Ectopic expression of C/EBPα in the lung epithelium disrupts late lung development

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Berg, Tove, Lukas Didon, and Magnus Nord. Ectopic expression of C/EBPα in the lung epithelium disrupts late lung development. Am J Physiol Lung Cell Mol Physiol 291: L683–L693, 2006.—The lung develops from the endoderm through a process of branching morphogenesis. This process is highly active during the pseudoglandular stage of lung development and continues into the canalicular stage, resulting in the formation of terminal sacs. CCAAT/enhancer binding proteins (C/EBPs) are transcription factors regulating central aspects of differentiation and proliferation. We report here the developmental expression of C/EBPα, -β, and -δ in the lung. C/EBPα exhibits a dynamic expression pattern and is first detected during the late pseudoglandular stage. At this stage, expression is observed in a subset of epithelial cells in the distal parts of the branching tubules. The expression of C/EBPα is confined to nonproliferating cells. To examine the role of C/EBPα in lung development, we generated transgenic mice ectopically expressing C/EBPα in the lung epithelium using the human surfactant protein C promoter. Lungs from these mice were of normal size but exhibited a phenotype characterized by fewer and larger developing epithelial tubules, indicating that the branching process was affected. No effects on overall proliferation or cellular differentiation were observed. When this phenotype was compared with that of mice carrying a targeted mutation of the Cebpa gene, the Cebpa−/− mice exhibited a similar developmental phenotype. In conclusion, our results show a role for C/EBPα in lung development and suggest a function in the later stages of lung branching morphogenesis.

CCAAT/enhancer binding proteins; transcription factors; expression pattern; branching morphogenesis; cellular differentiation

MOUSE LUNG DEVELOPMENT starts at embryonic day 9.5 (E9.5), when two primordial buds appear in the ventral foregut endoderm just anterior to the developing stomach. This is followed by growth and branching of the primitive lung endoderm in a process of branching morphogenesis dependent on reciprocal interactions between the endoderm and surrounding mesenchyme. It eventually leads to the development of the highly branched organ that constitutes the mature lung, where the conducting airways lead the inhaled air into the respiratory alveolar region. For reviews on lung development, see, for example, Refs. 9, 14, 24, 52, and 60.

Lung development can be histologically divided into four stages. The first stage, the pseudoglandular period, reaches from E9.5 to E16.5 in the mouse. During this period of active branching morphogenesis, the respiratory tree is formed from the primordial buds and lined with epithelial precursor cells. Coincident with growth and branching, the lung undergoes proximal-distal patterning. At the end of the pseudoglandular period, extensive cellular differentiation occurs. This results in a change of appearance, and structurally the developing lung is described as having entered the next histological stage, the alveolar period (E16.5–E17.5). In parallel with this burst of cellular differentiation in the late pseudoglandular and canalicular stages, the distal epithelium continues to branch through a process morphologically different from the preceding branching morphogenesis (45). This results in the formation of terminal sacs, and the following stage is referred to as the saccular, or terminal sac, period and spans E17.5 to postnatal day 5 (P5) in the mouse. During these later stages of lung development, differentiation of the epithelial precursor cells leads to the formation of the various cell types lining the proximal and distal airways. This differentiation is reflected by the onset of expression of differentiation-dependent genes necessary for respiration, most notably components of the surfactant system (35, 63). In parallel, future air spaces widen, interstitial tissue thins out, and the vasculature and capillary bed mature. Together, this serves to prepare the lung for respiration after birth. However, complete alveologenesis, with septation of the air spaces, finishes after birth during the fourth stage of lung development, the alveolar period (P5–P30 in the mouse).

CCAAT/enhancer binding proteins (C/EBPs) are a family of related basic region leucine zipper transcription factors regulating central aspects of differentiation such as gene expression and proliferation; for recent reviews, see, for example, Refs. 12 and 47. Typically, C/EBPs are involved in stimulating the transcription of genes characteristic of the mature differentiated organ, and their critical role in these processes has been well established in the liver and fat (7, 15, 27, 48). Studies in lung epithelial cell lines have shown that C/EBPs regulate the expression of several lung-enriched genes including surfactant proteins A and D (22, 49), the airway secretory protein secretoglobin 1a1/Clara cell secretory protein (CCSP) (38), and the cytochrome P-450 enzyme Cyp2b1 (11). All these genes exhibit a developmental expression pattern paralleling the extensive cellular differentiation occurring during the canalicular and saccular stages of lung development leading to the formation of the different specialized cell types of the proximal and distal lung.

Because of their highly related structure, considerable overlap in target gene specificity of the different C/EBPs exist, and different C/EBPs can, to a large extent, functionally replace

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each other with regard to their transactivating functions (47). In contrast, with regard to the effects of different C/EBP members on proliferation, a clear distinction is evident. Here, C/EBPα is a strong inhibitor of proliferation and is downregulated in proliferating cells, whereas C/EBPβ and C/EBPδ support proliferation (7, 34). These opposing roles are apparent during adipose differentiation, where C/EBPβ and C/EBPδ are expressed during mitotic expansion and C/EBPα is turned on later, inducing terminal differentiation (8, 57). Similar mechanisms are seen during liver regeneration, where C/EBPβ and C/EBPδ increase and C/EBPα decreases during the proliferative phase (17, 36).

In this study, we investigated the expression pattern of C/EBPα, C/EBPβ, and C/EBPδ during lung development. C/EBPα displayed a dynamic expression pattern with expression initially observed in a subset of cells in the distal parts of the branching tubules. By generating and studying lung development in a transgenic C/EBPα gain of function mouse model, in combination with a C/EBPδ loss of function mouse model, we found results indicating a role for C/EBPα in lung development with a function in the later stages of branching morphogenesis.

MATERIALS AND METHODS

Animals. To generate the construct for the lung-specific C/EBPα gain of function mouse [surfactant protein C (Sftpc)-Cebpa], we cloned an EcoRI fragment encoding murine C/EBPα from pCMV/EBPα (10) into the EcoRI site of the 3.7SPC/SV40 vector (a kind gift from Drs. Jeffrey A. Whitsett and Stephan W. Glasser, Cincinnati Children’s Hospital Medical Center). In the resulting construct, the 3.7-kb human SfpC promoter contained in the vector drives the expression of the Cebpa transgene only in embryonic lung epithelial cells. After linearization of the DNA construct with and NotI, C/EBPα and C/EBPβ were expressed during the embryonic stages described. Fixation was done with either PBS (pH 7.2) or PBS containing 4% paraformaldehyde or PBS with 4% paraformaldehyde plus 0.5% Triton (≥ E16.5) for 24 h at 4°C. After dehydration and paraffin embedding, 4-μm sections were cut and deparaffinized in xylene followed by rehydration through ethanol to water. Antigen retrieval was performed by microwaving in 10 mM citrate buffer (pH 6.0) for 30 min at 200 W. Endogenous peroxidase activity was retrieved by microwaving in 10 mM citrate buffer (pH 6.0) for 30 min at 200 W. Endogenous peroxidase activity was then quenched with 3% H2O2 in PBS for 10–15 min at room temperature. After being washed, sections were blocked in 5% serum in PBS with 0.3% Tween (PBS-T) for 1 h at room temperature. Sections were incubated with primary antibody overnight at 4°C, washed in PBS-T, and incubated with secondary antibody for 1 h at room temperature.

RESULTS

C/EBPα, C/EBPβ, and C/EBPδ are expressed during lung development. No detailed information exists on the developmental expression pattern of C/EBP transcription factors in lung development. In the lung, C/EBPα, C/EBPβ, and C/EBPδ are expressed (12), and we therefore investigated their expression pattern during lung development with immunohistochemistry. Lung sections of C57BL6 embryos from pseudoglandular, canalicular, and saccular stages of lung development were stained with antibodies for C/EBPα, C/EBPβ, and C/EBPδ.
We found the expression of both C/EBPα and C/EBPβ at the late pseudoglandular stage at E15.5, albeit with different patterns. At this stage, C/EBPα expression (see Fig. 1, A–C) was mainly detected in the epithelium, and here it was restricted to a subset of cells in the distal parts of the developing tubules. C/EBPβ was expressed in mesenchymal cells, and expression was also evident in most cells of the epithelium (Fig. 1). At E17.5, after the pseudoglandular-canalicular transition (Fig. 1E), the expression of C/EBPα was more widespread in the future alveolar region and also seen in a few cells in the epithelium lining the future distal conducting airways. At this stage, C/EBPβ continued to be expressed in mesenchymal cells as well as in most cells of the developing epithelium (Fig. 1J). During the saccular stage (Fig. 1F), the expression of C/EBPα was increased and started to resemble the adult expression pattern. At that time, C/EBPα exhibited higher levels of expression in the alveolar region as well as in the conducting airway epithelium (Fig. 1F). C/EBPβ and also C/EBPδ were expressed in the saccular stage lung, with expression detected in epithelial cells lining the developing conducting airways as well as in cells in the future alveolar region (Figs. 1, K and O). In the adult lung, low-level C/EBPα expression was detected in most epithelial cells lining the distal conducting airways. In the alveolar region, higher levels of expression were confined to cells suggestive of alveolar type II cells (Fig. 1G). Also, C/EBPβ (Fig. 1L) and C/EBPδ (Fig. 1P) were expressed in the alveolar region as well as in the conducting airways in the adult lung. Compared with C/EBPβ and C/EBPδ, C/EBPα exhibited a more dynamic expression pattern in the epithelium of the embryonal lung, and we thus included additional time points during development. Compared with the low-level expression detected at E15.5 (Fig. 1, A–C), the specific pattern of C/EBPα close to the distal tips was more pronounced 1 day later, at E16.5 (Fig. 1D). No C/EBPα expression could be detected at E13.5 (data not shown). In summary, C/EBPα exhibits a dynamic pattern of expression during lung development. Initially, during the later pseudoglandular stages, which are characterized by growth and branching of the epithelium, expression of C/EBPα is observed in a subset of cells in the distal tubules. Later, after the

Fig. 1. Developmental expression pattern of CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ, and C/EBPδ in the lung. Immunohistochemical staining (DAB; brown color) for C/EBPs during the pseudoglandular, canalicular, and saccular stages of lung development as well as in the adult lung is shown. Sections were slightly counterstained with hematoxylin. H: control excluding primary antibody. Numbers refer to embryonic days (E; plug day: E0.5). In A–F, I–K, and M–O, arrows point out the expression of respective C/EBPs in the distal, future respiratory epithelium; arrowheads indicate the expression in the proximal, future conducting airway epithelium; and open arrowheads indicate C/EBP expression in mesenchymal cells. In G, L, and P, arrows indicate the expression in the respiratory (alveolar) epithelium, and arrowheads indicate the expression in the conducting airway (bronchiolar) epithelium. Bars indicate magnification.
pseudoglandular-canonical transition, expression increases and exhibits a more widespread pattern in the developing lung epithelium, correlating with the extensive cellular differentiation occurring in this period (12).

Expression of C/EBPα in nonproliferating cells of the developing lung. C/EBPα is an inhibitor of proliferation and is absent from proliferating cells in other organs such as the liver and fat (34). To investigate the relationship between cellular proliferation and C/EBPα expression during lung development, we stained for C/EBPα together with PCNA as a marker for cell proliferation by immunofluorescence. As shown in Fig. 2, C/EBPα only rarely colocalized with PCNA in the growing epithelial tubules at E17.5. This suggests that C/EBPα is expressed only in growth-arrested cells within the epithelium, and this is in agreement with a role for C/EBPα as an inhibitor of proliferation in lung development as well (18). It is thus possible that the restricted expression of C/EBPα at these stages of lung development defines cells within the growing epithelial tubules that are not allowed to proliferate. This, together with the dynamic expression pattern of C/EBPα and the previously demonstrated role for C/EBPα in regulating lung-specific gene expression (12), suggested to us that C/EBPα has an important role in lung development.

Ectopic expression of C/EBPα affects lung development. To continue our investigations of the role of C/EBPα in lung development, we generated transgenic animals ectopically expressing C/EBPα in the lung epithelium. To drive the expression of the murine Cebpa gene, we used the human 3.7-kb Sftpc promoter (20, 62). This well-characterized promoter is specifically activated in the lung epithelium and gives expression from at least E10. Thus the use of this promoter to drive Cebpa provides an expression of C/EBPα starting at least 4 days earlier than normal but still epithelium specific. It also gives a more widespread expression than the discrete expression pattern normally seen in the distal epithelium during the later pseudoglandular stages. Sftpc-Cebpa transgenic mice were analyzed during the late pseudoglandular period. Of the 20 transgenic embryos analyzed at E15.5, 6 embryos were found to express the Cebpa transgene. In all cases, expression was specific for the developing lung epithelium, with the highest levels in the distal epithelium (see Fig. 4A). Lungs from Sftpc-Cebpa transgenic embryos showed an abnormal phenotype that was histologically characterized by a decreased number of growing epithelial tubules. The epithelial tubules were also larger in size compared with wild-type littermates (compare Fig. 3, A and B with C and D). However, the overall size of the lungs was not affected. Quantitative morphology corroborated that ectopic expression of C/EBPα reduced the number of growing airways and increased their mean area (Fig. 3E).

Effects of ectopic expression of C/EBPα on cellular differentiation. The first morphological sign of cellular differentiation in the developing lung epithelium is a proximal-distal patterning that results in a subdivision between the future conducting airway epithelium and alveolar epithelium, which occurs in mice at E14.5 (54). Still, an in vivo cell lineage study (42) has indicated that the epithelial precursors for the trachea and large bronchi versus more distal airways are segregated at an even earlier stage (before E11.5). Because C/EBPα is involved in controlling differentiation and differentiation-dependent gene expression in several organs, we investigated epithelial differentiation in Sftpc-Cebpa transgenic mice. No histological signs of affected differentiation were observed in the lungs of Sftpc-Cebpa mice (Fig. 3), and staining for the lung epithelial marker TTF-1 (Nkx2.1) was evident in all nuclei of the developing epithelial tubules (Fig. 4, C and D). The morphological proximal-distal differentiation occurring at E14.5 is accompanied by changes in gene expression (54). To investigate this differentiation step in the transgenic mice, their lungs were stained with antibodies against markers for proximal-distal differentiation. As seen in Fig. 4, E and F, staining for the distal marker pro-surfactant protein C was evident in the future alveolar region only, in both Sftpc-Cebpa mice and wild-type littermates, and expression of the proximal marker cytokeratin 8 was confined to the future conducting airway epithelium in both wild-type and transgenic lungs (Fig. 4, G and H). Thus this indicates that proximal-distal differentiation of the lung epithelium was intact. To investigate whether ectopic expression of C/EBPα resulted in premature differentiation, we analyzed the expression pattern of surfactant protein A as a marker for differentiation of the alveolar epithelium and secretoglobin 1A1/CCSP as a marker for the conducting airway epithelium using immunohistochemistry. Both of these are known target genes for C/EBPα in the lung epithelium, and

![Fig. 2. Expression of C/EBPαs and the proliferation marker proliferating cell nuclear antigen (PCNA) in the developing mouse lung. A: expression of C/EBPα at E17.5 detected by immunofluorescence with FITC (green fluorescence). B: expression of PCNA (detected by immunofluorescence with Cy3, red). C: merged image. The yellow color indicates coexpression of C/EBPαs and PCNA.](http://ajplung.physiology.org/)

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neither is normally expressed at E15.5. No premature expression of these genes could be seen in the lungs of Sftpc-Cebpa mice (data not shown). We also investigated the effects of ectopic C/EBPα/H9251 expression on the development of the mesenchyme. As seen in Fig. 4, I and J, staining for the mesenchymal marker α-smooth muscle actin showed a pattern reflecting the epithelial phenotype with fewer and larger developing tubules; however, within the mesenchymal compartment, the expression pattern was not affected. Moreover, morphometric quantification of the lung mesenchymal area showed that it was not altered in Sftpc-Cebpa transgenic mice compared with their wild-type littermates (Fig. 4R). Thus, together, this indicates that mesenchymal development was not primarily affected by transgenic overexpression of C/EBPα in the lung epithelium.

Several mechanisms could underlie the structural phenotype caused by the ectopic expression of C/EBPα, i.e., fewer and larger developing epithelial tubules. Because C/EBPα inhibits proliferation (34), one possibility could be that the ectopic expression of C/EBPα induces a general growth inhibition in the epithelium and thus affects development. This seems unlikely, because an overall growth inhibition would be expected to lead to smaller lungs, which was not observed. In addition, a general growth inhibition in the epithelium would be expected to result in an unaltered, or perhaps a decreased, number of tubules smaller in size instead of the observed decreased number of growing epithelial tubules larger in size. Still, to investigate whether an overall growth inhibition had occurred, we stained Sftpc-Cebpa transgenic and wild-type lungs for the proliferation markers phospho-histone H3, a marker for mitosis, and cyclin A, which is expressed in the S and G2 phases of the cell cycle. At E15.5, a large proportion of epithelial cells was proliferating as evidenced by cyclin A reactivity (Fig. 4, O and P). As shown in Fig. 4Q, the overall number of proliferating cells was not different between transgenic and wild-type lungs. As expected, a smaller fraction of cells was positive for the mitosis marker phospho-histone H3. Also with regard to this proliferation marker, no differences were seen between wild-type and transgenic lungs (Fig. 4, M and N). Because the expression of C/EBPα in lung cell lines has been reported to induce apoptosis (21), we also investigated apoptosis by TUNEL staining. However, no differences in the numbers of apoptotic cells could be detected between Sftpc-Cebpa and wild-type littermate lungs (data not shown). This shows that an
overall growth inhibition, or an altered rate of apoptosis, caused by ectopic expression of C/EBPα is not likely to underlie the observed phenotype with fewer and larger developing epithelial tubules. An alternative explanation could be that the branching process during the later stages of lung branching morphogenesis is affected by the ectopic C/EBPα expression. This would then cause insufficient formation of branches leading to the observed phenotype with fewer and larger epithelial tubules. We also investigated the deposition of the extracellular matrix protein fibronectin in the embryonic lungs. Fibronectin is secreted by the developing lung epithelium and has been shown to be important for lung branching morphogenesis. As seen in Fig. 4, K and L, fibronectin deposition in SftpC-Cebpa transgenic lungs displayed an abnormal pattern characterized by bundles of increased thickness around the enlarged developing epithelial tubules. Because an abnormal pattern of fibronectin deposition has previously been shown to be associated with disturbed branching, this adds support to the hypothesis that ectopic C/EBPα expression in the epithelium affects the later stages of branching morphogenesis.
genesis leading to insufficient formation of branches and the observed phenotype with fewer and larger epithelial tubules.

Mice lacking C/EBPα have defects in late embryonal lung development. To continue investigating the role of C/EBPα in lung development, we investigated lung development in mice carrying a targeted mutation of the gene for C/EBPα (Cebpa−/− mice). Cebpa−/− mice have developmental defects in their liver and adipose tissue and die within a few hours after birth due to hypoglycemia (18, 59). As previously described, a lung phenotype is present in Cebpa−/− mice at birth; their lungs exhibit alveolar abnormalities characterized by a hyperproliferation of epithelial cells (18). However, embryonal lung development in Cebpa−/− mice has not been studied in detail. We thus investigated the lungs of Cebpa−/− mice during the developmental period compared with wild-type and Cebpa+/− littermates. At E16.5, the first indications of a difference were visible, and, at E17.5, a phenotype could readily be observed in Cebpa−/− mice. This phenotype was characterized by a decreased number of developing airways (Fig. 5, A, B, D, and E). The airway spaces were also larger in size. The morphometric analyses shown in Fig. 5G further corroborated a structural phenotype characterized by fewer developing airways with an increased mean area. At later developmental stages, the lung phenotype seen at E17.5 was obscured by the hyperproliferation of epithelial cells in the distal respiratory portion of the developing lung. This latter phenotype has already been described in Cebpa−/− mice and most probably accounts for the immediate lethality observed in a fraction of newborn Cebpa−/− mice (18, 59). To investigate whether the absence of C/EBPα in Cebpa−/− mice affected proximal-distal patterning of the lung epithelium, the expression of pro-surfactant protein C was investigated. As seen in Fig. 5, C and F, staining for pro-surfactant protein C was evident in the distal, future alveolar region only in both Cebpa−/− mice and wild-type littermates. This indicates that proximodistal differentiation of the lung epithelium was intact. No phenotype was observed in heterozygous Cebpa+/− mice. Thus the lungs of Sftpc-Cebpa transgenic embryos and Cebpa−/− knockout embryos display a similar phenotype. This shows that both ectopic C/EBPα expression and loss of C/EBPα expression affect the later

![Image](http://ajplung.physiology.org/)

Fig. 5. Histology and immunohistochemistry of lungs from Cebpa−/− mice. A, B, D, and E: hematoxylin-eosin staining of E16.5 (A and D) and E17.5 (B and E) lungs from Cebpa−/− mice and WT littermates. C and F: Immunohistochemical staining (DAB, brown) for pro-SP-C as a marker for distal differentiation (sections were slightly counterstained with hematoxylin). Bars indicate magnification. G: Quantitative morphometry of lungs from Cebpa−/− mice. Numbers and mean areas of the developing epithelial tubules in E17.5 Cebpa−/− and WT littermate lungs were determined in hematoxylin-eosin-stained sections as described in MATERIALS AND METHODS. Values are means ± SD; n = 3–5. *P < 0.05.
stages of lung development. Together, this underlines the need for C/EBPα to be expressed at the right level, location, and time during lung development and demonstrates a role for C/EBPα in the later stages of lung development.

**DISCUSSION**

This study shows that the transcription factor C/EBPα exhibits a dynamic expression pattern during lung development. Expression is first detected during the late pseudoglandular stage in a subset of cells in the distal tubules, an area that actively grows and branches during this period, and expression is confined to nonproliferating cells. To examine the role of C/EBPα in lung development, we generated and studied mice ectopically expressing C/EBPα in the lung epithelium. At the late pseudoglandular stage, lungs from these transgenic mice were characterized by fewer and larger growing epithelial tubules. However, there were no signs of disturbed differentiation or overall proliferation. When we studied lung development in knockout mice lacking C/EBPα, we found a similar phenotype as in the Sftpce-Cebpa transgenic mice with fewer and larger developing airways. Together, these results indicate a role for C/EBPα in the later stages of lung development.

C/EBPα stimulates the transcription of many genes expressed in a tissue-specific and differentiation-dependent manner (5, 47). In addition, it has a role in regulating growth by inhibiting proliferation (34). This inhibition of proliferation appears independent of the transactivating functions of C/EBPα. The cell cycle inhibition is instead mediated via direct protein interactions with cell cycle regulators at several levels, for instance, by interacting with the cdk inhibitor p21 (55) and by binding to the cyclin-dependent kinases cdk2 and 4 (58) as well as to the E2F transcription factor (44). Findings indicating C/EBPα as a regulator of cell growth in the lung include observations that C/EBPα is absent in many cancer forms including lung cancers, suggesting that CEBPα is a potential tumour suppressor gene (21, 40). In this study, we found that C/EBPα is not normally expressed in proliferating cells, supporting a role for this transcription factor in the control of proliferation in the lung epithelium. However, overexpression of C/EBPα in the lung epithelium did not seem to have any inhibitory effect on overall proliferation. This indicates that forced expression of C/EBPα is not enough to cause complete block of proliferation on its own. Rather, it seems as if C/EBPα needs to act in concert with other factors that may not be expressed at these developmental stages. A possible such factor could be the cdk inhibitor p21. During lung development, p21 expression in the distal lung is first detected in the late pseudoglandular and canalicular stages (25). Because p21 is involved in mediating the antiproliferative effects of C/EBPα (56), overexpression of C/EBPα will not be able to arrest proliferation at earlier time points when p21 is absent, explaining why we failed to see an overall growth inhibition in Sftpce-Cebpa transgenic mice. Another possible explanation for the lack of growth inhibition could be that overexpression of C/EBPα causes the upregulation of other factors supporting proliferation, such as, for instance, CEBPβ and CEBPδ (7, 34). The opposing effect of CEBPβ and CEBPδ on proliferation could thus balance the growth inhibitory effect of C/EBPα, resulting in unchanged overall proliferation. However, we were unable to detect any upregulation of CEBPβ and CEBPδ in Sftpce-Cebpa transgenic lungs (data not shown), indicating that this mechanism does not explain the lack of growth inhibition in Sftpce-Cebpa transgenic mice.

In adipose cells as well as in cells of the myeloid lineages, liver, and lung, the induction of C/EBPα has been demonstrated to be sufficient to induce differentiation and/or to commit cells to differentiate into a predetermined fate (19, 21, 23, 26, 29, 46, 57). Lack of C/EBPα has previously also been found to cause failure of complete differentiation of alveolar type II cells that start to overgrow the alveolar region in Cebpa−/− mice (18). The period of extensive cellular differentiation occurring after the pseudoglandular-canalicular transition in the lung (9, 12, 35, 45) also corresponds well to the point when expression of C/EBPα increases and exhibits a more widespread pattern in the developing lung epithelium. We therefore examined at the ability of forced C/EBPα expression to promote differentiation of the lung epithelium. We stained for several markers for lung epithelial differentiation, including TTF-1, surfactant protein C, cytokeratin 8, surfactant protein A, and secretoglobin1a1/CCSP. However, we could not detect any differences with regard to the expression pattern of these molecular differentiation markers. This indicates that C/EBPα cannot induce differentiation on its own in the lung. The absence of stimulatory effects on surfactant protein A and secretoglobin1a1/CCSP was especially surprising because both of these genes have been demonstrated to be regulated directly by C/EBPα (13, 49), further underscoring that C/EBPα needs to act together with additional factors to promote cellular differentiation in the lung epithelium. Recently, the results of lung-specific inactivation of C/EBPα mice have been reported (1, 31). The resulting phenotype is characterized by neonatal lethality from respiratory failure and impaired lung maturation with a block of alveolar type II cell differentiation, decreased levels of surfactant proteins and lipids, and increased epithelial cell number in the alveolar region. Thus these loss of function models clearly show that C/EBPα is necessary for cellular differentiation of the distal lung epithelium. However, the present results, providing the corresponding gain of function model, indicate that even though C/EBPα is necessary for differentiation, it is not sufficient for lung epithelial differentiation.

Several mechanisms could underlie the structural phenotype caused by ectopic expression of C/EBPα with fewer and larger developing epithelial tubules. A general inhibition of growth, or an altered rate of apoptosis, seems not to be the underlying cause because no changes in overall proliferation, apoptosis, or lung size were observed. An alternative explanation for the decreased number of epithelial branches could be that the ectopic expression of C/EBPα interferes with the branching process. The restricted expression of C/EBPα close to the tips of the growing epithelial tubules, i.e., in locations of active branching, along with the abnormal pattern of fibronectin deposition, indicate that C/EBPα might have a role in the processes of branching. Because C/EBPα was found in nonproliferating cells, it is possible that C/EBPα, due to its antiproliferative activities, regulates the balance between dividing and nondividing cells in the distal parts of the growing epithelial tubules. The balance of cellular proliferation in the tubules is likely to be important for the branching process. Ectopic expression of C/EBPα could then be expected to disturb the balance between dividing cells and nondividing.
cells and result in a less controlled, more diffuse growth of the epithelium. This would lead to the establishment of fewer branches and the observed phenotype with fewer and larger epithelial tubules. Similarly, the lack of C/EBPα, as in Cebpa⁻/⁻ mice, would also be expected to disturb the balance of proliferation in the epithelial tubules, causing insufficient formation of branches and diffuse growth of the epithelium, subsequently leading to fewer and larger airways. This hypothesis thus provides a possible explanation for the similar phenotypes of the SftpC-Cebpa transgenic gain of function model and the Cebpa⁻/⁻ loss of function model. However, even though the present results are consistent with a hypothesis along these lines, further studies will be needed to establish the specific function for C/EBPα in lung branching morphogenesis.

Still, the findings that both overexpressing C/EBPα and losing C/EBPα expression disturbed branching indicate that C/EBPα needs to be expressed at the correct level, location, and time, underscoring the need for an exact and fine-tuned regulation of C/EBPα during lung development. This raises the question of which upstream regulators regulate C/EBPα during lung development. In the past few years, significant progress has been made in understanding the genetic and molecular control of lung branching (reviewed, for instance, in Refs. 9, 14, 24, 35, 52, and 60). Several key players that mediate epithelial-mesenchymal interactions have been identified, including members of the hedgehog (2, 30, 41), fibroblast growth factor (Fgf) (3, 43), and bone morphogenetic protein families (4, 61). The studies indicating a critical role for these factors have primarily focused on the earlier branching processes. Less is known about the function and expression pattern of these molecules during the branching morphogenesis occurring during the late pseudoglandular and canalaric stages. This branching also appears morphologically different from the branching morphogenesis of the preceding stages (45) and results in the formation of terminal sacs. Still, it is well possible that these factors continue to have a role throughout lung branching and therefore could be upstream regulators of C/EBPα expression. Alternatively, other factors could be of importance during the later stages of branching. Several members of the Wnt family of signaling molecules have been demonstrated to be important for lung development (28, 37, 53), and, using Wnt signaling reporter mice, dynamic Wnt expression was downregulated (39). Also, Wnt signaling reporter mice, dynamic Wnt signaling was recently demonstrated in the developing lung epithelium (39). Most notably, at E15.5–E18.5, Wnt signaling decreases specifically in the distal epithelium, in striking correlation with the onset of C/EBPα expression during lung development. This raises the question of which upstream regulators regulate C/EBPα during lung development. Further studies will be needed to elucidate the upstream regulators of the finely tuned expression pattern of C/EBPα necessary for a complete branching to occur during the later stages of lung development.

In conclusion, our results show a role for C/EBPα in lung development and suggest a function for this intracellular factor in the later stages of lung branching morphogenesis, and we speculate that this is achieved through the localized regulation of proliferation in the developing epithelium. A role for C/EBPα in branching could have possible general implications in mammalian development because this intracellular factor is expressed in several organs that develop through branching morphogenesis, such as the mammary gland and liver (12, 47).

Because our present results are centered on intracellular regulatory mechanisms, future studies mechanistically integrating these results with previous models of branching morphogenesis focused on extracellular signaling molecules are of central importance to further understand this fascinating developmental process.

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