Respiratory Failure Due to Differentiation Arrest and Expansion of Alveolar Cells following Lung-Specific Loss of the Transcription Factor C/EBPα in Mice¶

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The leucine zipper family transcription factor CCAAT enhancer binding protein alpha (C/EBPα) inhibits proliferation and promotes differentiation in various cell types. In this study, we show, using a lung-specific conditional mouse model of C/EBPα deletion, that loss of C/EBPα in the respiratory epithelium leads to respiratory failure at birth due to an arrest in the type II alveolar cell differentiation program. This differentiation arrest results in the lack of type I alveolar cells and differentiated surfactant-secreting type II alveolar cells. In addition to showing a block in type II cell differentiation, the neonatal lungs display increased numbers of proliferating cells and decreased numbers of apoptotic cells, leading to epithelial expansion and loss of airspace. Consistent with the phenotype observed, genes associated with alveolar maturation, survival, and proliferation were differentially expressed. Taken together, these results identify C/EBPα as a master regulator of airway epithelial maturation and suggest that the loss of C/EBPα could also be an important event in the multistep process of lung tumorigenesis. Furthermore, this study indicates that exploring the C/EBPα pathway might have therapeutic benefits for patients with respiratory distress syndromes.

Cell differentiation is a process of specialization that requires both the expression of tissue-specific genes and, in most cases, the achievement of a cell cycle-arrested state. This process depends in large part on the developmentally tightly regulated expression of transcription factors. A prototype transcription factor that couples differentiation to proliferation arrest during terminal differentiation is the CCAAT enhancer binding protein alpha (C/EBPα). C/EBPα belongs to the basic leucine zipper family of transcription factors and is expressed in, among other organs, adipose tissue, lung airway epithelium, and the myeloid lineage of the hematopoietic system, where it drives differentiation (6, 7, 31, 37, 42, 51, 75). In addition, C/EBPα is a potent inhibitor of proliferation. It directly interacts with cell cycle regulators and chromatin-remodeling proteins (27, 29, 38, 48, 59, 66, 70) and induces growth arrest even in tumorigenic cell lines expressing viral oncoproteins and lacking tumor suppressor proteins (26). These characteristics suggest that C/EBPα itself may play the role of a tumor suppressor, and several lines of evidence sustain this hypothesis. C/EBPα expression is lost in tumors from subsets of patients with breast, endometrial, skin, and lung cancers (16, 23, 57, 63). In addition, the growth inhibitory activity of C/EBPα needs to be blocked for liver tumors to develop (69), and disruption of the C/EBPα gene is a common event in leukemia (17, 39, 44, 45, 47, 78). However, in spite of the well-defined roles that C/EBPα is known to play in other systems, its role in airway epithelial differentiation and growth control remains poorly understood.

In the respiratory epithelium, C/EBPα is expressed in type II pneumocytes, which are cuboidal cells that store and secrete surfactant into the alveoli. Type II pneumocytes are derived from immature pre-type II cells shortly before birth during maturation of the lung epithelium (50, 73, 74). Some studies suggest that this process might be regulated by C/EBPα: (i) C/EBPα expression coincides temporally with surfactant production during terminal differentiation of type II alveolar cells (31); (ii) in addition, surfactant protein A and D expression increases during the differentiation of type II cells, and their gene promoters contain C/EBP binding sites, which are important elements regulating their expression (24, 31, 52); and (iii) although mice with a nonconditional targeted deletion of C/EBPα die perinatally due to hypoglycemia and impaired energy homeostasis (71), a subset show clinical symptoms of respiratory distress, displaying a primitive-appearing lung (14, 61). In spite of these findings, the role of C/EBPα as a molecular differentiation switch factor for alveolar cells in vivo still needs further characterization. Particularly, the mechanisms by which C/EBPα affects alveolar maturation are still largely unknown.

Lung cancer is the major cause of cancer deaths in the
United States, with a 5-year survival rate of only 15% (28). In the past 30 years, only minimal progress has been made in the treatment of this disease (5). Additional progress in the treatment of lung cancer will depend on a better understanding of the molecular events leading to lung neoplasias, as well as those sustaining the neoplastic phenotype. Interestingly, mice with a nonconditional deletion of C/EBPα show increased numbers of alveolar cells in the lungs (14, 61). Furthermore, we were able to determine that C/EBPα is down-regulated in the majority of lung cancer cell lines and in about half of non-small-cell lung cancer patients (23), which suggests a role for C/EBPα as a tumor suppressor in lung cancer. However, the mechanisms by which C/EBPα controls alveolar cell growth are unknown.

The current study was aimed at understanding the role of C/EBPα in regulating differentiation and cell growth in the respiratory epithelium in vivo. To avoid the effects of a systemic loss of C/EBPα (71), we used doxycycline-inducible Cre-driven excision to selectively and conditionally delete C/EBPα in respiratory epithelial cells in the mouse lung. Loss of C/EBPα in the respiratory epithelium leads to an arrest in the type II alveolar cell differentiation program, resulting in a lack of differentiated type I alveolar cells and surfactant-secreting type II alveolar cells, and culminates in respiratory failure at birth. This phenotype is accompanied by the down-regulation of many genes involved in airway differentiation and surfactant homeostasis. In addition, we were able to show for the first time in vivo that the loss of C/EBPα leads not only to an increase in the proliferation rate of alveolar cells, but also to a decrease in the numbers of apoptotic cells. Loss of these growth control mechanisms resulted in epithelial expansion with consequent loss of airspace. Taken together, our results corroborate the hypotheses not only that C/EBPα is a master regulator of differentiation and proliferation in the respiratory epithelium but also that it is a crucial factor in regulating alveolar cell survival. These findings suggest that loss of C/EBPα could be an important event in the multistep process of lung tumorigenesis.

MATERIALS AND METHODS

Mouse generation and genotyping. The generation of conditional C/EBPα knockout mice (C/EBPαloxP/loxP) (Fig. 1A) has been described previously (77). C/EBPαloxP/loxP mice were bred to surfactant protein C-reverse tetracycline transactivator (SPC-rTA)-transgenic mice and to tet(O7)CMV-Cre-transgenic mice (kindly provided by Jeffrey Whitsett) (Fig. 1B). The targeted C/EBPα allele, as well as the SPC-rTA transgene, were genotyped by PCR as described previously (65, 77). Genotyping of the tet(O7)CMV-Cre transgene was performed by amplification of a 365-bp fragment (oligonucleotide sequences used are listed in Table 1). Amplification was performed by denaturation at 94°C for 2 min, followed by 35 cycles of amplification at 94°C for 1 min, 55°C for 1 min, and 72°C for 40 s, with a final extension step of 5 min at 72°C.

Animal husbandry and doxycycline administration. Animals were housed under pathogen-free conditions according to the protocols approved by the Harvard University Institutional Animal Care and Use Committee. Doxycycline (Sigma, St. Louis, Mo.) was administered in the drinking water (1 mg/ml) of pregnant dams from conception to birth. Newborn mice not undergoing respiratory failure were sacrificed by decapitation. Adult mice were sacrificed by CO2 euthanasia.

Southern blot analysis. Fifteen micrograms of genomic DNA was digested overnight with BamHI (New England Biolabs), separated by 0.6% agarose gel electrophoresis, transferred to positively charged Biodyne nylon membranes ( Pall Corp., East Hills, NY) with 0.4 M NaOH overnight, and immobilized with a UV cross-linker (Stratagene, La Jolla, CA). The nylon membranes were then hybridized to an 875-bp PsI-XbaI DNA probe located outside the targeted allele (Fig. 1A) (77).

Flow cytometry. Fetal livers were isolated from pregnant mothers (day 19 of gestation) as previously described (75). Fetal livers from C/EBPα−/−, nonconditional C/EBPα mice (C/EBPα−/−), and their respective wild-type littersmates, were each made into a single-cell suspension. Cells were incubated for 1 h with fluorescein isothiocyanate-conjugated anti-mouse Gr1 antibody (catalog no. 553127; BD Pharmingen, San Diego, CA) and phycoerythrin-conjugated anti-mouse CD11b (integrin alpha-M chain, Mac-1; catalog no. 553311; BD Pharmingen, San Diego, CA) antibody and subsequently analyzed on a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ) according to standard protocols.

Histopathological analysis. Neonatal mice had their lungs dissected and fixed overnight in 10% formalin, or no dissection was performed prior to fixation. Lungs of adult mice were inflation fixed with 2.5 ml of 10% formalin. Fixed tissues were embedded in paraffin and sectioned at a 5-μm thickness. Tissue sections were stained with hematoxylin-eosin.

Antibodies and immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were immunohistochemically stained with the following antibodies: anti-C/EBPα (catalog no. sc-61; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:400, anti-SPC (rabbit polyclonal antibody kindly provided by Jeffrey Whitsett) diluted 1:2,500, anti-surfactant protein B (SPB) (catalog no. sc-13978; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500, and rat monoclonal anti-Ki-67 (clone TEC-3; DakoCytomation California Inc., Carpenteria, CA) diluted 1:25. Briefly, tissue sections were deparaffinized and endogenous peroxidase activity was quenched by incubation with 1% H2O2 for 30 min followed by 3% H2O2 for 5 min. Next, to achieve antigen retrieval, the tissue sections were immersed in 10 mM sodium citrate, pH 6.0, solution and boiled in a microwave oven for 10 min, followed by a 30-min cool down period. The slides were then stained using the Rabbit or Rat Vector Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

Periodic acid-Schiff staining. Tissue sections were deparaffinized, rehydrated, and stained using the periodic acid-Schiff staining system (Sigma, St. Louis, Mo.) according to the manufacturer's specifications.

Electron microscopy. Neonatal lungs were dissected and placed in cold phosphate-buffered saline. The lungs were then cut into 2- to 4-mm pieces and fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. This was followed by postfixation in 1% osmium tetroxide in 0.1 M cacodylate buffer (1 h, 4°C). The samples were subsequently dehydrated in ascending alcohols, cleared with propylene oxide, and infiltrated with a mixture of Epon resin and propylene oxide overnight. They were next infiltrated with pure Epon resin and polymerized at 60°C for 48 h. The hardened blocks were sectioned to a 70-nm thickness on a Reichert-Jung Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria). The sections were placed on nickel grids and stained for contrast with uranyl acetate and lead citrate. They were viewed and photographed on a JEOL 100CX electron microscope (JEOL USA Inc., Peabody, MA).

Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. Tissue sections were deparaffinized, rehydrated, and stained according to the ApopTag Plus in situ apoptosis detection kit (Upstate, Waltham, MA) instructions.

RNA isolation and real-time PCR analysis. RNA from total lung was isolated with TRI reagent (Molecular Research Center Inc., Cincinnati, OH) by following the manufacturer’s protocol. Real-time PCR analysis was performed in an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). For quantification using TaqMan probes, one-step reverse transcription-PCR was performed using TaqMan Universal One-Step Mastermix (Applied Biosystems, Foster City, CA), 100 ng of RNA sample, 400 nM of each gene-specific forward or reverse primer, and 200 nM of the gene-specific TaqMan probe. Alternatively, when TaqMan probes were not available, 1 μg RNA was reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA) and 10 μl of the resulting cDNA was used for amplification with SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 400 nM of each gene-specific forward or reverse primer (for primers and TaqMan probe sequences, see Table 1).

RNA microarray analysis. Total lung RNA was isolated as described above from seven lung-specific C/EBPα-deficient mice and nine control littersmates. Twenty micrograms of each sample was used for cRNA synthesis. Lung cRNA was then hybridized to mouse MOE430A gene chips (Affymetrix, Santa Clara, CA). The raw expression data consisted of the Affymetrix's scanner "signal" units and were processed from Affymetrix's GeneChip MAS5. These raw data were rescaled to account for different chip intensities. The top 10,000 genes were selected based on their ranking as measured by a median absolute deviation variation filter across all samples. From within this pool of 10,000 genes, those correlated with the class distinction of interest (genes from control mice [class 1] versus those
FIG. 1. Generation of mice with lung-specific conditional deletion of the transcription factor C/EBPα. (A) Generation of conditional C/EBPα mice. The targeted allele (C/EBPα^loxP^) used to generate C/EBPα-conditional mice is compared to the wild-type (wt) C/EBPα allele gene locus and with the excised allele (C/EBPα^loxP^). The position of the probe used for Southern blot analysis is indicated, as well as the expected sizes of BamHI restriction fragments. The positions of primers used for genotyping of the C/EBPα^loxP^ allele are indicated by arrows. B, BamHI restriction site. (B) Generation of transgenic mice with lung-specific Cre expression. The constructs used to generate transgenic mice for doxycycline-regulatable Cre expression in the lung are schematized. The SPC-rtTA transgene consists of 3.7 kb of the human SPC promoter, the 1-kb rTtA coding sequence, and a 0.45-kb simian virus 40 (SV40) polyadenylation signal (65). The Tet(O)7-CMV-Cre transgene consists of seven copies of the tet operator, a cytomegalovirus minimal promoter, the Cre recombinase coding sequence, and the MT-1 polyadenylation sequence (46). In the presence of doxycycline (Dox), rtTA binds to the tet operator and activates Cre recombinase expression (“tet-on” system). (C) The C/EBPα gene is excised in the lungs of C/EBPα^loxP^/loxP^ mice. Excision of the C/EBPα gene was evaluated by Southern blot analysis of genomic DNA from C/EBPα^loxP^/loxP^ mice, C/EBPα^loxP^/loxP^ mice, and control littermates as indicated. The sizes of the targeted (10.9-kb) and excised (4.7-kb) alleles are indicated. ΔrtTA, mice lacking the SPC-rtTA transgene; ΔCre, mice lacking the Tet(O)7-CMV-Cre transgene. (D) C/EBPα expression is decreased in the lungs of C/EBPα^loxP^/loxP^ mice and six control littermates was analyzed by quantitative real-time PCR analysis. “Control” indicates littermates that do not have at least one of the transgenic alleles and/or both targeted alleles. The mean expression level and standard deviation are presented as a percentage of 18S RNA expression. The asterisk indicates a significant difference (P value < 0.05) based on a two-tailed t test for samples of unequal variance. The P value for each sample is indicated.
from lung-specific/C/EBPα-deficient mice [class 2]) were identified by ranking them according to the \( P \) value of their signal-to-noise ratio (SNR) (60). For a given gene \( g \), the SNR of \( g \) is \( (\bar{x}_g - \bar{x})/(s_g + s_x) \), and \( \bar{x}_g \) and \( s_g \) denote, respectively, gene \( g \)'s sample mean and sample standard deviation within class \( i \) (class 1 or 2). A Monte Carlo simulation of the null distribution of the SNRs was performed by permuting the sample labels indicating class membership \( (n = 10,000) \). Empirical \( P \) values were then computed by comparing the observed SNRs to the corresponding null distribution of permuted SNRs. The \( P \) values thus obtained were corrected for multiple testing by the false discovery rate (FDR) procedure (1). Finally, genes with an FDR of >0.005 and/or change \((\alpha\)-fold\) of <2.0 were eliminated.

**RESULTS**

**Generation of mice with lung-specific and conditional C/EBPα deletions.** We have previously generated mice in which a conditional disruption of the C/EBPα gene was achieved by Cre-mediated excision (Fig. 1A) (77). To deliver Cre recombinase (Cre) specifically and conditionally to the lung epithelium, C/EBPα-conditioned mice (C/EBPα\(^{loxP/loxP}\)) were bred to two mouse transgenic lines (Fig. 1B): (i) transgenic mice expressing the reverse tetracycline transactivator under the control of the SPC promoter (SPC-rTAgTA mice), which drives rTAgTA expression specifically to the lung epithelium (46), and (ii) transgenic mice expressing Cre under the control of a tetracycline-regulatable element \( \text{[tet(O7)CMV-Cre]} \), which drives rtTA expression specifically to the lung epithelium compared to control littermates (Fig. 1D). In addition, excision was lung specific and was not detected in other organs (71), we sought to determine that the perinatal lethality of C/EBPα mice was mediated specifically by the loss of C/EBPα in the lung epithelium. Therefore, we analyzed C/EBPα expression by real-time PCR between C/EBPα\(^{Tg}\) mice and control littermates (mice that do not have at least one of the transgenic alleles and/or both targeted alleles) showed no difference in C/EBPα expression (data not shown), which indicates that excision of C/EBPα in these noninduced mice, although detectable, either is restricted to limited numbers of cells or occurs in cells that do not express C/EBPα. However, C/EBPα\(^{ΔΔ}\) mice displayed on average an 84% \((P = 0.0001)\) reduction in C/EBPα expression compared to control littermates (Fig. 1D).

**Lung-specific excision of the C/EBPα gene during development leads to respiratory failure at birth.** C/EBPα\(^{Tg}\) mice survived to adulthood, whereas 86% of C/EBPα\(^{ΔΔ}\) mice showed symptoms of respiratory distress accompanied by perinatal death. Because mice with a nonconditional deletion of C/EBPα (C/EBPα\(^{ΔΔ}\)) also die perinatally due to dysfunction in other organs (71), we sought to determine that the perinatal lethality of C/EBPα\(^{ΔΔ}\) mice was mediated specifically by the loss of C/EBPα in the lung epithelium. Therefore, we analyzed C/EBPα expression or function in the liver and in the hematopoietic system, where C/EBPα is known to play important roles (71, 75, 77). Consistently, C/EBPα\(^{ΔΔ}\) mice showed normal liver architecture (Fig. 2A) and contained levels of C/EBPα mRNA in the liver similar to those of control littermates (Fig. 2B). Nonconditional C/EBPα\(^{ΔΔ}\) mice, on the other hand, lost C/EBPα expression in the liver (Fig. 2B). In addition, in contrast to nonconditional C/EBPα\(^{ΔΔ}\) mice (75), granulocytes were found in the peripheral blood of C/EBPα\(^{ΔΔ}\) mice (Fig. 2C). Furthermore, we analyzed C/EBPα\(^{ΔΔ}\) mice fetal liver cells for the surface markers Gr1 and Mac1, which are found in differentiated granulocytes. Accordingly, while C/EBPα\(^{ΔΔ}\) mice lost the Gr1/Mac1-positive population (75), C/EBPα\(^{ΔΔ}\) mice showed unchanged numbers of Gr1/Mac1-

<table>
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<tr>
<th>Gene</th>
<th>PCR oligonucleotides (5′−3′)</th>
<th>TaqMan probe (5′−3′)</th>
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<tr>
<td>Cre</td>
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<td>N/A*</td>
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<tr>
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<tr>
<td>Ppp1r3c</td>
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<td>N/A</td>
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*NA, not applicable.*

that in the respiratory epithelium the level of excision is likely much higher. C/EBPα\(^{Tg}\) mice also displayed various levels of excision in the lungs (from 10 to 30%) (Fig. 1C), which is in agreement with the finding by Perl et al. (46) that SPC-driven rtTA is capable of activating doxycycline-independent Cre expression late in development. No excision was detected in mice that did not carry both transgenic alleles or at least one targeted allele. In addition, excision was lung specific and was not detected in other organs (Fig. 1C and data not shown). Interestingly, analysis of C/EBPα expression by real-time PCR between C/EBPα\(^{Tg}\) mice and control littermates (mice that do not have at least one of the transgenic alleles and/or both targeted alleles) showed no difference in C/EBPα expression (data not shown), which indicates that excision of C/EBPα in these noninduced mice, although detectable, either is restricted to limited numbers of cells or occurs in cells that do not express C/EBPα. However, C/EBPα\(^{ΔΔ}\) mice displayed on average an 84% \((P = 0.0001)\) reduction in C/EBPα expression compared to control littermates (Fig. 1D).
positive cells in their fetal livers compared to those in control littermates (Fig. 2D).

In contrast, the lungs of C/EBPα<sup>−/−</sup> mice showed remarkably abnormal histopathology, characterized by the following observations (Fig. 3A): (i) the alveolar walls were thicker due to prominent cellular accumulation; (ii) the increased cellular accumulation resulted in a reduction of airspace, sometimes completely obliterating the alveolar structure; (iii) differentiated type I and type II pneumocytes were lacking; and (iv) the alveoli were lined by cuboidal cells with a clear cytosol. In addition, immunohistochemical staining of the lungs of C/EBPα<sup>−/−</sup> mice shows, consistent with the high excision level observed by Southern blotting, an almost complete loss of C/EBPα protein expression (Fig. 3B).

Lung-specific excision of the C/EBPα gene during development leads to a block in the type II cell differentiation program. The presence of cuboidal cells lining the alveolar walls suggested that they were immature type II cells that could not differentiate into mature type II and type I pneumocytes. In order to investigate this hypothesis, we first confirmed that the abnormal cells were alveolar type II cells by staining them with the type II cell marker SPC (Fig. 4A). Next, we assessed the level of maturation: immature type II cells are glycogen rich, and as they differentiate, glycogen is converted into phospholipids and mobilized to the lamellar body (50). We were able to show that the cuboidal cells found in C/EBPα<sup>−/−</sup> mice had high glycogen content in the cytosol (Fig. 4B and C). In addition, they lacked lamellar bodies (Fig. 4B). As expected, numerous lamellar bodies were seen in alveolar cells isolated from control littermates. These results demonstrated that type II cells of C/EBPα<sup>−/−</sup> mice were immature, confirming our hypothesis of a differentiation arrest. Inadequate type II cell maturation is known to lead to surfactant deficiency and, consistent with the phenotype that we observed, respiratory distress within hours after birth (21). Further confirmation of our hypothesis comes from expression analysis of surfactant proteins, as well as hepatocyte nuclear factor 3β (HNF3β), a winged helix transcription factor regulated by C/EBPα in lung cells and also required for the transition to air breathing at birth (22, 68). As expected,

at least one of the transgenic alleles and/or both targeted alleles.

(B) C/EBPα RNA levels are unaffected in the livers of C/EBPα<sup>−/−</sup> mice. Quantitative real-time PCR analysis was performed using fetal liver RNA from C/EBPα<sup>−/−</sup> mice or C/EBPα<sup>−/−</sup> mice compared to that of their respective control littermates. C/EBPα expression is presented as a percentage of 18S RNA expression. This figure shows mean results plus standard deviations from six C/EBPα<sup>−/−</sup> mice and six control littermates (upper panel) or four C/EBPα<sup>−/−</sup> mice and five control littermates (two C/EBPα<sup>−/−</sup> and three C/EBPα<sup>−/−</sup> mice) (lower panel). The asterisk indicates a significant difference (P value < 0.05) based on a two-tailed t test for samples of unequal variance. The P value for each sample is indicated. (C) C/EBPα<sup>−/−</sup> mice have granulocytes in the periphery. Representative peripheral blood smears stained with hematoxylin from C/EBPα<sup>−/−</sup> mice and control littermates. Granulocytes are indicated by arrows. (D) C/EBPα<sup>−/−</sup> mice have normal granulocytic differentiation. Fetal liver cells from C/EBPα<sup>−/−</sup> mice or C/EBPα<sup>−/−</sup> mice compared to those of their respective control littermates were stained with the granulocytic markers Gr-1/Mac-1 and analyzed by flow cytometry analysis. The percentage of double-positive cells in each sample is indicated. mC/EBPα, mouse C/EBPα; WT, control wild-type littermate; KO, C/EBPα-knockout out sample.
FIG. 3. C/EBPαΔ/Δ mice show abnormal lung histopathology and decreased levels of C/EBPα protein in the lungs. (A) C/EBPαΔ/Δ mice have abnormal lung histopathology. Representative histological lung sections stained with hematoxylin-eosin of C/EBPαΔ/Δ mice and control littermates showing thickening of the alveolar walls (thin arrows), a lack of type I alveolar cells (arrowheads), and the presence of epithelial cuboidal cells with a clear cytosol (thick arrows). “Control” indicates littermates that do not have at least one of the transgenic alleles and/or both targeted alleles. The magnification used to photograph the sections is indicated. (B) C/EBPα protein expression is decreased in C/EBPαΔ/Δ mice. Immunohistochemistry for C/EBPα shows a loss of staining in the lungs of C/EBPαΔ/Δ neonates compared to that in control littermates (brown color staining). “Control” indicates littermates that do not have at least one of the transgenic alleles and/or both targeted alleles. The magnification used to photograph the sections is indicated.
C/EBPαΔ/Δ mice show a remarkable loss of SPB by immunostaining (Fig. 4D). In addition, the mRNAs for SPA and SPD, as well as the mRNA for HNF3β, were markedly down-regulated (Fig. 4E).

Alveolar cells of C/EBPαΔ/Δ mice have an increased proliferation rate. Because C/EBPα is a potent antimitotic factor, it is conceivable that the loss of C/EBPα in the lung epithelium would lead to increased proliferation. Consistent with this idea, the lungs of C/EBPαΔ/Δ mice display increased numbers of cells expressing the type II marker SPC (Fig. 4A). In addition, as stated earlier, C/EBPαΔ/Δ mice also show prominent cellular accumulation in the lungs, leading to a loss of airspace (Fig. 3A). In order to assess if the cellular expansion observed could be caused by increased proliferation, we performed immunostaining with an antibody for KI-67, a nuclear antigen present in proliferating cells (56). Our results show, as hypothesized, an increased number of proliferating cells in the lungs of C/EBPαΔ/Δ mice (Fig. 5).

Alveolar cells of C/EBPαΔ/Δ mice have decreased levels of apoptosis. Another important mechanism that controls cell growth is programmed cell death or apoptosis. It has been previously seen that expression of C/EBPα in lung cells triggers apoptosis (23). It is possible, therefore, that the loss of C/EBPα in the lung epithelium confers an antiapoptotic effect, leading to higher numbers of cells. In order to investigate this hypothesis, we used the TUNEL assay, a process that detects the DNA strand breaks associated with the apoptotic response (15). Indeed, C/EBPαΔ/Δ mice had very low numbers of apoptotic cells compared to the control littermates (Fig. 6).

Expression profiling identifies several differentially expressed genes in C/EBPαΔ/Δ mice. The phenotype of C/EBPαΔ/Δ mice led us to determine that C/EBPα plays an important role in the differentiation program and growth control of type II alveolar cells. In order to identify genes regulated by C/EBPα during this process, we compared the expression profiles of the lungs of C/EBPαΔ/Δ mice with those of control littermates using Affymetrix murine genome MOE430 gene chips (Affymetrix, Santa Clara, CA, USA). Analysis of the data resulted in the identification of 124 differentially expressed genes (Fig. 7) (for the full set, see Tables S1 and S2 in the supplemental material). Validation of the data set comes from the findings that Cre recombinase is the most significantly up-regulated gene in C/EBPαΔ/Δ mice and that C/EBPα itself is one of the most significantly down-regulated genes (Fig. 7).

Consistent with the phenotype and morphological findings with C/EBPαΔ/Δ mice, genes characteristic of differentiated pulmonary cells were markedly down-regulated (Fig. 7). For example, aquaporin 5 (Aqp5), a type I cell marker, and genes characterizedly expressed at high levels in type II cells, such as those encoding SPA and the ATP-binding cassette transporter ABCA3, were significantly down-regulated. In addition, the protein phosphatase 1 regulatory subunit 3C (Ppp1r3c) was one of the most significantly up-regulated genes. Ppp1r3c is involved in positively regulating glycogen synthesis and in preventing its breakdown (9, 18, 49), which could explain the glycogen-rich phenotype of the epithelial cells found in C/EBPαΔ/Δ mice.

Finally, genes associated with proliferation, tumor progression, and cell survival were up-regulated in C/EBPαΔ/Δ mice. Examples of proliferative genes include regulator of G protein signaling 2 (Rgs2) and G0/G1 switch gene 2 (G0s2), two genes found to be up-regulated by mitogenic stimuli in mononuclear cells (55, 58). Examples of genes associated with tumor formation and progression are T-cell lymphoma invasion and metastasis 1 (Tiam1) and proprotein convertase subtilisin/kexin type 6 (Pcsk6). Tiam1 is a Rac-specific guanine nucleotide exchange factor that directly mediates Ras activation of Rac (30) and is essential for the development of RAS-induced skin tumors (34), whereas Pcsk6 is a proprotein convertase that processes a variety of substrates, including matrix metalloproteinases, and not only is able to enhance the invasive ability of malignant cells, but also plays a significant role in converting noninvasive keratinocytes into malignant cells (33). Finally, an example of a cell survival gene up-regulated in C/EBPαΔ/Δ mice is Gli-1, a transcription factor downstream of sonic hedgehog signaling, which can act as an oncogene when aberrantly expressed (10, 40) and has been shown to positively regulate BCL2, a gene with antiapoptotic function (3).

In order to validate these findings further, we confirmed the expression change of a subset of targets by real-time PCR analysis. In addition to confirming the down-regulation of SPA (Fig. 3E), we confirmed the up-regulation of Ppp1r3c and Gli-1 (Fig. 8).

**DISCUSSION**

In the last few days of gestation, the alveolar epithelium of the rodent lung undergoes marked differentiation in preparation for air breathing. During this time, the immature glycogen-rich pre-type II cells that line the epithelium will become mature, surfactant-secreting type II cells. This process is characterized by ultrastructural and biochemical changes that include enhanced surfactant protein production, conversion of glycogen into phospholipids (an essential component of surfactant), and the appearance of the lamellar body, a unique organelle in which surfactant is stored (4, 50, 73, 74).

This process culminates with the appearance of surfactant in the alveolar fluid, which is essential for respiration after birth. Inadequate surfactant production leads to respiratory distress syndrome, a common disorder affecting preterm infants (8). Furthermore, surfactant lipid and protein deficiency is also observed in acute respiratory distress syndrome, a disease that has multiple etiologies and can affect infants, children, and adults (19).

Another important differentiation event takes place in the lung epithelium shortly before birth: the type II cells also differentiate into mature type I cells (13, 62, 72). Type I alveolar cells come into close contact with endothelial cells in the alveolar capillaries, forming an efficient gas exchange area. Differentiation of type II cells into type I cells is an important process that plays a role not only during intrauterine development, but also during restoration of the alveolar epithelium after injury. There is strong evidence that this differentiation process occurs via an intermediate cell type which exhibits both type II- and type I-specific characteristics (67) and that it may be reversible (12). The molecular events that direct the terminal differentiation of type II cells and type I cells are, nonetheless, not well understood.

A role for C/EBPα in this process of lung cellular differen-
tiation has been suggested by the phenotype of mice with a targeted deletion of C/EBPα. C/EBPα−/− mice die perinatally of hypoglycemia due to liver dysfunction (71). However, Flodby et al. (14) reported that a few of the C/EBPα−/− mice showed clinical symptoms of respiratory distress, displaying a primitive-appearing lung with an increased number of type II epithelial cells. In a second study examining the lungs of C/EBPα nullizygous mice, Sugahara et al. (61) demonstrated

FIG. 4. C/EBPαΔ/Δ mouse alveolar epithelial cells are immature type II cells. (A) C/EBPαΔ/Δ mouse alveolar cells have a type I cell origin. Immunohistochemistry for SPC shows strong staining of C/EBPαΔ/Δ mouse epithelial cells (brown color). (B) C/EBPαΔ/Δ mouse alveolar cells lack lamellar bodies. Electron microscopy analysis of the lungs from C/EBPαΔ/Δ mice shows cuboidal alveolar cells with round nuclei and glycogen inclusions (open arrowheads). The epithelium lacks type I cells and the alveolar cells lack lamellar bodies. Type I cells with flattened nuclei and long cytoplasmic extensions can be found in the control littermates. These cells contain numerous lamellar bodies (full arrowheads). The magnification used to photograph the sections is indicated. (C) C/EBPαΔ/Δ mouse alveolar cells are glycogen rich. Periodic acid-Schiff staining shows an increased glycogen content in the alveolar cells of C/EBPαΔ/Δ mice compared to that in control littermates (pink color). (D) Immunohistochemistry for SPB confirms that C/EBPαΔ/Δ mice show decreased SPB expression (brown color) compared to control littermates (littermates that do not have at least one of the transgenic alleles and/or both targeted alleles). (E) C/EBPαΔ/Δ mice have decreased expression of genes important for respiratory function at birth. Quantitative real-time PCR analysis of SPA, SPD, and HNF3β was performed using total lung RNA from six C/EBPαΔ/Δ mice compared to that of six control littermates (littermates that do not have at least one of the transgenic alleles and/or both targeted alleles). The mean expression level and standard deviation are presented as a percentage of 18S RNA expression. The asterisk indicates a significant difference (P value < 0.05) based on a two-tailed t test for samples of unequal variance. The P value for each sample is indicated.
an increase in alveolar cells positive for SPC, a type II cell marker. However, although the lungs of C/EBPα−/− were described as primitive, the stage of the differentiation block as well as the mechanism leading to it were not investigated, and despite the fact that both studies showed an increase in alveolar cells, the mechanism behind this expansion was not addressed. Finally, the importance of C/EBPα in the adaptation to air breathing was masked by the severe phenotype displayed by these mice (71).

This study was aimed at understanding how C/EBPα affects alveolar maturation and also how it affects alveolar cell growth. For that purpose, we generated mice with a conditional deletion of the transcription factor C/EBPα specifically in the lung epithelium (Fig. 1). Therefore, we avoided the unrelated serious consequences of a systemic deletion of C/EBPα (71). We were able to show that C/EBPα is a crucial factor in driving the maturation of the lung epithelium before birth. Most C/EBPαΔ/Δ mice undergo respiratory distress after birth, a phenotype that cannot be attributed to the loss of C/EBPα in other organs (Fig. 1C and 2). The respiratory epithelia of C/EBPαΔ/Δ mice lack type I cells (Fig. 3A) and are lined by immature type II cells that are glycogen rich and lack lamellar bodies (Fig. 4A to C). In contrast, the respiratory epithelia of the control littermates were characterized by epithelial cells displaying flattened nuclei and long cytoplasmic extensions typical of type I cells but also containing lamellar bodies typical of type II cells (Fig. 4B). This finding supports the hypothesis that during normal development, type II cells differentiate into type I cells via an intermediate cell type (67), a process that is blocked in C/EBPαΔ/Δ mice. Although conditional-deletion experiments with defined epithelial cultures would be required in order to more definitively assess the direct role of C/EBPα in type II cell differentiation, this study provides the first direct and unequivocal evidence that C/EBPα is required for terminal alveolar differentiation and the establishment of respiratory functions.

The importance of C/EBPα for the terminal differentiation of alveolar cells is further underscored by recent reports of three different mouse models with impaired alveolar differentiation. First, Wan et al. (68) showed that this complex differentiation program can also be regulated by HNF3β, a winged helix transcription factor implicated in endoderm development. Similarly to the mice described in this study, mice with lung-specific deletion of HNF3β showed incomplete alveolar cell maturation, leading to respiratory failure at birth. Interestingly, HNF3β and C/EBPα are expressed in the same cell
types in the lung epithelium, and we have recently shown that HNF3β expression can be regulated by C/EBPα in lung cells (22). In fact, we were able to show that expression of HNF3β was decreased in C/EBPα-deficient mice compared to that in control littermates (Fig. 4E). In addition, HNF3β-deficient mice share many gene targets in common with C/EBPα-deficient mice, such as stearoyl-coenzyme A desaturase 1 (SCD-1), SPA, and ABCA3 (Fig. 7). These results corroborate the idea that HNF3β and C/EBPα act in the same differentiation pathway in the lung epithelium and suggest that C/EBPα may lie upstream of HNF3β.

Second, trying to understand the role of Wnt signaling in lung development, Okubo and Hogan (43) generated transgenic mice expressing a constitutively active β-catenin–LEF1 fusion in the lung epithelium. Using this model, they showed that differentiated alveolar type II and type I cells are absent from the lungs and that the respiratory epithelium is lined by immature and hyperproliferative type II cells. Interestingly, expression profiling identified C/EBPα as one of the downregulated genes in the lungs of transgenic mice, which raises the possibility that the loss of C/EBPα expression could be the primary event leading to the block in type II differentiation program. Furthermore, additional evidence that Wnt signaling can regulate C/EBPα comes from studies of adipose tissue describing how Wnt signaling can block adipogenesis by inhibiting the induction of C/EBPα and other adipogenic factors (2, 54). In addition, the expression of Wnt receptors frizzled 1 (Fz1), 2 (Fz2), and 5 (Fz5) is high in preadipocytes and declines upon induction of differentiation (2). It is interesting that Fz2 is up-regulated in C/EBPα-deficient mice (Fig. 7), suggesting that the immature lung epithelium has a higher Wnt signaling activity and that C/EBPα plays a role in contributing to the inhibition of this pathway during lung epithelial differentiation.

Finally, Dang et al. (11) showed that ectopic expression of Notch3 in the lung epithelium also inhibits terminal epithelial differentiation in transgenic mice. Although an investigation of C/EBPα levels was not performed for these mice, there is evidence from other studies that notch signaling is able to block C/EBPα expression (53, 64), suggesting again that C/EBPα might be involved in mediating the differentiation block.

Because impaired differentiation of type II cells is associated with surfactant deficiency (50), it is straightforward to propose that C/EBPα is also an important factor in regulating surfactant production. This proposition is confirmed by our observations that the type II alveolar cells of C/EBPα-deficient mice lack
lamellar bodies, a surfactant-storing organelle (Fig. 4B); these cells also display a high glycogen content (Fig. 4B and C), which indicates that C/EBPα−/− mice lack the ability to convert glycogen into phospholipids, an important surfactant component. In agreement with these observations, many of the differentially expressed genes in the lungs of C/EBPα−/− mice are involved in lipid metabolism (Fig. 7). For instance, we observed the down-regulation of SCD1, a lipogenic enzyme that catalyzes the synthesis of monounsaturated fatty acids and is highly expressed in type II cells (36). In addition, we detected decreased expression of fatty acid binding protein 5 (Fabp5), a protein that mediates the synthesis of dipalmitoyl phosphatidylcholine (20), the main surfactant phospholipid. Consistent with these findings, other studies have suggested that C/EBPα is an important factor in promoting lipogenesis during fetal maturation of the lung (35, 36, 76).

![Expression profiling](image)

**FIG. 7.** Expression profiling identifies many differentially expressed genes in the lungs of C/EBPα−/− mice. The expression histograms of the top 50 up-regulated (A) and down-regulated (B) genes are shown (for the full gene set [n = 124], see Tables S1 and S2 in the supplemental material). Genes are ranked by FDR-corrected P values (see Materials and Methods and reference 1). Duplicated samples and genes of unknown function were removed from the list. “Control” indicates littermates that do not have at least one of the transgenic alleles and/or both targeted alleles.
In addition to showing a regulation of surfactant production through the regulation of lipogenesis, our results also show that C/EBPα can regulate some of the protein components of a surfactant: surfactant proteins A, B, and D are markedly down-regulated in C/EBPα/H9251/H9004 type II cells. In conclusion, our results show, for the first time in vivo, that the loss of C/EBPα leads to increased apoptosis in the lung adenocarcinoma cell lines leads to increased apoptosis supports this finding (23). Further corroboration of this finding comes from the increased expression in C/EBPα/H9251/H9004 mice of genes involved in survival signaling (Fig. 7 and 8), such as Gli-1 (3) and transforming growth factor beta 2 (Tgfβ2) (32).

In further confirmation that the loss of C/EBPα/H9251/H9004 mice are hyperproliferative. Furthermore, C/EBPα deficiency was shown to increase the proliferative potential of hepatocytes in vivo (14). Finally, disruption of C/EBPα expression or function is found in many different types of cancer (16, 17, 44, 45, 47, 57, 63, 69, 78), including non-small-cell lung tumors (23). Consistent with the above reports, we found that C/EBPαΔΔ mice show increased numbers of SPC-expressing cells in the lungs (Fig. 4A). This finding can be explained in three ways: (i) the immature type II cells simply continue to express SPC, in contrast to what is observed in normal type II cells, which lose SPC expression as they differentiate into type I cells; (ii) the immature type II cells have an increased proliferation rate; and (iii) the immature type II cells have a survival advantage. Of these possibilities, we think it less likely that the differentiation block itself is responsible for the increased numbers of SPC-positive cells; C/EBPαΔΔ mice display not only a differentiation block in type II cell program, but also cellular accumulation within the alveolar airspace.

In order to determine if an increased proliferation rate could explain the cell expansion, we performed staining with Ki-67, a nuclear antigen present in proliferating cells (Fig. 5). In agreement with the known antiangiogenic activity of C/EBPα, we were able to show that its loss results in an increased proliferation of alveolar cells and thereupon contributes to hypercellularity in the lungs. In agreement with this finding, C/EBPαΔΔ mice show an up-regulation of proliferation-promoting genes, such as Rgs2 and G6k2, as well as components of proliferative signaling pathways, such as Wnt (Fz2) and Sonic hedgehog (Gli-1) (Fig. 7 and 8).

We also investigated the possibility of increased survival as a potential mechanism contributing to cell expansion, even though very little is known about how C/EBPα affects survival signals. For that, we performed in situ TUNEL assays and found, for the first time in vivo, that the loss of C/EBPα leads to a marked inhibition of apoptosis (Fig. 6). Previous work from our laboratory showing that the expression of C/EBPα in lung adenocarcinoma cell lines leads to increased apoptosis supports this finding (23). Further corroboration of this finding comes from the increased expression in C/EBPαΔΔ mice of genes involved in survival signaling (Fig. 7 and 8), such as Gli-1 (3) and transforming growth factor beta 2 (Tgfβ2) (32).

In further confirmation that the loss of C/EBPα in the lung epithelium favors cell proliferation and survival, one adult C/EBPαΔΔ mouse developed hypercellular areas in the lungs that are correlated with C/EBPα loss (Fig. 9). In addition, a C/EBPαΔΔ female, which had not been treated with doxycycline during embryogenesis but had been induced with doxycycline during several breeding cycles, developed a papillary adenocarcinoma involving the major left lobe of the lung (not shown). C/EBPα expression was not detectable in the tumor tissue, and the tumor showed increased numbers of SPC- and Ki-67-positive cells compared to numbers of cells outside the neoplastic area. Although studies aimed at statistically evaluating the development of lung tumors in C/EBPαΔΔ mice are under way, these findings, coupled with the fact that C/EBPα is able to inhibit proliferation and promote apoptosis in the lung epithelium, suggest that the loss of C/EBPα might increase the susceptibility to lung cancer development and supports the
hypothesis that C/EBPα acts as a tumor suppressor for lung cancer in vivo.

In conclusion, we developed a lung-specific mouse model of conditional deletion of the transcription factor C/EBPα that identifies C/EBPα as a master regulator of type II cell differentiation and alveolar cell growth control in vivo. This model will be useful in future studies of lung development and carcinogenesis.

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