoglobin 1a1 (official gene name for CCSP)
Ser	Serine
SFPTC	Surfactant protein-C gene
SHH	Sonic hedgehog
SL2	Schneider's *Drosophila* line 2
SP	Surfactant protein
TBP	TATA box-binding protein
TBX	T-box protein
TF	Transcription factor
TGF	Transforming growth factor
Thr	Threonine
TNF	Tumor necrosis factor

INTRODUCTION

In order to supply the organism with oxygen the inner surface of the lung (the lung epithelium) is in direct contact with the outside environment. This also results in constant contact of the epithelium with damaging and transforming agents in the inhailed air. Thus mechanisms such as repair, regeneration and cell growth are normally occurring events in the adult pulmonary epithelium. By studying the differentiation of the pulmonary epithelium, and more specifically the role of the C/EBPs, we hope to gain a higher understanding of the processes regulating gene expression, cell growth and differentiation in lung. In the first part of this thesis we have investigated the role of C/EBP transcription factors in lung development. Since developmental programs are often reused during adult life in processes such as differentiation and regeneration, developmental mechanisms discovered could be of importance both in normal conditions as well as during disease in adult lung.

The second part of this thesis deals with glucocorticoids and the involvement of C/EBPs in glucocorticoid signaling. Glucocorticoids are steroid hormones known to be important during embryonic development of the lung. Their anti-inflammatory properties are also widely used in the clinic to treat inflammatory diseases in the lung, such as asthma. However, many questions remain about the molecular mechanism behind the effects of glucocorticoids in lung.

Lung physiology and histology

The lung is optimized for supplying the organism with oxygen. The architecture of the highly branched respiratory tree that leads the air into the alveoli provides a very large contact area between the inside of the body and the outside air, and is used to efficiently perform gas exchange. The respiratory tree is lined with a specialized epithelium consisting of several well-differentiated cells types. The upper bronchi are lined with ciliated pseudo-stratified epithelium containing scattered mucous producing goblet cells, which together form the mucociliary defense system. More distally, the ciliated cells and goblet cells decrease in number and serous secretory Clara cells appear. The Clara cells are proposed to have a protective role against inhaled toxins and oxidative stress (Stripp et al., 1995 and reviewed in Singh et al., 2000), and are stem cells for the bronchiolar epithelium during regeneration (Evans et al., 1978; Stripp et al., 1995). The cells produce of the Clara cell secretory protein (CCSP/CC16), also called Secretoglogin 1A1 (SCGB1A1) (Broers et al., 1992; Singh et al., 1986), and several cytochrome p450 enzymes (Hukkanen et al., 2002) involved in detoxification of

inhaled substances. In the most distal part of the lung, the alveoli are lined with two other types of epithelial cells, type I and type II cells. The flat alveolar type I cells are in close contact with an extensive capillary bed to facilitate gas exchange, and the cuboidal alveolar type II cells are responsible for the production of surfactant. The surfactant is a mixture of phospholipids and proteins, i.e. the surfactant proteins (SP) A, B, C and D (reviewed in Cardoso, 2000; Warburton et al., 2000 and Weaver et al., 1991). The primary function of the surfactant is to facilitate inhalation of air by lowering the surface tension in the air/liquid interface in the alveoli to prevent collapse during expiration, but also to facilitate expansion of the alveoli during inspiration. In addition, the surfactant has a role in host-immune defense and clearance. Besides being produced by alveolar type II cells the surfactant proteins SP-A, -B and -D are also produced and secreted by the Clara cells (Crouch et al., 1992; Kalina et al., 1992; Korfhagen et al., 1992; Phelps et al., 1991; Voorhout et al., 1992; Zhou et al., 1996b).

Lung development

The lung originates from the endoderm lining the primitive gut tube during organogenesis. The primitive gut tube that reaches from the anterior to the posterior of the embryo gives rise to the gastro-intestinal tract and the endodermal organs such as liver, pancreas, thyroid and lung (reviewed in Kaufman, 1992 and Wells et al., 1999). By interacting with the surrounding mesenchyme, the lung bud formed from the endodermal tube grows and branches to form the respiratory tree, a process referred to as branching morphogenesis. The whole process of development, from the formation of the lung primordium until formation of a fully mature lung is histologically divided into four stages: pseudoglandular, canalicular, saccular and alveolar, summarized in the table below.

Table 1. Histological stages of lung development

Stage:	Human	Mouse
Pseudoglandular	5-17 weeks	E9.5-16.5
Canalicular	16-26 weeks	E16.5-17.5
Saccular	24-38 weeks	E17.5-P(4/5)
Alveolar	36-to several years postnatally	P(4/5)-P14

Stages of fetal lung development in human (weeks) and mouse (days).
E (embryonic day), P (postnatal day)

The first stage, the *pseudoglandular period*, reaches from initiation of lung bud formation, occurring at embryonic day (E) 9.5 in mouse, until E16.5. This corresponds to week 5-17 in humans. During this period the main parts of the bronchial and respiratory tree are formed by branching morphogenesis. During the next *canalicular period* (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 (reviewed in Prodhan et al., 2002). During this stage the tubular branches lengthen, accompanied with widening of the distal airspaces and a decrease in mesenchymal mass. Parallel to the development of the airway tree the pulmonary vasculature develops. The two processes are intimately related and dependent on one another, and as a result of the decrease in mesenchymal mass the epithelial cells lining the prealveolar saccules and the mesodermally-derived pulmonary vasculature become more closely positioned to one another. This serves as a starting point for establishment of the future air-blood barrier. It also serves as a starting point for initiation of the cuboidal epithelium lining the distal airway region to differentiate into alveolar type I and type II cells and for maturation of the primitive capillary network.

The following s*accular stage* is characterized by the formation of prealveolar saccules or terminal sacs that later form the alveoli. This *saccular stage* spans through E17.5 to postnatal day (P) 5 in mice and weeks 24-38 in human embryonic development. During this stage of lung development, differentiation of the epithelial precursor cells occurs in the distal lung and the cells will start to resemble adult lung parenchyma cells. The differentiation is reflected by the onset of expression of differentiation-dependent genes necessary for respiration, for instance components of the surfactant system (reviewed in Mendelson, 2000 and Whitsett et al., 1998). In parallel, the capillary network continues to mature, together serving to prepare the lung for respiration after birth. Entering this stage is crucial for sufficient gas exchange and extra-uterine survival. Also preparation for alveolarization starts by deposition of elastic fibers (Wasowicz et al., 1996). *Alveolarization* is the final step in the development into a mature lung. During this stage septation of prealveolar saccules occurs to form alveoli, 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 (see table 1) (reviewed in Groenman et al., 2005; Hislop, 2005; Roth-Kleiner et al., 2003 and Ten Have-Opbroek, 1981).

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. How cells in the endoderm will respond to and interact with their surroundings depends on the context provided by the surrounding mesenchyme and neighboring cells, as well as the context within the responding individual cells. The actions of specific transcription factors or combinations of factors expressed in the cell, along with availability of DNA, will determine the transcription of various target genes and hence guide the cell down its developmental path.

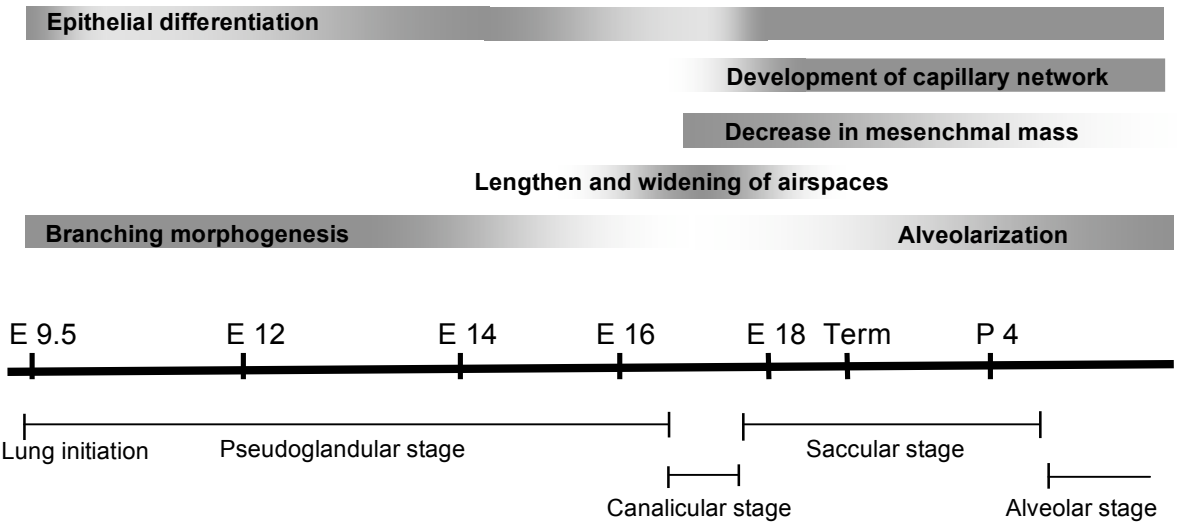

Figure 1. Histological and developmental processes during mouse lung development.

Time scale reaching from embryonic day (E) 9.5 to postnatal day (P) 4.

Gene regulation and transcription

The specificity of gene expression and transcription is controlled by regulatory transcription factors. By binding to sequence-specific binding sites in the promoter, the control region of transcription, the transcription factors influence the rate of transcription both positively and negatively (reviewed in Kornberg, 2005 and Roeder, 2005). In the cell, DNA is tightly packed around a core of histone proteins in chromatin fibers. For transcription to occur, chromatin remodeling is required so that the transcriptional machinery can gain access to the DNA. Binding of transcriptional regulators to the promoter and their interaction with or recruitment of co-regulators with chromatin remodeling functions makes the DNA accessible for transcription. Histone modifications are well-characterized; and different posttranslational

modifications such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation have been described and are linked to gene activation as well as silencing. Acetylation, carried out by histone acetyltransferases (HATs) for example, reduces the positive charge of the histone tails and weakens the interaction between histones and DNA. This facilitates binding of additional transcription factors and cofactors. The process is reversible and removal of the acetyl group by histone deacetylases (HDACs) compacts the DNA and silences transcription (reviewed in Peterson et al., 2004).

Regulatory information from transcription factors and cofactors assembled at the promoter is transmitted through the Mediator, an adaptor complex, to the general transcriptional machinery. The general transcriptional machinery, also called the pre initiation complex (PIC), includes the general initiation factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, which together with RNA polymerase II are responsible for initiation of transcription and synthesis of messenger RNA (reviewed in Kornberg, 2005 and Roeder, 2005). The contact between regulatory transcription factors and the general transcription machinery, the Mediator and chromatin remodeling co-activators can occur at many different levels, directly or indirectly. For instance the C/EBP transcription factors have been shown to interact with the Mediator (Mo et al., 2004) and with two essential components of the general transcription machinery, TBP and TFIIB (Nerlov et al., 1995; Pedersen et al., 2001). The transcription factor FOXA has been shown to bind compacted DNA and to open up the chromatin structure (Cirillo et al., 2002) and C/EBPs (Chen et al., 2001; Mink et al., 1997; Pedersen et al., 2001), POD1 (Hong et al., 2005) as well as the downstream effector of WNT signalling, LEF1 (Billin et al., 2000), have all been shown to interact directly with HATs and HDACs. Together these interactions provide the specificity in gene expression by recognition and binding of multiple transcription factors. Their combinatorial effects at the promoter add diversity to gene regulation, and mediate cell-type and developmental specific gene expression as well as spatial and temporal regulation.

Molecular regulation of lung development

Initiation of lung formation

After lung morphogenesis is initiated, the lung bud is formed from the ventral side of the anterior foregut endoderm. The position of lung bud formation is determined by a pre-established anterior-posterior axis, as well as a dorsal-ventral axis (reviewed in Perl et al., 2002 and Wells et al, 1999). The molecular mechanisms behind the initiation events are not yet fully understood. Interplay between the endoderm and the surrounding tissue, together with inductive signals from the cardiac mesoderm, takes

part in establishing and maintaining the lung-specific domain. Fibroblast Growth Factors (FGFs*) produced by the cardiac mesoderm establish a concentration gradient in the mesenchyme surrounding the area of the prospective lung bud. The proximity of the primitive gut endoderm to the embryonic heart will determine the strength of the signal received by the endoderm. At a certain threshold level of FGFs, lung specification of the endoderm occurs (Serls et al., 2005). Recent data suggests that retinoic acid induces expression of *Fgf10** in the surrounding mesoderm (Desai et al., 2004) possibly through activation of the transcription factors TBX4 and TBX5 that are involved in regulating *Fgf10* (Cebra-Thomas et al., 2003). Retinoic acid could therefore be one of the first determinants of lung formation.

Other factors that are found to be expressed early in the lung specific domain of the endoderm are the transcription factors GLI, NKX2.1 and members of the Forkhead family of transcription factors (FOX), as well as the secreted molecules Sonic Hedgehog (SHH) and Bone Morphogenetic protein 4 (BMP4). Of these factors only GLI has been shown to be an absolute requirement for initiation of the lung bud shown by the absence of lung bud formation in double mutant $Gli2^{-/-}/Gli3^{-/-}$ mice (Motoyama et al., 1998). GLI2 and GLI3 are both downstream effectors of SHH. However, the phenotype of the $Shh^{-/-}$ mutant is not as severe as the phenotype for the double *Gli* mutant. Lung formation is initiated in *Shh* mutants but the $Shh^{-/-}$ mice then fail to undergo branching morphogenesis (Litingtung et al., 1998). Null mutants for *Bmp4* die between embryonic days 6.5 and 9.5 (Winnier et al., 1995) and therefore it has not been possible to investigate the role of BMP4 in lung bud initiation. NKX2.1 is a homeodomain transcription factor, also named Thyroid transcription factor-1 (TTF-1), which is specifically expressed in lung, thyroid and specific regions in the diencephalon of the brain (Lazzaro et al., 1991). In the lung, *Nkx2.1* has been detected in the ventral endoderm as early as E8.25 (Serls et al., 2005) i.e. before the first visible sign of lung bud formation. This indicates that the lung specific region has started to be defined within the endoderm at this developmental stage before any morphological signs of lung bud initiation appear. Despite the expression pattern of NKX2.1, and that NKX2.1 is absolutely necessary for lung development (Kimura et al., 1996), it is not critical for formation of the lung bud, which is formed even in mice that lack expression of NKX2.1 (Kimura et al., 1999). In addition to NKX2.1, several members of the

* According to the Guidelines for Human Gene Nomenclature, *HUGO Gene Nomenclature Committee* (Wain et al., 2002), all gene names are written in *italics*. Mouse genes have first letter capitalized and rest lower case letters (e.g.: *Fgf10*). Genes of other mammalian species are written with all letters capitalized (e.g. *FGF10*). All proteins names, regardless of species will be written with all letters capitalized non-*italics* (e.g.: FGF10), following recommendations in the Guidelines for Human Gene Nomenclature.

Forkhead gene family members expressed in lung have been suggested to be important for lung initiation based on their early expression in the endoderm, including FOXA1 (HNF3α) and FOXA2 (HNF3β) (Besnard et al., 2004). However, their function in lung initiation has not been possible to establish as knockout mice for *Foxa2* have defects in the endoderm before onset of lung formation (Weinstein et al., 1994) and knockout mice for *Foxa1* show no lung phenotype (Kaestner et al., 1999; Shih et al., 1999). A reason for the lack of phenotype in the $Foxa1^{-/-}$ mice could be that FOXA1 and FOXA2 have partially redundant functions and thus could compensate for one another. That they have partially overlapping functions in lung have been shown in lung specific double knockout mice, which also have proven that both factors are important for lung development (Wan et al., 2005; Wan et al., 2004). Another member of this family, FOXF1, has also been shown to be important for early lung formation. Lungs from $Foxf1^{+/-}$ mice exhibit lobe fusions (Lim et al., 2002; Mahlapuu et al., 2001) and reduction of *Foxf1* expression also causes down regulation of several factors that are important for lung initiation and early development such as *Fgf10*, *Bmp4* and *Gli3* (Lim et al., 2002). In addition, pulmonary expression of *Foxf1* was undetectable in $Shh^{-/-}$ mice embryos, which suggests that *Shh* signaling is essential for *Foxf1* expression (Mahlapuu et al., 2001).

Branching morphogenesis

After the initial formation of the lung bud in mouse (E9.5), one left and four right primary bronchi are formed at E11 defining the five lung lobes. Through dichotomous branching at the tip of each developing duct the airway branches are formed. This process is referred to as branching morphogenesis and is a highly genetically controlled mechanism of repetitive growth and branching of lung endoderm into the surrounding mesenchyme. Interacting signals between the endoderm and the surrounding mesenchyme guide the branching epithelium. Several key-factors have been identified including growth factors, transcription factors and components of the extracellular matrix (reviewed in Cardoso, 2000 and Warburton et al., 2005). A general model has been proposed for how this dichotomous branching morphogenesis is regulated. In this model, a local source of FGF10 in the mesenchyme acts as a chemo-attractant for the epithelium that expresses the receptor for FGF10, FGFR2. As the epithelium grows towards the source of FGF10, expression of SHH is induced in the epithelium. The release of SHH in turn down-regulates the expression of FGF10 in the mesenchyme as the bud approaches the source of FGF10. When FGF10 expression is reduced at its original position the expression moves laterally to form new signaling centers at the

sides. These new signaling centers now direct outgrowth and formation of two new lung buds (Bellusci et al., 1997; Hogan, 1999; Metzger and Krasnow, 1999).

By using genetically modified mouse models, components of several other major signaling pathways have also been shown to take part in branching morphogenesis including members of the Transforming Growth Factor-β (TGF-β) superfamily and the WNT family. Knockout mice for *Wnt5a* (Li et al., 2002) exhibit a stimulated branching along with increased expression of *Shh* and *Fgf10*, indicating that WNT5A has a negative effect on branching. Also TGF-β1 has a negative influence on branching, and endogenous TGF-β1 has been shown to inhibit branching both in explant cultures (Bragg et al., 2001; Zhao et al., 1998) and in transgenic mice (Zhou et al., 1996a). Expression of TGF-β1 colocalizes in time and space with the extracellular matrix proteins collagen I and III, fibronectin and glycosaminoglycans surrounding non-branching epithelial regions such as the stalks and clefts of the primitive airways (Heine et al., 1990). As deposition of fibronectin has been reported to be involved in cleft formation during branching (Sakai et al., 2003) and as TGF-β stimulates production of fibronectin (Maniscalco et al., 1994) this also suggests TGF-β to be a possible regulator of branch point formation (Heine et al., 1990). TGF-β might also function as an upstream inhibitor of BMP4 which, in contrast to TGF-β1, has a positive influence on branching with an effect locally restricted to the branching tips of the airways (Bellusci et al., 1996; Bragg et al., 2001; Shi et al., 2001; Weaver et al., 1999). Other factors found to stimulate branching morphogenesis are the secreted factor Epidermal growth factor (EGF) (Miettinen et al., 1997), Retinoic acid (possibly acting through upregulation of EGFR) (Schuger et al., 1993), the transcription factor HOXB5 (Volpe et al., 2000) and the transcription factor GATA-6 (Keijzer et al., 2001). Also lung specific double mutants for *Foxa1* and *Foxa2* in the lung have diminished branching (Wan et al., 2005). However, how the external paracrine and autocrine signaling is intracellularly transduced to functionally link with transcriptional regulation is not as well understood.

Epithelial cell differentiation

As development and branching morphogenesis proceed, the epithelium matures and differentiates into multiple cell types. The first visual sign of cell differentiation in the lung epithelium is the appearance of a cuboidal epithelium in the prospective respiratory portion at E14.5 in mouse (corresponding to weeks 11-12 in human). This event is followed by additional flattening of the epithelium seen around E16.5 (week 16 in human) that primes for differentiation of alveolar type I and type II cells (Ten Have-

Opbroek, 1981) and expression of cell type specific proteins, important for lung function, such as the surfactant proteins. The importance of cell-cell contact and cell-extracellular matrix interactions for the cellular differentiation to occur and for maintaining the cells differentiated state is illustrated by the culturing of isolated primary alveolar type II cells. In culture the primary cells rapidly dedifferentiate and their expression of SP-A, SP-B and SP-C is decreased, whereas the expression is sustained in cultures surrounded by fetal lung fibroblasts and collagen (Shannon et al., 1992; Shannon et al., 2001). A variety of extracellular signals, coming from neighboring cells and the extracellular matrix, are intracellularly transduced in the epithelium by transcription factors together promoting tissue and cellspecific gene expression (reviewed in Cardoso, 2000; Costa et al., 2001 and Warburton et al., 2000). Their exact temporal and spatial expression is crucial in controlling differentiation of the multipotent progenitors into the different specialized cell types constituting the adult lung.

Several of the secreted factors that have a role in branching as discussed above also take part in differentiation of the pulmonary epithelium, for instance FGF, BMP, TGF-β and WNT. BMP-4 and WNT signaling are important for differentiation of the distal epithelium and disruption of BMP4 and WNT-signaling pathways repress differentiation of the distal airway epithelium and cause expansion of the proximal epithelium into the distal region (Bellusci et al., 1996; Lu et al., 2001; Mucenski et al., 2003; Shi et al., 2001; Shu et al., 2005; Shu et al., 2002; Weaver et al., 1999). TGF-β1, on the other hand, is doing the opposite, 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; Maniscalco et al., 1994; Zeng et al., 2001). 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 et al., 1995). Although much remains unknown about how the effect of the secreted factors is transduced at the transcriptional level, many transcription factors have been shown to be involved in lung cellular differentiation, such as FOXA1, FOXA2, FOXJ1 (Chen et al., 1998; Wan et al., 2005), MASH-1 (Borges et al., 1997; Ito et al., 2001), GATA-6 (Liu et al., 2002b), NKX2.1 (Minoo et al., 1999; Yuan et al., 2000; Zhou et al., 1996b) and POD1 (Quaggin et al., 1999).

NKX2.1 is a transcription factor that is expressed in all pulmonary epithelial cells during early morphogenesis. As development progresses, expression of NKX2.1 becomes restricted to alveolar type II cells and proximal Clara cells (Ikeda et al., 1995; Lazzaro et al., 1991). Targeted disruption of the *Nkx2.1* gene has also demonstrated that

NKX2.1 is essential for terminal cell differentiation, as *Nkx2.1*$^{-/-}$ mice lack expression of CCSP/ SCGB1A1 and the surfactant proteins SP-A and SP-C (Kimura et al., 1996; Kimura et al., 1999; Minoo et al., 1995; Minoo et al., 1999). The expression of several of these markers is also directly regulated by NKX2.1 including CCSP/SCGB1A1 (Ray et al., 1996; Zhang et al., 1997b), SP-A (Bruno et al., 1995), SP-B (Bohinski et al., 1994; Margana et al., 1997), SP-C (Kelly et al., 1996) and SP-D (Dave et al., 2004). NKX2.1 also regulates the expression of *Wnt7b* that is important for lung development and cellular differentiation (Weidenfeld et al., 2002), and is suggested to be an upstream regulator of *BMP4* (Minoo et al., 1999; Zhu et al., 2004). Together this indicates a central role for this transcription factor in controlling lung cellular differentiation.

Several of the transcription factors identified are also more or less cell type specific and the absence of the respective transcription factors leads to absence of the corresponding cell type. Due to their specific expression, these transcription factors can also be used as markers to determine the origin of cells such as Mash-1/hASH1 (pulmonary neuroendocrine cells) (Borges et al., 1997; Ito et al., 2001) and Foxj1 (ciliated cells) (Blatt et al., 1999; Brody et al., 2000; Chen et al., 1998; Tichelaar et al., 1999a; Tichelaar et al., 1999b). However, their specific gene targets and their exact mechanisms in cell linage specification remain to be identified. Other transcription factors as GATA-6 have been identified to be important for differentiation of both proximal and distal epithelial cell types (Liu et al., 2002b). Lack, or reduced levels, of GATA-6 leads to reduced expression of the genes encoding SP-C, *Sftpc,* and *Ccsp/Scgb1a1* (Keijzer et al., 2001; Koutsourakis et al., 2001; Liu et al., 2002a; Yang et al., 2002). The transcription factor POD-1 has also been shown to be essential for epithelial differentiation and *Pod*$^{-/-}$ mice show proximalization of the lung epithelium with increased expression of *Ccsp/Scgb1a1*, reduced levels of *Bmp4*, lack of alveolar type II cells as well as lack of alveoli structures (Quaggin et al., 1999). However, in contrast to the other transcription factors mentioned, POD-1 is exclusively expressed in the mesenchyme, showing the importance for POD-1 in mediating the epithelial-mesenchymal interactions (Hidai et al., 1998) and also illustrating the importance of the mesenchyme for epithelial differentiation.

Alveolar period

Alveolarization is the final step in development into a mature lung and starts just prior to birth and continues at least for 3 years after birth in humans, whereas it occurs entirely postnatally in mice. Alveolarization includes formation of interalveolar walls, i.e. septation of the air spaces, which drastically increases the surface area of the lung.

One of the key-factors that control alveolarization is the Platelet derived growth factor-A (PDGF-A). Knock out mice for *Pdgfa* lack alveolar myofibroblasts and as a consequence no elastin fibers necessary for septation are produced (Lindahl et al., 1997). Hence, alveolarization is not completed which results in an emphysema-like phenotype. In addition to PDGF-A, double mutants for *Fgfr3* and *Fgfr4* also show defects in alveolar septation, but in contrast to the *Pdgfa* null mice, *Fgfr3* and *Fgfr4* double mutants show elevated elastin production (Weinstein et al., 1998) suggesting another mechanism of action. Retinoic acid and its receptors have also been shown to have a role in septation. Knockout mice for the receptors *Rarg* and *Rxra* have impaired alveolarization and diminished elastin deposition (McGowan et al., 2000). In addition, treatment with retinoic acid has been shown to increase postnatal septation and alveolar formation (Massaro et al., 1996) and to induce formation of new septa in adult rats with elastase-induced emphysema. These results provided hope of a non-invasive cure for human emphysema patients (Massaro et al., 1997). However recent studies performed have not been able to reproduce this effect (March et al., 2004).

CCAAT/Enhancer Binding Proteins

The CCAAT/enhancer binding proteins (C/EBPs) are a family of regulatory transcription factors whose expression has been linked to development, cellular differentiation, and regulation and expression of tissue specific genes. The family consists of six members; C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ, and C/EBPζ (reviewed in Ramji et al., 2002). The different members exhibit different modes of regulation and have individual stage-specific expression patterns. C/EBPα, C/EBPβ and C/EBPδ are expressed in several organs such as liver, adipose tissue, intestine, lung, adrenal gland, placenta, breast, and white blood cells of the myelocytic lineage (Antonson et al., 1995; Birkenmeier et al., 1989; Buck et al., 2003; Cao et al., 1991; Darlington, 1999; Darlington et al., 1998; Descombes et al., 1990; Flodby et al., 1996; Grimm et al., 2003; Lane et al., 1999; Poli, 1998; Sugahara et al., 2001; Wang et al., 1995; Williams et al., 1991; Zahnow, 2002; Zhang et al., 1997a). Here, C/EBPα is primarily expressed in non-dividing, fully differentiated cells (Antonson et al., 1995; Birkenmeier et al., 1989; Umek et al., 1991), which suggests C/EBPα to be an important regulator of differentiation. In contrast to C/EBPα, C/EBPβ and C/EBPδ are expressed in proliferating cells and their expression is also induced by inflammatory stimuli (Akira et al., 1990; Alam et al., 1992; Kinoshita et al., 1992; Poli et al., 1990). C/EBPε is primarily expressed in myeloid and lymphoid cells (Antonson et al., 1996;

Chumakov et al., 1997; Yamanaka et al., 1997) whereas C/EBPγ and C/EBPζ are ubiquitously expressed (Roman et al., 1990; Ron et al., 1992).

C/EBP structure and function

C/EBPs are classified as basic-leucine zipper (bZIP) transcription factors based on the basic-leucine zipper domain in their C-terminus (figure 2). The leucine zipper domain enables dimerization between family members and both homo- and heterodimers can be formed, which contact DNA through the basic region. The basic region/DNA-binding region is highly homologous within the family and as a consequence the dimers exhibit similar target gene specificities and a consensus DNA-binding sequence has been identified: 5´ ATTGCGCAAT 3´ (Osada et al., 1996 and reviewed in Ramji et al., 2002). The N-terminus of the C/EBPs harbors the transactivation domain, which interacts with, or recruits, co-activators and co-repressors. In contrast to the DNA-binding domain, the transactivation domain is less well conserved leading to different transactivation and/or repression potential of the different C/EBPs and dimers (reviewed in Ramji et al., 2002).

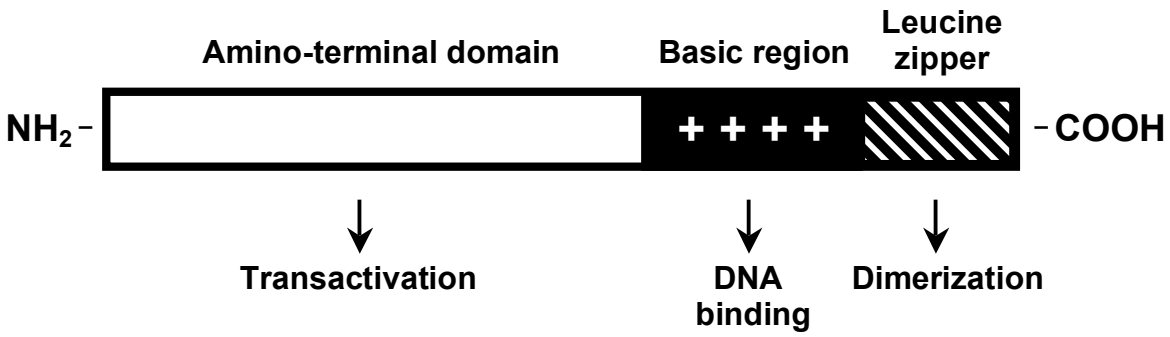

Figure 2. Basic structure of CCAAT Enhancer Binding Proteins

The complexity of the response to C/EBPs is further increased by the fact that the C/EBPs also have the ability to dimerize with other transcription factors belonging to the bZIP family such as FOS/JUN (AP-1) (Hsu et al., 1994) and ATF/CREB (Vallejo et al., 1993), as well as to interact with non-bZIP factors. C/EBPβ for instance has been shown to interact with the p50 subunit of NF-κB, the glucocorticoid receptor and the retinoblastoma (RB) protein (Boruk et al., 1998; Chen et al., 1996; LeClair et al., 1992 and reviewed in Ramji et al., 2002). In addition, C/EBPα and C/EBPβ also exist in different isoforms generated through leaky ribosomal scanning. When leaky ribosomal scanning occurs translation is not only initiated at the first AUG but also at additional

downstream AUGs (Touriol et al., 2003). The different lengths of the translational products that are produced give rise to full-length and truncated proteins with different properties. The full-length protein of C/EBPα contains three transactivation domains (TEI-III) whereas the shorter isoform contains only one transactivation domains and therefore functions as a weaker transactivator (Lin et al., 1993; Nerlov et al., 1995; Ossipow et al., 1993). The *Cebpb* mRNA gives rise to at least three different isoforms of which the two longest forms, LAP* and LAP, function as activators and the shortest isoform, LIP, lacking the complete transactivation domain, functions as a repressor (Calkhoven et al., 2000; Descombes et al., 1991; Eaton et al., 2001).

Regulation and posttranslational modifications

The activity of the C/EBP proteins is tightly controlled. Several posttranslational modifications such as phosphorylations, sumoylations and acetylations (Eaton et al., 2003; Subramanian et al., 2003; Xu et al., 2003) have been described to affect both the DNA binding (Ray et al., 1994; Tang et al., 2005; Trautwein et al., 1994) and transcriptional activity (Nakajima et al., 1993; Piwien-Pilipuk et al., 2001; Sterneck et al., 1998; Trautwein et al., 1993; Wegner et al., 1992), as well as nuclear import, export (Buck et al., 2001; Metz et al., 1991; Sprott et al., 2002; Yin et al., 1996) and intra-nuclear localization (Piwien Pilipuk et al., 2003). Phosphorylations have also been shown to regulate the ability of C/EBP to interact with other proteins (Mo et al., 2004; Wang et al., 2005). These modifications have been characterized mainly for C/EBPβ, but C/EBPα and C/EBPδ have also been shown to be modulated by phosphorylations affecting DNA binding, transactivation, conformation and localization (Billiard et al., 2001; Hemati et al., 1997; Mahoney et al., 1992; Ross et al., 1999; Svotelis et al., 2005).

Cell cycle regulation

C/EBPα, C/EBPβ and C/EBPδ have opposing roles during proliferation. In contrast to C/EBPβ and C/EBPδ that supports proliferation, C/EBPα is a strong inhibitor of proliferation (Cao et al., 1991; Flodby et al., 1993; Hendricks-Taylor et al., 1995; Mischoulon et al., 1992; Soriano et al., 1998; Umek et al., 1991). C/EBPα is down regulated in proliferating cells and knockout mice for *Cebpa* show increased proliferation in liver and lung (Flodby et al., 1996). In addition, many cancer forms lack expression of C/EBPα suggesting that C/EBPα might function as a tumor suppressor (Halmos et al., 2002; Pabst et al., 2001; Shim et al., 2005; Xu et al., 2001). The growth

inhibitory effects of C/EBPα are mostly independent of gene activation and are instead mediated through protein-protein interactions with several proteins involved in cell-cycle regulation. C/EBPα has for instance been described to bind to the cell cycle inhibitor p21, which has a stabilizing effect on p21 and protects the protein from degradation (Harris et al., 2001; Timchenko et al., 1997; Timchenko et al., 1996). C/EBPα has also been shown to cooperate with p21 to inhibit the cell cycle activator CDK2, a Cyclin dependent kinase (Harris et al., 2001; Timchenko et al., 1997; Timchenko et al., 1996), or to directly bind to both CDK2 and 4 and inhibit their function (Wang et al., 2001). This anti-proliferative effect of C/EBPα has recently been shown to be under post-translational control. In the proliferating liver, phosphorylation of C/EBPα reduces the interaction between C/EBPα and CDK2 and 4. Instead this phosphorylated form of C/EBPα binds to and inhibits the growth inhibitor RB. Altogether the phosphorylation neutralizes the growth inhibitory effect of C/EBPα, allowing the liver to respond to a proliferative signal (Wang et al., 2005).

C/EBP in development and differentiation

The expression of C/EBPs has in many organs been linked to development, cellular differentiation, and regulation and expression of tissue specific genes. Knockout mice for C/EBPα have defects in liver and white and brown adipose tissue, and die within a few hours after birth due to hypoglycemia (Wang et al., 1995 and reviewed in Lekstrom-Himes et al., 1998). However, a few mice die from respiratory failure and it has been shown that *Cebpa*$^{-/-}$ mice exhibit under-developed lungs with hyperproliferation of alveolar type II cells at birth (Flodby et al., 1996). In contrast to the *Cebpa*$^{-/-}$ mice, double knockout mice for *Cebpb* and *Cebpd* have no demonstrated lung phenotype, but have defects in white adipose tissue and lack brown adipose tissue (Tanaka et al., 1997). The importance of C/EBPs for adipose differentiation has been thoroughly investigated *in vitro* and has been shown to be regulated by a cascade of C/EBPα, C/EBPβ and C/EBPδ (Yeh et al., 1995). C/EBPβ and C/EBPδ are the first to be detected in preadipocytes but their expression diminishes as differentiation proceedes and is at later stages replaced by C/EBPα (Cao et al., 1991; Yeh et al., 1995). Ectopic expression of C/EBPα has also been shown to arrest mitotic growth (reviewed in Umek et al., 1991) and to, alone, initiate differentiation of preadipocytes into adipocytes (Freytag et al., 1994; Lin et al., 1994). Similarly, abrogation of C/EBPα expression prevents terminal adipocyte differentiation (Lin et al., 1992; Samuelsson et al., 1991).

Studies in mice have also shown the importance of C/EBPs in liver proliferation and differentiation. Following partial hepatectomy, expression of *Cebpa* mRNA decreases and levels of *Cebpb* and *Cebpd* mRNAs increase (Flodby et al., 1993; Michalopoulos et al., 1997; Mischoulon et al., 1992; Skrtic et al., 1997) showing opposing functions for the C/EBPs during proliferation in the liver. Lack of C/EBPα in *Cebpa*$^{-/-}$ mice also disturbs the architecture of the liver and the mice lack expression of several genes encoding enzymes controlling the gluconeogenetic pathway (Flodby et al., 1996; Wang et al., 1995) showing the need for C/EBPα in hepatocyte differentiation.

C/EBP in lung

Transcripts of *Cebpa, Cebpb* and *Cebpd* have been found in lung during late development with increasing expression towards birth (Barlier-Mur et al., 2003; Breed et al., 1997; Rosenberg et al., 2002). However, the individual expression patterns of the C/EBPs during development have not been investigated at the protein level. In the adult lung C/EBPα, C/EBPβ and C/EBPδ are expressed in alveolar type II cells and C/EBPδ and lower levels of C/EBPα are expressed in Clara cells of the bronchiolar epithelium (Cassel et al., 2000b; Lag et al., 2000; Li et al., 1995; Nord et al., 1998; Rosenberg et al., 2002; Sugahara et al., 1999). The C/EBPs regulate the expression of several lung-enriched genes encoding SP-A (Rosenberg et al., 2002), SP-D (He et al., 2002), CCSP/SCGB1A1 (Nord et al., 1998) and the P450-enzyme CYP2B1 (Cassel et al., 2000a; Luc et al., 1996). Expression of C/EBPα is lost rapidly when primary lung epithelial cells are cultured *in vitro*. This correlates with loss of expression of the differentiation marker CCSP/SCGB1A1 in cultured Clara cells (Nord et al., 1998). Expression of C/EBPα has also been found to correlate with expression of surfactant proteins in cultured alveolar type II cells (Li et al., 1995). Together with the knockout phenotype of C/EBPα, these findings indicate that C/EBPα could be important for lung epithelial differentiation.

Glucocorticoids

Glucocorticoids (GC) are steroid hormones and are involved in regulation of various biological processes such as metabolism, stress and growth. In addition, glucocorticoids have anti-inflammatory and immunosuppressive effects. The lipophilic properties of the hormones enable free diffusion into the cytoplasm where they bind to and activate the glucocorticoid receptor (GR). After translocation into the nucleus the complex can either activate or repress transcription by direct binding to target gene promoters. In

addition to this classical pathway of gene regulation, the glucocorticoid-receptor complex also interferes with other signaling pathways by direct protein-protein interactions resulting in either positive or negative influences on gene transcription (reviewed in De Bosscher et al., 2003).

The anti-inflammatory and suppressive effects on immune responses are thought to occur mainly through transcriptional repression of inflammatory genes, such as proinflammatory cytokines and adhesion molecules. In most cases this is due to a repressive interaction between the glucocorticoid receptor and the transcription factors NF-κB and AP-1, both central activators of inflammatory genes. This repressive effect can occur through direct binding of GR with NF-κB and AP-1 with or without DNA contact. The protein interaction may prevent the complex from binding DNA, mask the transactivational domains of the proteins, or involve repressive effects on chromatin structure such as recruitment of co-repressors (reviewed in Schoneveld et al., 2004). The mechanisms behind the antiproliferative effects of glucocorticoid hormones are still unclear but some effects are mediated by transcriptional inhibition of cyclins and cyclin-dependent kinases (CDKs) as well as induction of transcription of CDK inhibitors, such as p21 and p27 (Corroyer et al., 1997; Corroyer et al., 2002; Rhee et al., 1995; Rogatsky et al., 1997; Sanchez et al., 1993). The antiproliferative effects of glucocorticoids are of clinical importance, as inhibition of growth is linked to several side effects of long-term treatment with glucocorticoids such as osteoporosis and atrophy of the skin (reviewed in Schacke et al., 2002).

Glucocorticoids in lung

Glucocorticoids are among the most widely prescribed anti-inflammatory drugs, frequently and effectively used in the treatment of various inflammatory lung disorders (reviewed in Barnes et al., 2003). Glucocorticoids also have an important role during lung development, and mice lacking the glucocorticoid receptor develop highly immature lungs with increased number of proliferating mesenchymal and epithelial cells and the mice die shortly after birth due to respiratory failure (Cole et al., 1995). Treatment with glucocorticoids has also been shown to accelerate lung differentiation and maturation, and to enhance the production of surfactant proteins (Boggaram et al., 1989; Boggaram et al., 1991; Liley et al., 1989; O'Reilly et al., 1989) and CCSP/SCGB1A1 (Hagen et al., 1990). However, no functional binding sites for the glucocorticoid receptor have been found within the promoters of these genes (Breed et al., 1997; Karin, 1998). This suggests that alternative mechanisms apart from direct DNA binding by the glucocorticoid receptor account for the stimulated expression of the surfactant protein and CCSP/SCGB1A1 encoding genes. This highlights that much

still remains unknown about the molecular mechanisms of glucocorticoid action in the lung.

Mice expressing a glucocorticoid receptor with a mutation in the dimerization domain have been generated. This domain is believed to be a prerequisite for DNA binding and for direct regulation of gene transcription. In contrast to the $Gr^{-/-}$ mice, mice carrying this mutated receptor survive after birth and do not have any respiratory problems (Reichardt et al., 1998). This indicates that the critical function of glucocorticoids for lung development is mediated by means other than direct DNA binding, for instance direct protein-protein interactions. However, studies have shown that liganded-glucocorticoid receptor, under certain circumstances, does bind to DNA without dimerization to so called glucocorticoid receptor half-sites (Adams et al., 2003). Whether a specific subset of genes, important for lung maturation, is controlled by glucocorticoid half-sites is not known.

C/EBPs and glucocorticoids

In bronchial smooth-muscle cells in the lung, activated glucocorticoid receptor has been shown to form a complex with C/EBPα on the p21 promoter leading to transactivation of p21 and growth arrest (Roth et al., 2002; Rudiger et al., 2002). This phenomenon has possible implications in the treatment of asthma as it has been found that bronchial smooth muscle cells from subjects with asthma lack C/EBPα and thus the glucocorticoid-C/EBPα complex (Roth et al., 2004). These observations could provide an explanation for the increased proliferation of smooth-muscle cells that can occur in patients with asthma and could also explains the failure of glucocorticoids to inhibit proliferation *in vitro* of smooth-muscle cells isolated from asthmatics (Roth et al., 2004). In addition, glucocorticoid treatment has been demonstrated to upregulate C/EBPδ and C/EBPβ in liver, fat, muscle and osteoblasts (Cao et al., 1991; MacDougald et al., 1994; Matsuno et al., 1996; Penner et al., 2002; Yang et al., 2005) and thereby stimulate expression of a subset of glucocorticoid target genes in these tissues (McCarthy et al., 2000; Gotoh et al., 1997). Together this suggests that glucocorticoid signaling via C/EBPs could be a potential mechanism for glucocorticoid action in lung.

SPECIFIC AIMS

The aims of the present investigation have been:

To investigate the developmental expression of C/EBPα in lung, and its role in cellular differentiation in the lung epithelium (paper I and II).

To investigate the role of C/EBP transcription factors in glucocorticoid signaling in lung epithelial cells (paper III and IV).

COMMENTS ON METHODOLOGY

Cell cultures

SL2 cells

The Schneider's Drosophila line 2 (SL2) used in paper I was established from *Drosophila melanogaster* embryos (Schneider, 1972). The cell line is relatively easy to grow and handle, and has been repeatedly used for expression of mammalian proteins. As they accept transfection with multiple constructs, they have also been used for studies of signal transduction, especially as many pathways and processes are more highly conserved between *Drosophila* and mammals, than between mammals and prokaryotes. However, the SL-2 lack many homologues of transcription factors ubiquitously expressed in mammals. Still, this could be useful when studying the transcriptional activities of transcription factors as it ensures less interference by endogenous factors compared with using a mammalian cell system (Suske, 2000). For our investigations of combinatorial effects of transcription factors on the *Ccsp/Scgb1a1* promoter the SL-2 cells thus provided a suitable cell model.

H441-cells

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; ATCC). The Calu-1 cells are derived from a squamous cell carcinoma (Fogh, 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 CCSP/SCGB1A1 but do support transcription of the *Ccsp/Scgb1a1* 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 III and IV to study the involvement of C/EBPs in glucocorticoid regulation of the *Ccsp/Scgb1a1* gene.

Primary cell cultures

In paper III and IV primary cells from rat and mouse were used, respectively. Cells from both species were isolated through protease digestion via tracheal instillation. To investigate the effect of glucocorticoids on CCSP/SCGB1A1, produced by Clara cells, the isolated epithelial cells were fractionated by size via centrifugal elutriation to enrich for a population of primary Clara cells (paper III). This gives a Clara cell fraction that is 40–50% pure. The remaining cells are mainly alveolar type II-cells. In paper IV the effect of glucocorticoids on C/EBPs in the epithelium were investigated in isolated primary cells from mouse. For these experiments we used all epithelial cells isolated from the protease digestion. The results obtained from glucocorticoid treating this mixed population of epithelial cells thus represent the overall response of the lung epithelium to dexamethasone. To further characterize if different epithelial cells respond differently to glucocorticoids, additional fractioning of the cells would be needed.

In paper IV human primary epithelial cells were also used. The cells were isolated from brush biopsies taken from two healthy human subjects via fiberoptic bronchoscopy as described in (Riise et al., 1994). The biopsies were taken from the right main bronchus and three consecutive brushings from an approximately 5 cm^2 intrabronchial mucosal area. The brush was then agitated in a vortex in 1 mL phosphate-buffered saline solution to collect the cell suspension for analysis. Approximately one million cells are obtained by this method and of which 90% are of epithelial origin (Riise et al., 1992). This number of cells enables preparation of nuclear extracts for further analysis by electrophoretic mobility shift assay (EMSA). The method provides the advantage of studying not only the presence of C/EBPs, but also to the DNA-binding activity of C/EBPs in human epithelial cells.

Mouse models

Even though mice are different from humans in many ways, they exhibit a high degree of physiological, as well as very close genetic similarities. Well established methods for genetic manipulations, as well as the possibility of using inbred mice that are genetically identical, makes mice the most common model system used for investigating the function of specific genes in development and physiology. To address the developmental importance of the C/EBPs and the role of C/EBPs in cellular differentiation we thus used mice as our animal model system. The C57BL/6 strain is one of the most widely used inbred strains in many areas of biomedical research including developmental biology. In paper II this strain was used for examining the

wild type expression of the C/EBPs during development. Furthermore, mouse strains overexpressing and lacking C/EBPα were used to examine the role of C/EBPα in lung development. The specific promoter (SFTPC) chosen for generation of transgenic mice overexpressing C/EBPα in lung, as well as the *Cebpa*$^{-/-}$ knockout mouse strain lacking C/EBPα are described in relevant sections of Results and Discussion below.

RESULTS AND DISCUSSION

Lung development is regulated by interactions between the endoderm and surrounding mesenchyme. This extracellular signaling eventually activates specific combinations of transcription factors regulating gene expression and thus guiding cellular differentiation. Compared to the molecular knowledge of how the first phases of lung development is controlled, less is know about the final steps in pulmonary branching and cellular differentiation, in mice occurring in the last days prior to birth. By investigating gene regulatory mechanisms active during this period, we hope to gain a greater understanding of the molecular basis of gene expression, cell growth and differentiation in the developing lung. Developmental programs of cellular differentiation are often reused in adult life in processes such as regeneration and repairation after injury. Thus, knowledge of how these processes are controlled could help us to understand how regeneration and differentiation of the epithelium after injury is achieved, as well as how normal cell turn over in the adult pulmonary epithelium is regulated.

C/EBPs in pulmonary gene regulation and development (Papers I and II)

As development proceeds, the cells of the lung differentiate to form the different cell types important for the function of the mature organ (Breeze et al., 1977). The level of differentiation and formation of individual cell types during development can be followed by looking at expression of lung specific and/or cell type specific proteins. The onset of expression of the genes encoding these proteins therefore reflects the ongoing cellular differentiation. By studying the regulation of these genes an insight into the molecular mechanisms behind the cellular differentiation can be gained. From previous studies of the regulation of one such gene, *Ccsp/Scgb1a1*, results indicating that C/EBP transcription factors could be involved in regulating cellular differentiation in the lung have been found (Nord et al., 1998).

C/EBPα, C/EBPβ and C/EBPδ are expressed in the adult lung epithelium (Cassel et al., 2000b; Lag et al., 2000; Li et al., 1995; Nord et al., 1998; Rosenberg et al., 2002; Sugahara et al., 1999). Due to high homology in their DNA-binding region they all recognize the same sequence and can activate similar sets of genes (Osada et al., 1996 and reviewed in Ramji et al., 2002). In addition to *Ccsp/Scgb1a1* (Cassel et al., 2000b), C/EBPs regulate the expression of several lung-specific genes with a differentiation dependent expression pattern such, as *Sftpa, Sftpd* and *Cyp2b1* (Cassel et al., 2000a; He

et al., 2002; Luc et al., 1996; Nord et al., 1998; Rosenberg et al., 2002). In several other organs where C/EBPs are expressed they not only regulate the expression of tissue-specific genes but also have a role during development. Knockout mice for *Cebpa* display a lung phenotype at birth with hyperproliferation of alveolar type II cells indicating that C/EBPα is necessary for differentiation of the lung epithelium (Flodby et al., 1996; Wang et al., 1995). Also when growing primary Clara cells in culture they rapidly dedifferentiate and in parallel to loss of expression of CCSP/SCGB1A1, the cells also lose the expression of C/EBPα (Nord et al., 1998). This implies that C/EBPα could be important for keeping these cells in a differentiated state and maybe be involved in the onset of expression of *Ccsp/Scgb1a1* and other genes whose expression, like *Ccsp/Scgb1a1,* is dependent on the differentiation status of the epithelium. However, no detailed information about the developmental expression pattern of C/EBPs during lung development exists.

Developmental expression

In paper II the developmental expression patterns of C/EBPα, C/EBPβ and C/EBPδ were investigated by immunohistochemistry. C/EBPα, C/EBPδ and C/EBPβ were detected during mouse lung embryogenesis from the late pseudoglandular stage in both mesenchyme and epithelium. Their expression then increased through the saccular stage and in parallel progressively became epithelium specific. In adult lung, expression was found in cells in the alveolar region as well as in cells lining the conducting airways. Our results are in agreement with the few existing publications on developmental expression of C/EBPs. In microarray analysis of developing mouse lung *Cebpb* and *Cebpd* have been detected in the branching region whereas *Cebpa* could not be detected at E11.5 (Lu et al., 2004). In whole rat lung, mRNA expression of *Cebpa*, *Cebpb* and *Cebpd* has been seen to increase just prior to birth, dropping around birth after which it increased again (Barlier-Mur et al., 2003).

As the C/EBPs were expressed during development and their expression increased during the final maturation process just prior to birth, their expression patterns correlated well with the onset of expression of *CCSP/SCGB1A1* and other lung specific genes with a differentiation dependent expression pattern such as *Sftpa*, *Sftpd* and *Cyp2b1*. This leads us to the hypothesis that C/EBPs could be the involved in initiating the high-level CCSP/SCBG1A1 expression seen late in lung development.

C/EBPα and NKX2.1 synergistically transactivate the CCSP promoter

The most proximal part (around 170bp) of the *Ccsp/Scgb1a1* promoter drives lung-specific expression of the *Ccsp/Scgb1a1* gene (Ray et al., 1995; Stripp et al., 1992). This promoter fragment, in addition to C/EBPs (Cassel et al., 2000b), is regulated by several other transcription factors such as FOXA1, FOXA2 and NKX2.1 (Ray et al., 1996; Sawaya et al., 1994; Toonen et al., 1996). As discussed above, expression of CCSP/SCGB1A1 is differentiation-dependent and is turned on late in lung development, in mouse around day E17 (Zhou et al., 1996b). This late onset cannot be explained by the expression patterns of NKX2.1, FOXA1 or FOXA2, which all are expressed from at least when the lung bud is formed (E9.5 in mouse) and then continue to be expressed throughout development (Besnard et al., 2004; Zhou et al., 1996b). Thus it seemed plausible that the combinatorial activity of C/EBPs and these other transcription factors could be involved in the onset of high-level expression of the *Ccsp/Scgb1a1* gene.

By transfecting the *Drosophila* cell line SL-2 with expression plasmids encoding the transcription factors, FOXA, NKX2.1, C/EBPα, C/EBPδ and a reporter plasmid containing a 172bp long proximal fragment of the *Ccsp/Scgb1a1* promoter we examined combinatorial activities in *Ccsp/Scgb1a1* gene regulation (paper I). The SL-2 cell line lacks many homologues of mammalian transcription factors and therefore provides a low background for studies of co-operativity in transcriptional regulation. We found that NKX2.1 together with C/EBPα had a strong synergistic effect on *Ccsp/Scgb1a1* promoter activity, and that these two factors together activated the promoter almost 50-fold. This induction can be compared with the activation of the promoter using each of them alone where NKX2.1 activated the promoter 3.5-fold, and C/EBPα activated the promoter 1.5-fold. This strong synergistic effect was not seen with any other combination of transcription factors tested. Most notable was that C/EBPδ and NKX2.1 did not have the same activation potential as C/EBPα and NKX2.1. As the two family members have very similar DNA-binding regions the difference is probably due to the more diverse transactivation domains of C/EBPδ and C/EBPα.

To characterize the mechanism behind the synergistic effect between C/EBPα and NKX2.1, we performed additional transfection studies using expression plasmids encoding truncated forms of each transcription factor. The results show that the synergistic effect is indeed dependent on the transactivation domain located in the N-terminus of both factors. The deletion of residue 1-127 of C/EBPα and residues 46-152 of NKX2.1 had the most striking effects. In addition, the deletion of 46-152 on NKX2.1

also took away the additive effect of the two factors. While the results from this paper do not provide a definite mechanism for how the synergistic effect between NKX2.1 and C/EBPα is carried out they do demonstrate the involvement of the transactivation domains of both factors. This result is not surprising as the deleted domains of C/EBPα and NKX2.1 are necessary for these factors to promote transcription (De Felice et al., 1995; Nerlov et al., 1995; Tell et al., 1998). The synergy was also dependent on both binding sites for NKX2.1, however, some synergy between the two factors still occurred after both C/EBP binding sites were mutated. In addition, we could see that both factors can bind simultaneously at the promoter. Although evidence for a direct interaction between C/EBPα and NKX2.1 is not provided in this paper, the data indicates that some kind of protein-protein interaction might be involved. Results from pull-down assays also reveal that an interaction between C/EBPα and NKX2.1 does occur in solution (Nord, unpublished observation). C/EBPα has previously been shown to directly interact with another member of the homeodomain factor-family, PIT-1. In this case the interaction with PIT-1 helped to recruit C/EBPα to the nucleus (Day et al., 2003; Enwright et al., 2003). It is possible that a direct interaction between C/EBPα and NKX2.1 aids recruitment to the promoter by forming a more stable complex, and thus promotes more efficient recruitment of co-activators and the general transcription machinery.

C/EBPα in lung development

The expression pattern of C/EBPα together with the cooperative action of C/EBPα and NKX2.1 indicated that this synergy could be a major determinant for the onset of *Ccsp/Scgb1a1* expression during development. This suggested a role for C/EBPα in controlling cellular differentiation of the lung epithelium, and as C/EBPα has previously been shown to promote differentiation in other organs we further characterized the role of C/EBPα during lung development. When the expression pattern of C/EBPα was investigated in more detail, a very specific expression pattern was revealed. The expression of C/EBPα was turned on weakly at E15.5 in just a subset of cells in the distal tips of the growing and branching epithelial tubules. The specific pattern suggested, that C/EBPα could be involved in branching morphogenesis. However, in previous studies of the lungs of *Cebpa*$^{-/-}$ mice, no indications of disturbed branching had been seen. Instead, these mice had overproliferation of alveolar type II cells in the distal region indicating a role for C/EBPα in differentiation of the distal lung epithelium. There was no obvious change in the expression pattern of CCSP/SCGB1A1 in these mice, which could be expected if C/EBPα is necessary for

expression of the *Ccsp/Scgb1a1* gene (Flodby et al., 1996). However, the levels of expression were not analyzed and as C/EBPs are highly homologous especially in their DNA-binding region, and thus capable of activating almost identical genes, it is possible that the role of C/EBPα to promote *Ccsp/Scgb1a1* expression is compensated for by C/EBPβ or C/EBPδ in these mice. To what extent this occurs in the *Cebpa*$^{-/-}$ mice has however not been addressed. Paper I showed that the strong synergistic effect was specific to the transactivation domain of C/EBPα and that the synergistic effect could not be replaced by C/EBPδ or a hybrid protein consisting of the DNA-binding region of C/EBPα and the transactivation domain of C/EBPδ. However, C/EBPβ and NKX2.1 do have some synergistic activity, although not as strong as C/EBPα and NKX2.1 (Cassel, unpublished observations) and possibly C/EBPβ and C/EBPα have some redundant activities in the lung epithelium. Thus the presence of C/EBPβ in the epithelium at these developmental stages could compensate for the loss of C/EBPα. That C/EBPδ and C/EBPα did not show the same synergistic activity together with NKX2.1 in this assay shows that although the C/EBPs do display similar or identical DNA-binding specificity, they do differ in their regulation of target genes. Furthermore, transgenic knock in mice where the *Cebpa* gene functionally has been replaced by *Cebpb* partly rescues the phenotype of the *Cebpa*$^{-/-}$ mice in the liver and white adipose tissue, providing further evidence that C/EBPβ has the possibility to take over C/EBPα functions in other organs. However, the excess of C/EBPβ in these mutants leads to an increased mass of brown adipose tissue and suggests that the timing and level of expression of C/EBPs are of importance (Chen et al., 2000; Jones et al., 2002). To further investigate the role of C/EBPα in lung development we continued by generating a g*ain-of-function* mouse model that ectopically expressed C/EBPα in order to complement the results from the *Cebpa*$^{-/-}$ *loss-of-function* mice.

Ectopic expression of C/EBPα affects lung development

Gain-of-function mice were generated (paper II) by using the human surfactant protein C promoter to control the *Cebpa* gene (*SFPTC-Cebpa* mice). The *SFPTC*-promoter has been shown to give a lung epithelial specific expression of many different transgenes from at least E11 of lung development. The expression is restricted to the lung epithelium with higher expression in the distal region (Okubo et al., 2004; Wert et al., 1993). When lungs from the transgenic mice were examined at E15.5, C/EBPα expression was readily detected in most cells of the distal lung epithelium. Histological studies revealed a defect in lung development characterized by fewer and larger developing epithelial tubules than their wild type littermates. The transgenic lungs did,

however, not differ in size from lungs of their wild type littermates. Moreover, the number of proliferating cells at this particular stage of lung development was not altered using Cyclin A as a marker of proliferation. Thus, the phenotype cannot be explained by a general inhibition of growth that might be expected from the anti-proliferative activity of C/EBPα. As C/EBPα mediates growth inhibition through interacting with proteins involved in cell-cycle regulation, such as CDK2, CDK4, p21 and p53 (Harris et al., 2001; Timchenko et al., 1997; Timchenko et al., 1996; Wang et al., 2001), the lack of growth inhibitory effect in these mice could possibly be due to limited expression of such a factor. P21 for instance, is not expressed until the late pseudoglandular and canalicular stages (Ikoma et al., 2001; Berg, unpublished observations).

To investigate proximal- distal cell differentiation, expression of SP-C was examined and found to be unaltered. Likewise did we not detect any differences in SP-A or CCSP/SCGB1A1 expression. These two proteins were used as markers to investigate whether forced expression of C/EBPα resulted in premature differentiation, as SP-A or CCSP/SCGB1A1 normally are not expressed at this early stage (Korfhagen et al., 1992; Zhou et al., 1996b). This indicates that ectopic expression of C/EBPα from an earlier time-point than normal did not cause premature maturation of the lung epithelium as could have been expected from the results in paper I. Instead the phenotype of the *SFPTC-Cebpa* transgenic mice, with fewer and larger developing epithelial tubules, together with the expression pattern of the endogenous C/EBPα close to the tips, indicates that the tempo-spatial expression of C/EBPα is important for correct development during the late pseudoglandular stage.

C/EBPα$^{-/-}$ have defects in late development

Knockout mice for *Cebpa* have previously been generated, see above, but as these mice have not been studied in detail during the embryonic period, we investigated the lungs of the *Cebpa$^{-/-}$* mice prior to birth (paper II). Around day E17.5 the first phenotype was observed in the *Cebpa$^{-/-}$* mice. This phenotype was characterized by a decreased number of growing epithelial tubules also larger in size, similar to the morphological changes seen in the transgenic mice overexpressing C/EBPα. In agreement to what has previously been observed for newborn *Cebpa$^{-/-}$* mice, there was no change in SP-C expression. This indicates that proximal-distal differentiation of the lung epithelium is still intact even though C/EBPα is missing. That the lungs of the *SFPTC-Cebpa* transgenic embryos and the *Cebpa$^{-/-}$* knockout embryos displayed a similar phenotype

further underlines the need for C/EBPα to be expressed at the right level, but also at the right time and place, during development.

C/EBPα involved in late lung branching morphogenesis?

C/EBPα is a strong inhibitor of proliferation and in paper II we observed that most of the cells that expressed C/EBPα at E17.5 do not proliferate. This suggests that C/EBPα under normal conditions in lung, similarly to what has been seen in other organs, is expressed in non-proliferating cells and possibly could have a role in regulation of cell growth during development. Based on these observations a model for how C/EBPα could modulate branching was proposed in paper II. In this model, C/EBPα, due to its antiproliferative properties, helps to define cells close to the tips of the growing epithelial tubules that are to stop proliferating. Cells not expressing C/EBPα would then be allowed to proliferate and to expand into the surrounding mesenchyme forming new branches (figure 3).

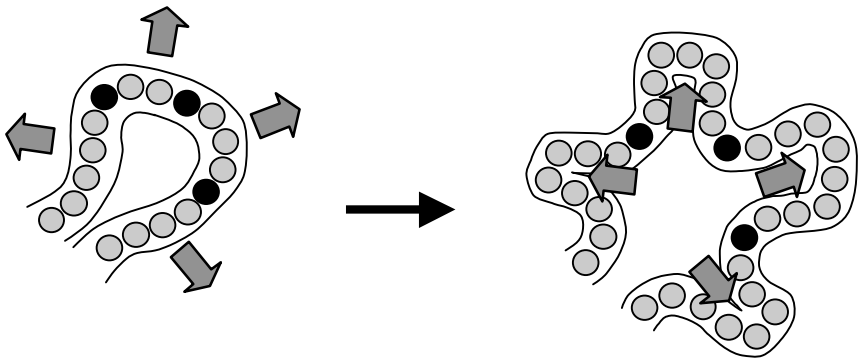

Figure 3. Hypothetical model for how C/EBPα could be involved in controlling branching morphogenesis during late lung development

A subset of cells express C/EBPα (black nuclei) in the growing epithelial tubules. Cells expressing C/EBPα do not proliferate whereas neighbouring cells continue to proliferate and expand into the surrounding tissue, forming new branches. The C/EBPα expressing cells in this case mark non-proliferative areas that are not actively growing and hence are involved in branch-point definition.

In this way, the specific expression of C/EBPα could help to define branch points by determining which cells are to stop proliferating and thus regulate the ratio between non-dividing cells and dividing cells in the tips of the growing-epithelial tubules. Overexpressing, or losing C/EBPα expression, could then disturb the fine balance required for adequate branch formation, resulting in diffuse growth of the epithelium and establishment of fewer branch points. This would lead to the observed phenotypes

with fewer and more dilated epithelial tubules seen in both the *gain-of-function* and the *loss-of-function* mouse models. However, this model is based on the assumption that regulation of proliferation in the epithelium is important in the process of branching morphogenesis at the later stages of lung development. On the other hand, no model for how branching and branch point formation is regulated at these developmental stages yet exists and even though C/EBPα is expressed in cells that do not proliferate, the data provided in paper II is not enough to conclusively say that this is the case. Also, ectopic expression of C/EBPα did not cause a significant inhibition of general lung growth, indicating that our model for the formation of fewer and bigger airways must be due a more subtle difference in proliferation, locally restricted to the epithelial tips. Since the effect of ectopic expression of C/EBPα was not known, all *SFPTC-Cebpa* pups were taken before term and no line of *SFPTC-Cebpa* mice was established. Possibly the *SFPTC-Cebpa* phenotype will, in contrast to the *Cebpa*$^{-/-}$ mice, allow for post-natal survival. If so, looking at the effect of ectopic expression of C/EBPα at earlier stages and simultaneously performing double stainings for a marker of proliferation and C/EBPα could be informative. This would further address the effects of overexpression of C/EBPα on branching and growth of the epithelium, and determine if the processes of growth and branching are independent or dependent events.

C/EBPα in cellular differentiation and gene regulation

After the pseudoglandular-canalicular transition, the expression of C/EBPα expands from just a subset of cells in the tips of the growing epithelial tubules into being expressed in almost all cells lining the conducting airways as well as some cells in the future respiratory region. The expression thus resembles the expression pattern of C/EBPα seen in the adult lung where C/EBPα is expressed in most cells in the conducting airways and in alveolar type II cells. This expansion correlates well with the extensive cellular differentiation occurring in this period (reviewed in Cardoso, 2000; Cassel et al., 2003; Mendelson, 2000 and Prodhan et al., 2002) and the onset of C/EBPα target gene expression including *Ccsp/Scgb1a1, Sfpta* and *Sfptd* (Cassel et al., 2000b; He et al., 2002; Rosenberg et al., 2002). As implied by paper I, the combinatorial activity of the transcription factors C/EBPα and NKX2.1 gives rise to high-level expression of *Ccsp/Scgb1a1*. However, as NKX2.1 is an early marker of lung differentiation and is expressed throughout lung development its expression pattern cannot explain the late appearance of CCSP/SCGB1A1 (figure 4) (Lazzaro et al., 1991). Instead, the relatively late onset of high expression of C/EBPα spoke in favor of C/EBPα being the final factor needed for onset of *Ccsp/Scgb1a1* expression (figure 4) and differentiation of the distal epithelium. In addition to the lung epithelium,

NKX2.1 has been reported to be expressed in the thyroid and restricted regions in the fore-brain. Hence, the combination of NKX2.1 and C/EBPα is also rather unique for the lung epithelium. No detailed information exists of the expression of C/EBPα in the brain and since no expression of C/EBPα has been reported in the thyroid this specific combination of transcription factors could be involved in the lung specific expression of *Ccsp/Scgb1a1* and other genes that similar to *Ccsp/Scgb1a1* also are regulated by both factors, for instance the gene encoding SP-D, *Sfptd* (figure 4).

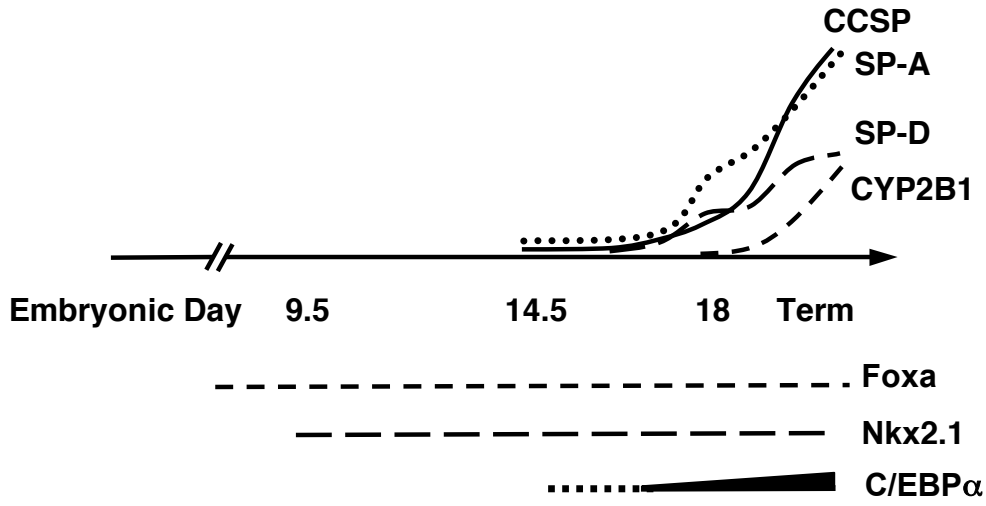

Figure 4. Is C/EBPα involved in the onset of differentiation dependent gene expression in lung development?

Illustration of the relative expression of genes during embryonic development that are important for the function of the lung after birth (y-axis). Their expression is related to the expression of transcription factors involved in their regulation (x-axis). Numbers indicate days of embryonic development in mouse. Does the relatively late onset of C/EBPα explain the late onset of expression of the Clara cell secretory protein?(Figure based on Crouch et al., 1991; Fanucchi et al., 1997; Farrell et al., 1990; Lazzaro et al., 1991; Li et al., 1995; Ogasawara et al., 1991; Shimizu et al., 1991; Ten Have-Opbroek, 1981; Ten Have-Opbroek and De Vries, 1993; Zhou et al., 1996b)

However the results in paper II do not strengthen this hypothesis as the *SFPTC-Cebpa* transgenic mice, ectopically expressing C/EBPα from an earlier time point, did not show any obvious signs of pre-mature epithelial differentiation or expression of CCSP/SCGB1A1 or SP-A. Therefore we conclude that overexpression of C/EBPα is not enough to promote cellular differentiation and *Ccsp/Scgb1a1* expression in the lung on its own. Probably additional factors needed for high level *Ccsp/Scgb1a1* expression are lacking in the lung epithelium at this particular developmental stage. Additionally, losing C/EBPα as in the *Cebpa*$^{-/-}$ mice already previously described, did not disrupt the expression of CCSP/SCGB1A1 as could be expected from the strong synergistic effect

of C/EBPα and NKX2.1. Several explanations for this are plausible. One possibility could be that C/EBP family members have redundant functions and that other family members can take part in or replace the function of C/EBPα as discussed above. Another possible explanation for the lack of effect on CCSP/SCGB1A1 expression in these mice could be that C/EBPα plays a bigger role in *Ccsp/Scgb1a1* regulation after birth. Normally the maximal levels of CCSP/SCGB1A1 are not reached until several days postnatally (Cardoso et al., 1993; Ten Have-Opbroek et al., 1993) and as the *Cebpa*$^{-/-}$ mice not are viable (Wang et al., 1995) the effect of lack of C/EBPα on adult CCSP/SCGB1A1 levels cannot be examined. In addition, differentiation of the distal lung epithelium occurs even in the absence of C/EBPα, as both alveolar type I and type II cells are present in the *Cebpa*$^{-/-}$ mice. This indicates that C/EBPα is not an essential requirement for initial differentiation of the distal pulmonary epithelium to occur. Still, the phenotype in the *Cebpa*$^{-/-}$ mice with overexpansion of alveolar type II cells suggests that C/EBPα is important for keeping these cells in a differentiated and non-dividing state. Construction of a lung-specific knockout mouse for *Cebpa* could be a way to further investigate the importance of C/EBPα in cellular differentiation and gene expression in the adult lung.

C/EBPs in glucocorticoid signaling (Paper III and IV)

Glucocorticoids act both through classical gene activation/repression by forming a ligand-receptor complex that directly binds to DNA, and by interfering with other pathways via protein-protein interactions (reviewed in De Bosscher et al., 2003). During development glucocorticoids are of critical importance and defects in the glucocorticoid signal result in immature lungs and mice without the glucocorticoid receptor die directly after birth due to respiratory failure (Cole et al., 1995). Glucocorticoids are also used in the clinic to speed up lung differentiation in premature infants in order to avoid respiratory stress syndrome due to immature lungs (reviewed in Purdy et al., 2004). As glucocorticoids also have strong anti-inflammatory properties they are widely used in treatment of various inflammatory lung disorders (reviewed in Barnes et al., 2003). However, glucocorticoid treatment also causes unwanted systemic effects, for instance on metabolism and growth (reviewed in Schacke et al., 2002). As outlined in the introduction, unclear issues remain about the mechanisms of glucocorticoid signaling in the lung. Considering the wide use of glucocorticoids in treatment of lung disorders, increased knowledge about glucocorticoid mechanisms of action in lung are of importance.

C/EBPβ and C/EBPδ mediate glucocorticoid induction of CCSP/SCGB1A1

In other organs such as liver and fat, a positive interplay between C/EBPs and glucocorticoids has been demonstrated (Cao et al., 1991; MacDougald et al., 1994; Matsuno et al., 1996) which lead us to the hypothesis that C/EBPs could be involved in glucocorticoid signaling in the lung epithelium. In paper III we investigated the effect of glucocorticoids on *Ccsp/Scgb1a1* expression in the human lung epithelial cell line NCI-H441. A 172bp fragment of the proximal *Ccsp/Scgb1a1* promoter coupled to a luciferase reporter gene was used in transfection studies. When cells were treated with dexamethasone, a glucocorticoid homologue, the activity of the promoter was increased. As the *CCSP/Scgb1a1* promoter does not contain any functional binding sites for the glucocorticoid receptor, we inactivated the two C/EBP binding sites by site-directed mutagenesis. This abolished the glucocorticoid induction, indicating that the C/EBP-sites are necessary for the effect of glucocorticoids. To study whether C/EBPs are involved in the activation of other glucocorticoid-responsive genes expressed in lung, we studied the *CYP2B1* promoter (Ronis et al., 1999). This gene has not been shown to contain any functional GR-bindingsites but does have one functional C/EBP-binding site in the proximal promoter (Park et al., 1996). Treatment with dexamethasone increased the promoter activity of the *CYP2B1* gene as well, and the induction was similarly abolished after mutating the C/EBP-binding site. This suggests that C/EBPs have a more general role in glucocorticoid activation of gene expression in the lung epithelium. By electrophoretic mobility shift assay (EMSA), we could show that binding of C/EBPβ and C/EBPδ to a C/EBP consensus-binding site was increased after treatment with glucocorticoids. However, the C/EBPβ and C/EBPδ protein levels did not increase, indicating that protein synthesis could not explain the increased binding. In paper IV we continued to investigate the underlying mechanism behind the increased DNA binding of C/EBP by using cyclohexamide to block protein synthesis. Cyclohexamide did not affect the ability of glucocorticoids to increase binding of C/EBPβ and C/EBPδ, showing that the effect is independent of protein synthesis. This result is in contrast to what is seen in skeletal muscle and hepatocytes where glucocorticoids increase the DNA binding of both C/EBPβ and C/EBPδ by increasing the expression of C/EBPβ and C/EBPδ (Matsuno et al., 1996; Penner et al., 2002; Yang et al., 2005). Possibly this phenomenon is specific to the lung epithelium, which could provide a pootential target for development of drugs specifically active in lung with less metabolic side effects in liver and muscle.

When the time frame of the increased DNA binding was investigated by EMSA, we found that it was increased as early as after ten minutes of glucocorticoid treatment.

Moreover, the increase in binding showed dose-response and experiments using RU486, a glucocorticoid receptor antagonist, showed that the rapid increase in binding of C/EBPβ and C/EBPδ was mediated via the glucocorticoid receptor. That the effect was rapid and independent of protein synthesis indicated that the effect of glucocorticoids is direct and possibly involves some kind of post-translational modification of C/EBPβ and C/EBPδ.

Glucocorticoids increase binding of C/EBP through post-translational modification

Post-translational modifications of C/EBPs could result in increased affinity for DNA, increased nuclear import, altered intra-nuclear localization or protein-protein interaction between C/EBPs and other proteins. All scenarios mentioned could in theory cause increased binding of the C/EBPs to their target promoters, independent of new protein synthesis. For instance, both C/EBPβ and C/EBPδ can directly interact with the glucorticoid receptor (Boruk et al., 1998). However, we have been unable to find any co-interaction between C/EBPs and the glucocorticoid receptor, using EMSA studies or immunoprecipitation (Berg, unpublished observations). In paper IV we also examined the localization of the C/EBPs after glucocorticoid treatment using Western blot and immunofluoresence. However, the localization of C/EBPs seemed not to be influenced by glucocorticoids and no effects on nuclear localization or intra-nuclear localization could be found.

Post-translational modifications such as phosphorylations, acetylation and sumoylation has been shown to modulate transactivation, DNA binding and localization of the C/EBPs (reviewed in Ramji et al., 2002). So far nothing is known about whether glucocorticoids influence these modifications. When using an antibody specifically recognizing C/EBPβ phosphorylated on Thr^{235} we saw that this form of C/EBPβ was increased after glucorticoid treatment. This site of phosphorylation in human C/EBPβ corresponds to phosphorylation of Thr^{188} in mouse (Piwien-Pilipuk et al., 2002). Phosphorylation at this site has been shown to be important for C/EBPβ to acquire DNA-binding activity during adipocyte differentiation (Park et al., 2004; Tang et al., 2005) as well as to stimulate its transactivating activity (Nakajima et al., 1993). Based on these observations it is possible that increased phosphorylation of this residue could account for the increased binding and transactivation at the *Ccsp/Scgb1a1* and *CYP2B1* promoters seen in paper III. The Thr^{235} phosphorylation has been previously shown to be mediated by ERK 1/2. However, using an antibody that specifically recognizes activated ERK 1/2, we showed that ERK 1/2 was not activated by glucocorticoids. This

was confirmed by blocking MEK 1/2, the upstream activator of ERK 1/2, with a chemical inhibitor. Blocking MEK 1/2 did not affect the ability of glucocorticoids to increase the binding activity of C/EBPβ. In an attempt to further characterize the signaling pathways that could be involved, different chemical inhibitors for several kinase signaling pathways, as well as a broad-spectrum phosphatase inhibitor, were used. However, none of them blocked the ability of dexamethasone to induce C/EBP-binding. Therefore it could be that the mechanism behind the phosphorylation of residue Thr^{235} on C/EBPβ includes a kinase not covered by the chemical inhibitors used in this study. An alternative mechanism is that glucocorticoid activation facilitates phosphorylation of C/EBPβ by an already activated kinase. Thus, the glucocorticoid pathway, when activated, could facilitate an interaction between C/EBPβ and a kinase/phosphatase or by other means make C/EBPβ more accessible for modifications. In this case it would be possible for increased phosphorylation on C/EBPβ to occur even if the kinase itself is not activated, as the basal activity of the kinase would be enough to promote increased phosphorylation. Hence, an involvement of ERK in the glucocorticoid pathway cannot be ruled out even though increased activation of ERK was not detected after glucocorticoid treatment. Whether the increased phosphorylation on Thr^{235} contributes to the increase in DNA binding by C/EBPβ remains to be established, as well as what causes the increase in binding by C/EBPδ. Studying different phosphorylation mutants of C/EBPβ could be used to address this but it is possible that glucocorticoid stimulation activates several pathways leading to multiple phosphorylations or dephosphorylations of the C/EBPs.

Even though the NCI-H441 cell line is described as being of Clara cell origin, it is derived from a human adenocarcionoma as described above, and does not therefore fully reflect a differentiated Clara cell. However a similar DNA-binding profile as observed in the NCI-H441 cells, with prominent binding of C/EBPβ and binding of C/EBPδ to a lesser extent, was observed in primary lung epithelial cells from both human and mouse. Glucocorticoid treatment also increased binding of C/EBPβ and C/EBPδ in the primary mouse epithelial cells, and increased binding of these factors after glucocorticoid treatment was also observed in the human lung cell line A549, which is of alveolar type II cell origin (Berg, unpublished observations). These results further support the relevance of our findings in the NCI-H441 cell line, and validate the use of NCI-H441 cells to model glucocorticoid signal transduction in human airway epithelial cells. Together the data provided in papers III and IV indicate a previously unknown role for C/EBP transcription factors in glucocorticoid signaling in the lung epithelium.

Glucocorticoids are used effectively in the treatment of various inflammatory lung disorders, especially asthma. In contrast to asthma, glucocorticoid treatment of the inflammatory lung disorder chronic obstructive pulmonary disease (COPD) is less effective (reviewed in Caramori et al., 2003). A decreased DNA-binding activity of C/EBPβ in the lung epithelium of smokers with chronic bronchitis and COPD has recently been reported (Didon et al., 2005). It is possible that the decreased activity of C/EBPβ in the lungs of COPD patients could play a role in the relative glucocorticoid unresponsiveness seen in this group of patients.

CONCLUSIONS

Paper I

C/EBPα and NKX2.1 synergistically transactivate the *Ccsp/Scbg1a1* promoter.

The lung-specific combination of C/EBPα and Nkx2.1, together with their strong synergistic activity, suggests that these transcription factors could be responsible for the lung specific, high-level expression of CCSP/SCGB1A1 in the adult lung epithelium.

Paper II

C/EBPα has a role in the late stages of lung development

During lung development, C/EBPα exhibits a dynamic expression pattern correlating with cellular differentiation. *Loss-of-function* and *gain-of-function* mice display similar developmental phenotypes with fewer and larger epithelial tubules. However, no effects on cellular differentiation were seen. This indicates that the locally restricted expression and activity of C/EBPα in time and space are important for lung development.

Paper III and IV

C/EBPβ and C/EBPδ have a role in glucocorticoid signaling in the lung epithelium.

C/EBPβ and C/EBPδ mediate glucocorticoid induction of the *Ccsp/Scgb1a1* and *CYP2B1* genes in lung epithelial cells. This occurs through glucocorticoid-induced post-translational modifications resulting in increased DNA binding.

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