Conditional deletion of Abca3 in alveolar type II cells alters surfactant homeostasis in newborn and adult mice

Valérie Besnard,1* Yohei Matsuzaki,1,2* Jean Clark,1 Yan Xu,1 Susan E. Wert,1 Machiko Ikegami,1 Mildred T. Stahlman,3 Timothy E. Weaver,1 Alan N. Hunt,4 Anthony D. Postle,4 and Jeffrey A. Whitsett1

1The Perinatal Institute and Section of Neonatology, Perinatal and Pulmonary Biology, Cincinnati Children’s Hospital Medical Center, Department of Pediatrics and University of Cincinnati College of Medicine, Cincinnati, Ohio; 2Department of Pediatrics, School of Medicine, Keio University, Tokyo, Japan; 3Department of Pediatrics, Vanderbilt University Medical Center, Nashville, Tennessee; and 4Division of Infection, Immunology, and Immunity, School of Medicine, University of Southampton, Southampton, United Kingdom

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Am J Physiol Lung Cell Mol Physiol 298: L646–L659, 2010. First published February 26, 2010; doi:10.1152/ajplung.00409.2009.—ATP-binding cassette (ABC) proteins are members of a large family of transporters that are required for surfactant function and lipid metabolism (7), allowing surfactant homeostasis in the postnatal lung. Maintenance of surfactant lipid content during lung injury may involve intracellular sensing and extracellular signals capable of detecting and regulating lipid content and trafficking.

ABC proteins are members of a large family of transporters associated with ATP-dependent translocation of various substrates across cellular membranes. ABCA3 is expressed in various organs, including lung, brain, and kidney (32, 44). In the lung, Abca3 mRNA increases dramatically prior to birth (44). Abca3 gene expression is regulated by glucocorticoids (53) and cis-acting cassettes that mediate pulmonary cell-and lipid-sensitive pathways (7), allowing surfactant homeostasis at birth and thereafter. Abca3 is a 1,704-amino acid and 190-kDa protein, highly expressed in alveolar type II cells, where it is present in the limiting membrane of lysosomal-derived intracellular inclusions termed lamellar bodies (LBs) (32, 52, 55). Abnormalities in lipid content and function were observed in surfactant from patients with ABCA3-related pulmonary disease (20). Deletion of the Abca3 gene in mouse models resulted in respiratory failure after birth, which was caused by the absence of surfactant in the alveoli. Loss of mature LBs in Abca3 gene-deleted mice was also observed and was consistent with findings in human infants with mutations in ABCA3 (2, 13, 19, 23). Taken together, ABCA3 is required for LB formation and pulmonary surfactant function. While deletion of the Abca3 gene in mice demonstrated its requirement at birth, little is known about the effects of Abca3 deficiency in adult lung function. In this study, the Abca3 gene was conditionally deleted in respiratory epithelial cells. Deletion of Abca3 altered lung lipid content and synthesis. Maintenance of surfactant function in Abca3-deleted mice after birth

PULMONARY SURFACANT REDUCES surface tension at the air-liquid interface in the alveolus, thereby maintaining lung volumes during the respiratory cycle. Surfactant consists of lipids and associated proteins that are required for surfactant function. Notably, surfactant proteins (SP) B and C (encoded by SFTPB and SFTPC) and ATP-binding cassette (ABC) A3 (ABCA3) play critical roles in surfactant synthesis and function. Various autosomal-recessive mutations in SFTPB and ABCA3 cause fatal respiratory distress in human newborns due to the lack of surfactant (20, 42). In some cases, ABCA3 mutations are associated with interstitial lung diseases in older children (12, 17, 54). Histological analysis of lungs of such patients showed interstitial fibrosis. Heterozygosity for ABCA3 mutations increased the severity of interstitial lung disease associated with mutations in SFTPC, indicating that alterations in ABCA3 may influence the pathogenesis of lung diseases (10).

Coordination of the synthesis and packaging of surfactant components is essential for the transition to air breathing at birth and thereafter. Surfactant content and function are regulated at multiple levels, including synthesis, processing, intracellular transport, assembly, and storage of surfactant components, surfactant secretion, and catabolism of lipids and proteins. Expression of surfactant proteins and regulation of lipid metabolism during development are dependent on a number of transcription factors that are expressed in respiratory epithelial cells (6, 14, 15, 29, 30, 47). Less is known regarding control of surfactant lipid homeostasis in the postnatal lung. Maintenance of surfactant lipid content during lung injury may involve intracellular and extracellular sensing modules capable of detecting and regulating lipid content and trafficking.

ABC proteins are members of a large family of transporters associated with ATP-dependent translocation of various substrates across cellular membranes. ABCA3 is expressed in various organs, including lung, brain, and kidney (32, 44). In the lung, Abca3 mRNA increases dramatically prior to birth (44). Abca3 gene expression is regulated by glucocorticoids (53) and cis-acting cassettes that mediate pulmonary cell- and lipid-sensitive pathways (7), allowing surfactant homeostasis at birth and thereafter. Abca3 is a 1,704-amino acid and 190-kDa protein, highly expressed in alveolar type II cells, where it is present in the limiting membrane of lysosomal-derived intracellular inclusions termed lamellar bodies (LBs) (32, 52, 55). Abnormalities in lipid content and function were observed in surfactant from patients with ABCA3-related pulmonary disease (20). Deletion of the Abca3 gene in mouse models resulted in respiratory failure after birth, which was caused by the absence of surfactant in the alveoli. Loss of mature LBs in Abca3 gene-deleted mice was also observed and was consistent with findings in human infants with mutations in ABCA3 (2, 13, 19, 23). Taken together, ABCA3 is required for LB formation and pulmonary surfactant function. While deletion of the Abca3 gene in mice demonstrated its requirement at birth, little is known about the effects of Abca3 deficiency in adult lung function. In this study, the Abca3 gene was conditionally deleted in respiratory epithelial cells. Deletion of Abca3 altered lung lipid content and synthesis. Maintenance of surfactant function in Abca3-deleted mice after birth

* V. Besnard and Y. Matsuzaki contributed equally to this work.

Address for reprint requests and other correspondence: J. A. Whitsett, Cincinnati Children’s Hospital Medical Center, Section of Neonatology, Perinatal and Pulmonary Biology, 3333 Burnet Ave., Cincinnati, OH 45229-3039 (e-mail: jeff.whitsett@chmc.org).
was associated with compensatory lipid synthesis in nontargeted type II cells, revealing a novel compensatory system that senses surfactant deficiency caused by cell-selective deletion of *Abca3*.

**MATERIALS AND METHODS**

**Gene construction for Abca3 floxed mouse.** To generate a conditional *Abca3* floxed mutant allele, a 14.4-kb region of the mouse gene was subcloned from a positively identified bacterial artificial chromosome (BAC) clone from inGenious Targeting Lab (Stony Brook, NY) and used to construct the targeting vector. The construct was designed such that the short-homology arm extended 1.9 kb 3' of the loxP-floxed neomycin (Neo) cassette, in intron 7/8. The long-homology arm extended 12.5 kb 5' of the Neo cassette, and a single loxP site was inserted in intron 3/4, 5' of exon 4. The target region was 4.6 kb and included exons 4, 5, 6, and 7 (Fig. 1A). A targeting construct was generated with Neo-resistance (neoR) gene as a selective marker. The linearized targeting construct was electroporated into eukaryotic stem cells and grown in the antibiotic G418. Surviving clones were screened for homologous recombination by Southern and PCR analysis. Ten positive clones were detected in 288 samples analyzed. Eukaryotic stem cells from three positively identified loxP-floxed *Abca3* clones (*Abca3flx*) were injected into blastocysts to generate chimeric mice, and germ-line transmission of the *Abca3flx* allele was achieved from all clones injected and backcrossed with C57BL6 for at least three generations.

**SP-C-rtTA (line 2)/tetO-CMV-Cre/Abca3flx/flx** triple-transgenic mice were generated as described previously (34, 35, 39, 49). In the presence of doxycycline, exons 4, 5, 6, and 7 of the *Abca3* gene were permanently deleted from respiratory epithelial cells prior to birth (*Abca3/H9004/H9004/H9004* mice; Fig. 1A). For genotyping, DNA was purified from the
tail of experimental mice, and PCR was performed for Abca3^{flx/wt}, SP-C-CreTA, and (tetO)-CMV-Cre genes using the following primers: 5'-AGC ACT TTT CCC TCT GTG AG-3' and 5'-TGC CCA CCC ACC ATG CT-3' for Abca3^{flx/wt}, 5'-GAC ACA TAT AAG ACC CTG GCTA-3' and 5'-AAA ATC TTC TCG CCT TTC CC-3' for SP-C-CreTA, and 5'-TGC CAC CAA GTG ACA GCA ATG-3' and 5'-AGA GAC GGA AAT CAA TCG CTTCG-3' for (tetO)-CMV-Cre. Abca3-deleted transgenic (Abca3^{flx/flx}) mice and non-deleted (SP-C-CreTA^{wt/wt}) Abca3^{flx/wt}, control) littermates were used for the experiments. FVB/N-TgN(EIIa-Cre)C5379Lmgd mice carrying a Cre transgene, under the control of the adenovirus Elia promoter, which targets expression of Cre recombinase to the early mouse embryo, were purchased from the Jackson Laboratory (Bar Harbor, ME). Double-transgenic mice were generated by mating Elia-Cre^{wt/wt} /Abca3^{flx/wt} to Abca3^{flx/flx} mice. Abca3^{flx/flx} littermates lacking the Cre gene served as controls.

Animal husbandry and doxycycline administration. Mice were maintained in a pathogen-free environment in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Cincinnati Children’s Hospital Research Foundation. All animals were housed in humidity- and temperature-controlled rooms on protocols approved by the Institutional Animal Care and Use Committee were then fed normal food.

All experiments are representative of findings from at least four clonal; Seven Hills Bioreagents), and cleaved caspase-3 (1:1,000, Seven Hills Bioreagents), FOXA2 (1:4,000 and 1:8,000, rabbit polyclonal; AB3428, Chemicon), ABCA3 (1:1,000, rabbit polyclonal; L648 CONDITIONAL DELETION OF Abca3 IN AJP-Lung Cell Mol Physiol • VOL 298 • MAY 2010 • www.ajplung.org

Tissue preparation, histology, and immunohistochemistry. Lung tissue preparation and immunohistochemistry were performed essentially as described previously (28, 46). Tissue sections were stained with hematoxylin and eosin. Primary antibodies were used at the following dilutions: surfactant protein (SP)-B (1:2,000, rabbit polyclonal; Chemicon, Temecula, CA), pro-SP-C (1:4,000, rabbit polyclonal; AB3428, Chemicon), ABCA3 (1:1,000, rabbit polyclonal; Seven Hills Bioreagents), FOXA2 (1:4,000 and 1:8,000, rabbit polyclonal; Cleaved caspase-3 (1:1,000, rabbit polyclonal; R & D Systems, Minneapolis, MN). The secondary antibody was goat anti-rabbit IgG (1:200; Vector, Burlingame, CA). All experiments are representative of findings from at least four Abca3^{flx/flx} and control mice. Lung sections were stained with orcein to detect elastic fibers (Poly Scientific, Bay Shore, NY).

Transmission electron microscopy. Lung tissue from postnatal day 0 (PND0) and 9-mo-old Abca3^{flx/flx} mice and littermate controls was fixed in modified Karnovsky’s fixative consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer and 0.1% calcium chloride (pH 7.3). Adult tissue was stained en bloc with 4% uracil acetate, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated, and embedded in epoxy resin (EMbed 812, Electron Microscopy Sciences, Fort Washington, PA), as previously described (36). Ultrathin sections were postfixed with uracil acetate and lead citrate and viewed in Morgagni 268 and Hitachi 7600 transmission electron microscopes (TEM), and digitized images were collected with an Advantage Plus 2K × 2K TEM equipped with a charge-coupled device digital camera (Advanced Microscopy Techniques, Danvers, MA).

Morphometry. Morphometric measurements were performed on 9 mo-old mice of both genotypes. The overall proportion [%fractional (F) area] of respiratory parenchyma and air space was determined by a point-counting method (50). Measurements were performed on two sections taken at intervals throughout the right, left upper, and right lower lobes. Slides were viewed with an ×20 objective. Images (fields) were acquired randomly with a digitalized camera and quantified using MetaMorph imaging software (version 7.5, Molecular Devices, Downingtown, PA). A computer-generated, 121-point lattice grid was superimposed on each field, and the number of intersections (points) falling over respiratory parenchyma (alveoli and alveolar ducts) or air space was counted. Points falling over bronchioles, large vessels, and smaller arterioles and venules were excluded from the study. %Fx area was calculated by dividing the number of points for each compartment (n) by the total number of points contained within the field (N) and then multiplying by 100: %Fx area = n/N × 100. Fifteen random fields per section were analyzed to gather the data. The x- and y-coordinates for each field measured were selected by a random-number generator (Microsoft Excel 2003, Redmond, WA).

The number of ABCA3-stained cells was determined by a point-counting method. Five mice of both genotypes were studied. Measurements were performed throughout the left, right upper, and right lower lobes, and the number of ABCA3-stained cells was counted manually. The density of ABCA3-stained cells was calculated by dividing the number of ABCA3-stained cells by alveolar area (mm²). Fifteen fields per section were analyzed. The x- and y-coordinates for each field measured were selected by a random-number generator.

Bronchioles, large vessels, and smaller arterioles and venules were excluded.

The cross-sectional area of LBs in alveolar type II cells from 9-mo-old control and Abca3^{flx/flx} mice was determined from ultrathin (90-nm-thick) sections cut from one to two Epon blocks from each group (n = 1 for control and n = 3 for Abca3^{flx/flx} mice) and transferred to 200-mesh copper grids. Ten electron micrographs were randomly selected from one to two sections per block. Each electron micrograph was coded according to genotype and digitally acquired at a final magnification of ≥10,000. The cross-sectional areas of all LBs were determined using the region measurement function in MetaMorph imaging software (version 7.5).

The percentage of type II cells with or without LBs was assessed by TEM of tissue from 9-mo-old control and Abca3^{flx/flx} mice. A total of 292 type II cells found in 1–2 grids from 3 different Abca3^{flx/flx} mice were analyzed.

Isolation of alveolar type II epithelial cells. Alveolar type II cells were isolated from 9-mo-old control and Abca3^{flx/flx} mouse lung with use of collagenase and differential plating, as described previously (38). Type II cells were used 2 h after isolation for RNA analysis.

Flow cytometry analysis and sorting. After isolation, alveolar type II cells were stained for 10 min in the presence of 1 μM Nile red (NR). Cells were then washed three times in PBS and brought to a concentration of 4 × 10⁵ cells/ml in sorting buffer [1× PBS (Ca²⁺/Mg²⁺ free), 1 mM EDTA, 25 mM HEPES (pH 7.0), 1% fetal bovine serum, 50 U of penicillin/ml, and 50 μg of streptomycin/ml]. Cells were sorted and analyzed by a FACSARia II sorter (Becton Dickinson, San Jose, CA) within 2 h of staining. Excitation was at 533 nm using a yellow-green laser, and emission was collected at 628 nm. A more intense red fluorescence of the phospholipid-enriched cells is obtained, because the emission maxima wavelength of phospholipids stained with NR is longer than that observed for neutral lipids (9, 21). NR-positive cells were initially gated on the basis of side scatter and mean fluorescence intensity of the NR signal. Data were exported and analyzed with FACSDiVa software (Becton Dickinson).

Bronchoalveolar lavage fluid. Nine-mo-old mice (n = 5/group) were anesthetized and killed by exsanguination. Tracheas were cannulated, and five 1-ml aliquots of 0.9% NaCl were flushed into the lungs and withdrawn by syringe three times for each aliquot. The volumes of recovered bronchoalveolar lavage (BAL) fluid from all the groups were similar. After centrifugation, BAL cells were counted using a hemocytometer to determine total BAL cell concentration. BAL cells were used for RNA extraction, or, after cytospin, cells were stained to determine differential cell counts (Diff-Quik, Dade Behring, Miami, FL).

RNA extraction and RT-PCR. RNA was extracted from whole lung homogenate of PND0 Abca3^{flx/flx} and control mice or isolated lung alveolar type II cells or BAL cells of adult mice with use of RNeasy Protect Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s
protocol. Samples were obtained from five control or Abca3+/- mice. Quantitative PCRs using Taqman probes (Applied BioSystems, Foster City, CA) were performed with primer sets specific for Abca3 (Mm00550501_m1), surfactant protein (Sftp) SftpA (Mm00499710_m1), SftpB (Mm00455681_m1), SftpC (Mm00488144_m1), fatty acid synthase (Fasn, Mm01253292_m1), stearyl-CoA desaturase (Scd1, Mm01197142_m1), lysophosphatidylcholine acyltransferase 1 (Lpcat1, Mm00461015_m1), fatty acid-binding protein 5 (Fabp5, Mm00783731_s1), choline phosphate cytidylyltransferase 1 (Pcyt1a, Mm00447774_m1), napsin A aspartic peptidase (Napsa, Mm00492829_m1), steroidalogenic acute regulatory domain protein (StarI2) (StarD2, Mm00476629_m1), StarD7 (Mm00460536_m1), StarD9 (Mm00626196_m1), StarD10 (Mm00502345_m1), Abca1 (Mm00442646_m1), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Hmgcs1, Mm00241111_m1), nuclear factor of activated T cells (Nfatc3, Mm01249200_m1), NK2 homeobox 1 Nkx2.1 (Mm00447558_m1), forkhead box A2 (Foxa2, Mm01976556_s1), CCAAT enhancer-binding protein-α (Cebpα, Mm01265914_s1), macrophage inflammatory protein 1α (Mip1α, Mm00441258), transforming growth factor-β1 (Tgfb1, Mm03024053), TNFα (Tnfα, Mm00443258_m1), IL-1β (Il-1b, Mm01336189_m1), and matrix metalloproteinase 12 (Mmp12, Mm00838401_g1). A probe set for 18S rRNA was used as the normalization standard. The PCR and relative quantifications were performed using 25 ng of cDNA per reaction in a real-time PCR system (model 7300, Applied BioSystems). Relative quantification data from quantitative PCR analysis were statistically analyzed using Student’s t-tests.

Western blot analysis for ABCA3. ABCA3 expression was quantified by Western blot analysis in whole lung homogenate. Samples were obtained from PND0 control and Abca3+/- mice (n = 3 for each genotype). Proteins were extracted using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL), diluted in Laemmli buffer, 7 ml of 0.5 M sucrose buffer, and 3 ml of 0.3 M sucrose. The supernatant was strained through lens paper. The crude lung extract overlaid by a sucrose gradient consisting of 3 ml of 0.7 M sucrose buffer, 7 ml of 0.5 M sucrose buffer, and 3 ml of 0.3 M sucrose. The tubes were centrifuged for 3 hr at 104,000 g at 4°C in a Beckman L-70 ultracentrifuge with an SW28 swinging-bucket rotor. After centrifugation, the LB fraction was collected from the top and stored at −80°C. 

Analysis of surfactant components. Saturated phosphatidylcholine (Sat PC) was measured in homogenized whole lung tissue from PND0 mice (n = 4-6 mice for each genotype) as previously described (3). The phospholipids in chloroform-methanol extracts of homogenized lung were separated by two-dimensional thin-layer chromatography for compositional analyses as previously described (26). The spots were visualized with iodine vapor, scraped, and assayed for phosphorus content as previously described (26).

Electrospray ionization-mass spectrometry of phospholipid molecular species and phosphatidylcholine synthesis. Mice were injected intraperitoneally with 1 mg of methyl-D3-choline chloride (Cambridge Isotope Laboratories, Andover, MA) 3 hr before they were killed. Phospholipids were extracted from BALF, lavaged lung tissue, and LBs with chloroform and methanol according to Bligh and Dyer (8) after addition of the following internal standards: phosphatidylcholine (PC14:0/14:0, 10 pmol), phosphatidylethanolamine (PE14:0/14:0, 4 pmol), phosphatidylglycerol (PG14:0/14:0, 2 pmol), and phosphatidylserine (PS14:0/14:0, 2 pmol). Electrospray ionization-mass spectrometry of phospholipids was performed as previously described (36). In addition, incorporation of methyl-D3-choline as a measure of PC synthesis in the various tissue fractions was determined by precursor scan of mass-to-charge ratio (m/z) 193 (P193) expressed relative to total PC, determined as the sum of precursor scan of m/z 184 (P184) and P193 (5). The volumes of recovered BALF from all the groups were similar. Protein concentration was determined using a Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard.

RNA microarray analysis. For Affymetrix MicroArray, E18.5 lung RNAs from Abca3+/- mice and littermate controls were prepared using the RNaseasy Protect Mini kit according to the manufacturer’s protocol. RNA was treated with DNase at room temperature for 15 min. Lung cRNA was hybridized to the Murine Genome 430 2.0 Array (consisting of 45,000 probe sets representing >34,000 mouse genes; Affymetrix) using the manufacturer’s protocol. Affymetrix MicroArray Suite version 5.0 was used to scan and quantitate the gene chips using default scan settings. Normalization was performed using the Robust Multichip Average model, which consists of three steps: background adjustment, quartile normalization, and summarization (24, 25). Microarray analysis was performed with the software package BRB Array Tools, developed by the Biometric Research Branch of the National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html). Differentially expressed genes were identified using a univariate F test and permutation test (n = 100) with a significance level of 0.05. Data were prefiltred by excluding probe sets as follows: 1) those whose expression differed <1.2 from the median in 80% of the samples, 2) those whose expression data were missing in >50% of the samples, or 3) those with >70% of absent calls by the Affimetrix algorithm in six samples. Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes pathway analysis were performed using the publicly available web-based tool DAVID (Database for Annotation, Visualization, and Integrated Discovery) (16). Gene frequency and probability in each functional category were calculated by Fisher’s Exact test using Mouse Genome 430 2.0 as background control. Biological association networks were built using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

Statistical analysis. Student’s t-test was used to determine the levels of difference between groups. Values for all measurements are expressed as means ± SE, and P values for significance are indicated for each experiment. 

RESULTS

Abca3 is required for postnatal adaptation to air breathing. Abca3 mRNA was previously detected in alveolar type II cells in the murine lung, as well as in other organs, including brain and kidney (32, 44). To identify the role of Abca3 in alveolar type II cells, triple-transgenic SP-C-rtTA3 expressing TetO-Cre39/40, and Abca33/4 mice were produced; in these mice, Abca3 was selectively and permanently deleted in the respiratory epithelium following administration of doxycycline to the dam from E6.5 to E14.5 (Fig. 1A). Analysis of Abca3 gene expression demonstrated a dramatic reduction of Abca3 mRNA.
and ABCA3 protein in newborn (PND0) Abca3Δ/Δ mice (Fig. 1, B–E); 67% of PND0 Abca3Δ/Δ mice died at birth. The lungs of the Abca3Δ/Δ mice that died at PND0 failed to inflate. Atel-ectasis was observed (Fig. 1, F–G). Immunostaining for mature SP-B and proSP-C were similar in Abca3Δ/Δ and control mice (Fig. 1, H–K). To confirm the targeting of the conditional Abca3 floxed mutant allele, Ella-Cre<sup>gmi</sup>, Abca3<i>flx</i>/flx mice wherein Abca3 is deleted prior to implantation in the uterine wall, died soon after birth from respiratory failure, confirming the recombination of the Abca3<sup>flx</sup> allele and supporting its requirement for the transition to air breathing at birth (data not shown).

Adult Abca3Δ/Δ mice develop emphysema. Approximately 33% of the Abca3Δ/Δ mice survived postnatally and to 9 mo of age. Postnatal survival was better in females than males (Fig. 2A). The efficiency of Abca3 deletion in alveolar type II cells in surviving mice at 9 mo was assessed by quantitative RT-PCR (qRT-PCR) and immunohistochemistry. Both Abca3 mRNA and the number of cells stained for ABCA3 were decreased by ~50% in type II cells isolated from adult Abca3Δ/Δ mice compared with control littersmates (Fig. 2, B and D), indicating the heterogeneity of Abca3 targeting. Deletion of Abca3 in adult mice altered surfactant-associated protein mRNA expression. While a marked decrease in Sftpb was observed in Abca3Δ/Δ lungs, Sftpc and Napsa were similar in both groups of mice (Fig. 2C). In contrast, Sftpa1 was significantly increased in Abca3Δ/Δ mice compared with control littersmates.

Surviving Abca3Δ/Δ mice developed normally after birth. While alveolarization was unchanged at 4 wk of age, air space enlargement was observed in 9-mo-old Abca3Δ/Δ mice (Fig. 3, A–D). The relative proportion of respiratory parenchyma (Fig. 3E) and air space (Fig. 3F; fractional area) were altered in 9-mo-old Abca3Δ/Δ mice compared with control littersmates, with a significant increase in the fractional area of air space (< 0.002) in Abca3Δ/Δ mice. The enlargement of alveoli was associated with alterations in the distribution and structure of elastic fibers. Thickened elastin fibers were observed surrounding enlarged air spaces in Abca3Δ/Δ mice, in contrast to fine elastin fibers in septae of normal lung (data not shown). Inflammation was not readily apparent in the Abca3Δ/Δ mice, and total cell counts were similar in lung lavage fluid (Fig. 3G), wherein monocytes/macrophages were the main cell population. Inflammatory cytokine mRNA levels in Abca3Δ/Δ mice were not statistically different from those in control littersmates (Fig. 3H). There was no histological or serological evidence of infection, and sentinel mice did not harbor viral or bacterial pathogens.

Deletion of Abca3 alters lamellar body formation and surfactant composition. In newborn mice, in contrast to control littersmates, LBs were not observed in type II cells from PND0 Abca3Δ/Δ mice that died at birth from respiratory distress (Fig. 4, A and B). Alveolar type II cells from PND0 Abca3Δ/Δ mice contained more glycogen, and some contained atypical lipid structures. Secreted surfactant and tubular myelin were absent from the alveoli of the newborn Abca3Δ/Δ mice, while evidence of type I cell injury and early hyaline membrane formation, consistent with respiratory distress syndrome, was also detected by TEM (data not shown).

Numerous LBs were observed in 9-mo-old control mice (Fig. 4C). Although the average LB cross-sectional area was similar between control and Abca3Δ/Δ mice, variations in size and number of LBs were seen in the Abca3Δ/Δ mice (see supplemental Fig. 1 in the online version of this article). In Abca3Δ/Δ mice, two distinct subsets of type II cells were altered. One subset of type II cells contained no LBs (68%), but liposome-like vesicular structures of intermediate electron density with electron-dense inclusions were observed (Fig. 4, E and F). In addition, this subset contained variable numbers of lipid inclusions that were absent from the controls (Fig. 4, G and H). The second subset of type II cells contained normal (26%) and “giant” (6%) LBs, either single lamellar-like bodies or larger structures containing several LBs (Fig. 4, D, I, J; see supplemental Fig. 1). These data support the concept that changes in surfactant lipid homeostasis in nondeleted type II cells may compensate for the absence of LBs in cells in which Abca3 was deleted.

Effect of deletion of Abca3 on phospholipid composition and synthesis. Consistent with the ultrastructure alterations observed in newborns, Sat PC (Fig. 5A), PC, and, to a lesser extent, PG content were decreased in lungs from PND0 mice.
Abca3/H9004/H9004 mice (Fig. 5, B and C); lung lysophosphatidylcholine and phosphatidylethanolamine contents were increased. Despite the altered morphology of type II cells in the adult Abca3/H9004/H9004 mice, the concentration of total PC in lavaged lungs and LBs, calculated as the sum of individual molecular species determined by electrospray ionization-mass spectrometry, was not significantly different in adult Abca3/H9004/H9004 and control mice (Fig. 6). The concentration of total PC was, however, decreased by 50% in BALF from adult Abca3/H9004/H9004 mice. Both groups of mice showed a comparable sequential enrichment pattern of surfactant-specific PC species from lavaged lungs, through LBs to BALF. Comparison of profiles of PC synthesis and concentration indicated that acyl remodeling mechanisms were also unaltered in Abca3/H9004/H9004 compared with control mice (data not shown). PC synthesis, calculated as the sum of methyl-D9-choline incorporation into PC molecular species after 3 h of labeling, was decreased by 20% in lavaged lung in Abca3/H9004/H9004 mice, from 1.28 ± 0.06 to 1.00 ± 0.12% total PC (mean ± SE, P < 0.05; Fig. 6). Incorporation of stable isotope into PC in isolated LBs was 1.73 ± 0.10% total PC in controls and was significantly decreased in lungs of Abca3/H9004/H9004 mice to 1.11 ± 0.10% total PC (mean ± SE, P < 0.001). In contrast, methyl-D9-choline enrichment in BALF PC was essentially identical in Abca3/H9004/H9004 mice and controls, although newly synthesized PC was considerably less abundant in BALF than in lavaged tissue or LB at 0.34 ± 0.07 vs. 0.31 ± 0.06% total PC. PG content was markedly decreased in LBs and BALF fractions, but not in lung parenchyma (Fig. 6). In agreement with previous analyses (37), this detailed lipidomic characterization showed that disaturated PG species consistently represented

Fig. 3. Adult Abca3/H9004/H9004 mice develop emphysema. Lung sections of Abca3/H9004/H9004 (B and D) and control littermates (A and C) were prepared from 4-wk-old (A and B) and 9-mo-old (C and D) mice and stained with hematoxylin and eosin. While no histological abnormalities were observed in 4-wk-old mice, Abca3/H9004/H9004 mice developed emphysema by 9 mo of age (D, inset). Photomicrographs are representative of ≥4 individual mice at each time. Scale bars, 500 μm. Magnification of inset is 3 times the original magnification. Changes in fractional areas (%Fx area) of respiratory parenchyma (E) and air space (F) were determined in 9-mo-old Abca3/H9004/H9004 and control littermates. Values are means ± SE. *P < 0.001. G: cell populations in bronchoalveolar lavage (BAL) fluid (BALF) from adult Abca3/H9004/H9004 and control littermates. BALF cell counts were similar in Abca3/H9004/H9004 mice and control mice. mRNAs for selected cytokines were assessed by quantitative RT-PCR in isolated BAL cells from lungs of adult Abca3/H9004/H9004 and control littermates and normalized to β-actin mRNA, indicating no evidence of activation of inflammation (H). Values are means ± SE of 5 animals per group. *P < 0.01 vs. control littermates. NS, no statistical difference.
Deletion of *Abca3* influences lamellar body (LB) formation in adult type II epithelial cells. Electron microscopy was used to study lung sections from PND0 control (A) and *Abca3*−/− (B) and adult control (C) and *Abca3*−/− (D–J) mice. Type II cells from PND0 control mice contained numerous LBs (arrow; A, inset). LBs were absent in PND0 *Abca3*−/− mice. Glycogen (Gly) was increased in type II cells from PND0 *Abca3*−/− mice (B). Membranous structures were present in the cytoplasm of type II cells from PND0 *Abca3*−/− mice (B, inset). In adult mice, sizes and numbers of LBs were consistent in type II cells from control mice (C). D–J: heterogeneity of LB size and numbers in type II cells from *Abca3*−/− mice. Subsets of type II epithelial cells from *Abca3*−/− mice lacked LBs and contained abnormal electron-dense bodies (arrows, E and F) or contained multiple LBs (D) or abnormal (*, I) or extremely large (J) LBs. Lipid vesicles were visualized in cytoplasm of subsets of type II epithelial cells from *Abca3*−/− mice (arrowhead, G and H). Original magnification: ×10,000 (A–C), ×20,000 (E, G, and H), ×40,000 (D and F). N, nucleus; m, mitochondria.
<20% of PG in tissue, LB, and BALF of all mice. The decrease in PG16:0/16:0 content was accounted for by increased longer-chain PG species (see supplemental Fig. 2, A and B). Analysis of the “minor components” of surfactant showed alterations in phosphatidylinositol (PI). PI was increased twofold in lung tissue and LB fractions. Notably, content of PI16:0/22:6 increased in the progression from tissue, through LB to BALF. The content of PI16:1/18:1, PI16:0/18:1, PI18:1/18:2, and PI18:1/18:1 was increased in Abca3ΔΔA compared with control mice at the expense of longer-chain PI species (see supplemental Fig. 2, C–E).

**Deletion of Abca3 influences genes regulating lipid synthesis and transport in type II epithelial cells.** RNA was extracted from whole lung homogenate from E18.5 Abca3ΔΔA and control mice. RNAs were hybridized to the murine genome MOE430 chip. Data were analyzed using the publically available web-based tool DAVID. RNA microarray analysis of E18.5 Abca3ΔΔA mice revealed that deletion of Abca3 influenced a number of mRNAs related to lipid metabolism (Table 1). To determine whether deletion of Abca3 in respiratory epithelial cells altered expression of genes regulating lipid homeostasis, expression of mRNAs of genes associated with lipid synthesis and transport was assessed by qRT-PCR (Fig. 7A). A marked decrease in mRNAs influencing fatty acid (Scd1 and Lpce1) and cholesterol (Hmgcs1 and Abca1) metabolism was observed in the lungs of newborn Abca3ΔΔA mice, perhaps indicating response to altered lipid handling in the absence of ABCA3. In contrast, Petlta mRNA coding for the choline cytidylyltransferase-α (CCTα) protein was upregulated in lungs from PND0 Abca3ΔΔA mice.

Expression of mRNAs of genes associated with lipid synthesis, transport, and secretion in alveolar type II cells isolated from 9-mo-old mice was assessed by qRT-PCR (Fig. 7). A marked decrease in mRNAs influencing fatty acid metabolism (Scd1, Fasn, Fabp5, Lpca1, and Petlta; Fig. 7B) and cholesterol metabolism (Hmgcs1 and Abca1; Fig. 7C) was observed in total type II cells of 9-mo-old Abca3ΔΔA mice. Levels of mRNAs encoding a family of lipid transfer proteins, Stard2, Stard7, Stard9, and Stard10, were assessed by qRT-PCR (Fig. 7D). Stard2 was significantly upregulated in total type II cells from Abca3ΔΔA mice compared with control, whereas Stard9 and Stard10 were slightly reduced.

To determine whether the alterations in mRNA expression of genes associated with lipid metabolism are triggered by cell death in response to Abca3 deletion in the respiratory epithelium, apoptosis was assessed by immunohistochemistry using cleaved caspase-3 as a marker. No differences in cleaved caspase-3 staining were observed in lungs of 4-wk-old and 9-mo-old Abca3ΔΔA mice compared with control littermates (data not shown), indicating that alveolar type II cell survival was not affected by the deletion of Abca3.

To assess the potential compensatory regulation of gene expression between the two populations of alveolar type II cells in adult Abca3ΔΔA mice, type II cells were isolated from the lung and then distinguished by flow cytometry using NR. Highly enriched populations of NR-positive (NR+) and NR weakly (NR+) stained cells were obtained by excluding from the sort cells in the region of overlap between the two peaks. NR is a fluorescent hydrophobic probe that stains both neutral lipid and phospholipid inclusions using specific filter packages. When cells were subjected to flow cytometric analysis under red fluorescence conditions (>630 nm), two cell populations stained with NR were observed in Abca3ΔΔA mice; only one cell population (NR+) was present in control mice. In Abca3ΔΔA mice, the phospholipid-loaded (NR+) cells (undeleted Abca3 cells) were differentiated by stronger fluorescence intensity, which was similar to findings in Abca3ΔΔA cells in control mice. Abca3ΔΔA cells displayed weaker fluorescence (NR++; Fig. 8A), because these cells lack LBs and NR is taken up by cell membrane lipids as well as by intracellular lipid droplets. Expression of mRNAs of genes associated with surfactant, lipid synthesis, transport, and secretion was assessed by qRT-PCR in NR+ and NR++ type II cells. Sfip1 was significantly increased in NR+ compared with NR++ and control type II cells. A marked decrease in mRNAs influencing fatty acid (Scd1, Fasn, and Lpca1) and cholesterol (Hmgcs1; Fig. 8C) metabolism was observed in NR+ type II cells isolated from 9-mo-old Abca3ΔΔA mice compared with NR++ type II cells, supporting a decrease in the activity of surfactant synthesis pathways in the targeted cells. In contrast, Fasn, Scd1, and Hmgcs1 mRNA expression was increased in NR++ cells compared with control type II cells, perhaps indicating a selective compensatory lipid synthesis pathway in ABCA3-sufficient cells. On the other hand, mRNA expression of other genes (Sfip1, Petlta, Fabp5, and Abca1) was similarly decreased in NR+ and NR++ type II cells, indicating that compensatory mechanisms were partial in response to deletion of Abca3. Finally, Stard2 was significantly increased in Abca3-deficient cells compared with Abca3-sufficient cells, whereas Stard9 was slightly reduced (Fig. 8D).

**Deletion of Abca3 alters the transcriptional network associated with lipid homeostasis in alveolar type II cells.** Nfatc3, Cebpα, and Foxa2 mRNAs, genes known to influence expression of genes regulating surfactant lipid homeostasis, were significantly reduced in NR+ type II cells from Abca3ΔΔA mice.
Fig. 6. Phospholipid composition and synthesis are altered in adult Abca3Δ/Δ mice. Relative distribution of the phospholipid classes PC, phosphatidylglycerol (PG), and phosphatidylinositol (PI) in lung tissue (after lavage), isolated LBs, and BALF from Abca3Δ/Δ and control littermates were analyzed by electrospray ionization-mass spectrometry. There was a reduction in total PC (BALF) and PG (BALF and LB) and an increase in total PI in the Abca3Δ/Δ mice. Synthesis of PC was determined from incorporation of methyl-D9-choline into intact molecular species and expressed as fractional enrichment of labeled substrate relative to total PC. This analysis showed that while the rate of PC synthesis was decreased in lavaged tissue and LBs and despite the lower PC content in BALF, synthesis and secretion of BALF PC from functional type II cells were unaltered in Abca3Δ/Δ mice. Sums of individual molecular species from each phospholipid class are expressed as means ± SE of 6–8 animals per group.

(Fig. 9A). Nkx2.1 mRNA was similar in NR++ and NR+ cells. Immunostaining for FOXA2 was readily detected in type II cells in control mice (Fig. 9, B and C). In contrast, numbers and intensity of FOXA2 staining of type II cells were decreased in Abca3Δ/Δ mice (Fig. 9, D and E).

DISCUSSION

Abca3, a lipid transporter protein that is highly expressed in the lung alveolar type II cells (32, 52, 55), is critical for surfactant lipid homeostasis and for adaptation to air breathing.

Germ-line deletion of the mouse Abca3 gene caused respiratory distress immediately followed by death at birth (2, 13, 19, 23), and severe mutations in ABCA3 caused respiratory failure in newborn infants (42). Since chronic lung disease was reported in older patients (11, 17), we used a conditional system to selectively delete Abca3 in alveolar epithelial cells of the mouse lung to assess the long-term effect of Abca3 deficiency in the lung.

In the present work, respiratory epithelium-specific, conditional deletion of Abca3 caused respiratory distress and death,
confirming previous findings that Abca3 was critical for adaptation for air breathing (2, 13, 19, 23). In mice that died at birth, Abca3 mRNA was reduced by 85–90% and was associated with the absence of LBs in type II epithelial cells. Thickenened mesenchyme and glycogen accumulation in Abca3Δ/Δ mice with neonatal lethality, a few ABCA3 mutations are associated with interstitial lung disease in older children who survive beyond the perinatal period (12, 17, 54). The heterozygosity mutation was associated with a more severe clinical presentation in the case of a SFTP mutation, indicating the importance of ABCA3 in alveolar homeostasis that may influence the pathogenesis of lung disease (10). Expression of genes involved in lipid metabolism was decreased during the perinatal period as well as in adult Abca3Δ/Δ mice. Expression of genes dedicated to the biosynthesis of fatty acids and phospholipids was decreased, consistent with altered lipid content in the lungs of newborn Abca3Δ/Δ mice. Decreased expression of mRNAs related to lipid metabolism [acyl-CoA oxidase 1 (Acox1), Lpcat1, Sftp1a, lipase (Lipg), glycerophosphodiester phosphodiesterase domain-containing protein 2 (Gdpd2), pancreatic lipase-related protein 1 (Pnlli1p1), delta-like 1 (Dlk1), and patatin-like phospholipase domain-containing protein 2 (Pnpla2)] was observed at E18.5. Such changes in gene expression may represent an indirect

### Table 1. Alterations in gene expression after deletion of Abca3 in type II epithelial cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference Sequence ID</th>
<th>Description</th>
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<td>Lipase, endothelial</td>
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<tr>
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<td>Scd5</td>
<td>1434520_at</td>
<td>Sterol-C5-desaturase homolog</td>
<td>+1.712</td>
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Differences in mRNAs in the lung of embryonic day 18.5 Abca3Δ/Δ and control mice were identified through microarray analysis. Genes most changed after deletion of Abca3 are shown. Differentially expressed genes were identified using a univariate F test and a permutation test (n = 100) with a significance level of 0.05. Abca3Δ/Δ mice developed emphysema, perhaps indicating that ABCA3 deficiency resulted in cell injury and lung remodeling. Although the majority of mutations in ABCA3 are associated with neonatal lethality, a few ABCA3 mutations are associated with interstitial lung disease in older children who survive beyond the perinatal period (12, 17, 54). The heterozygosity for an ABCA3 mutation was associated with a more severe clinical presentation in the case of a SFTP mutation, indicating the importance of ABCA3 in alveolar homeostasis that may influence the pathogenesis of lung disease (10). Expression of genes involved in lipid metabolism was decreased during the perinatal period as well as in adult Abca3Δ/Δ mice. Expression of genes dedicated to the biosynthesis of fatty acids and phospholipids was decreased, consistent with altered lipid content in the lungs of newborn Abca3Δ/Δ mice. Decreased expression of mRNAs related to lipid metabolism [acyl-CoA oxidase 1 (Acox1), Lpcat1, Sftp1a, lipase (Lipg), glycerophosphodiester phosphodiesterase domain-containing protein 2 (Gdpd2), pancreatic lipase-related protein 1 (Pnlli1p1), delta-like 1 (Dlk1), and patatin-like phospholipase domain-containing protein 2 (Pnpla2)] was observed at E18.5. Such changes in gene expression may represent an indirect
effect of ABCA3 deficiency and alteration in cellular lipids or, alternatively, a generalized delay in type II cell maturation. Gene Ontology and network analysis on mRNAs that were altered >1.5-fold suggested “lipid/sterol metabolism” as a major group of associated genes influenced by Abca3 deletion. Expression of genes regulating lipid synthesis (Lpcat1, Sedl, Abca1, and Hmgcs1) was decreased at PND0 in Abca3Δ/Δ mice, consistent with the RNA microarray data. In contrast, Pcyt1a was increased in PND0 Abca3Δ/Δ lungs, perhaps indicating a compensatory role for CCTαs in PC synthesis after birth. Lung epithelial cell-specific deletion of Pcyt1a resulted in severe respiratory failure at birth due to a major defect in phospholipid synthesis and LB formation (45). In the adult Abca3Δ/Δ type II cells, decreased expression of several genes, including Sedl, Fasn, Lpcat1, and Pcyt1a, as well as Hmgcs1 (a gene critical for cholesterol synthesis), also was observed, demonstrating that chronic deletion of Abca3 altered lipid biosynthesis in type II epithelial cells after birth. Expression of a number of lipid transporters, including the fatty acid-binding protein 5 (Fabp5) and cholesterol transporter ATP-binding cassette A1 (Abca1), was decreased in type II cells of Abca3Δ/Δ mice. For most of these genes, levels of mRNA were reduced by 40–70%, consistent with the loss of ABCA3 in 50% of type II cells in Abca3Δ/Δ mice. This inhibition of lipid biosynthesis and transport may serve to counterregulate surfactant production, since it can no longer be packaged and secreted properly in the alveolar space. Alternatively, cell injury related to the lack of ABCA3 may initiate an inhibitory effect on lipid biosynthesis. While Sftpa1 mRNA was decreased in newborn Abca3Δ/Δ lungs, it was increased in ABCA3-deficient type II cells in adult Abca3Δ/Δ mice, perhaps indicating a compensatory role for SFTPA1 in the formation of tubular myelin in the ABCA3-deficient cells.

Lungs of surviving Abca3Δ/Δ mice contained two distinct populations of alveolar type II cells: one lacking LBs and the other containing LBs of various size. Absence of LBs related to the deletion of Abca3 in type II cells was consistent with previous mouse and human studies (2, 13, 19, 20, 23, 42). The presence of large atypical LBs and ABCA3 protein in a subset of type II cells indicates both targeting and compensatory responses to the lack of surfactant synthesis in coexisting Abca3 targeted cells, a concept supported by analysis of Sftpa1 mRNA levels and ABCA3 expression by immunohistochemistry. Mouse Abca3Δ/Δ cells lacking LBs contained structures of intermediate electron density with electron-dense inclusions scattered throughout the cytoplasm of type II cells, similar to findings in patients with ABCA3 mutations (11, 18, 40, 42, 43). Abca3Δ/Δ cells lacking LBs were enriched in atypical lipid vesicles that appear to contain neutral lipids based on their staining characteristics. In contrast, alveolar type II cells wherein deletion of Abca3 did not occur contained heterogeneous populations of LBs, some of which were enlarged.

Fig. 8. Deletion of Abca3 induces compensatory mechanisms in both type II cell populations present in adult Abca3Δ/Δ mice. Flow cytometric data of alveolar type II cells from adult control (A) and Abca3Δ/Δ (B) mice stained with Nile red (NR) showed 1 cell population with high fluorescence intensity (NR++) and 1 cell population with lower fluorescence intensity (NR+). mRNAs were extracted from alveolar type II cells isolated from adult control or Abca3Δ/Δ mice and sorted according to NR fluorescence intensity. SSC-A, side-scatter area. Quantitative RT-PCR was performed to estimate gene mRNA levels for surfactant-associated proteins (C; Sftpa1, Sftpb, and Sfpc), lipid metabolism (D; Fasn, Sedl, Lpcat1, Pcyt1a, Fabp5, Abca1, and Hmgcs1), or members of the START domain superfamily (E; Stard2, Stard7, Stard9, and Stard10). Sftpa1 and Stard2 were significantly increased in NR+ cells compared with control and NR++ cells. Fasn, Sedl, and Hmgcs1 were significantly reduced in NR+ cells compared with control and NR++ cells. Fasn, Sedl, and Hmgcs1 were significantly increased in NR++ cells compared with control cells. Results were normalized to 18s rRNA and are representative of 4 animals per group. *P < 0.05 and **P < 0.01 vs. NR++ cells. $P < 0.05 and §§P < 0.01 vs. control cells.
abnormal accumulation of LBs may indicate the existence of a compensatory pathway that serves to enhance surfactant production. Fasn, Scd1, and Hmgs1 mRNAs were increased in type II cells from Abca3 Δ/Δ mice, wherein deletion of Abca3 did not occur, providing further support for the concept of a compensatory pathway maintaining surfactant synthesis. It is unclear whether the lack of ABCA3 in a subset of cells or compensatory changes in ABCA3-sufficient cells influences cell injury and leads to emphysema in the Abca3 Δ/Δ mice. Nonuniform exogenous expression of Sftpb in distal respiratory epithelial type II cells of Sftpb Δ/Δ mice, resulting in the presence of both Sftpb-deficient and -sufficient cells, was associated with air space enlargement and alterations in LB structure, phenotype similar to the adult Abca3 Δ/Δ mice (33). These data suggest that focal variations of surfactant production and secretion may be responsible for alterations in lung structure and function and that they may be partially corrected by SFTPB- or ABCA3-sufficient cells.

Deletion of Abca3 altered Sat PC levels and phospholipid composition in newborn and adult Abca3 Δ/Δ mice. The decreased PC and PG content in newborn Abca3 Δ/Δ lungs is consistent with previous reports in Abca3 Δ/Δ mice (2, 13, 19). PC and PG contribute to surfactant function, and thus their dramatic reduction in newborn Abca3 Δ/Δ mice is likely directly correlated with respiratory distress and death at birth. Our results and other studies demonstrate that deletion of Abca3 in alveolar type II cells from newborn mice reduces surfactant PC, especially short acyl chain species, which are important for maintaining normal lung function (4, 36). In the surviving Abca3 Δ/Δ mice, while PC was significantly reduced in BALF, no differences were observed in the lung tissue (after lavage) and LB fractions, perhaps indicating differences in secretion or recycling. In agreement with the morphological analysis, two metabolically distinct LB populations could be distinguished in the adult Abca3 Δ/Δ mouse lung. The methyl-D9-choline incorporation data suggest that one subset of Abca3 Δ/Δ mouse lung LBs accumulates and secretes PC at a rate similar to that of control mice. The second subset of LBs contains a more metabolically inert pool of PC that is not secreted. The lower PC content of BALF in adult Abca3 Δ/Δ mice is likely directly correlated with respiratory distress and death at birth. Quantitative RT-PCR was performed to estimate gene mRNA levels for transcription factors known to play a critical role in lung homeostasis (Nkx2.1, Foxa2, and Cebpa). Results were normalized to 18S rRNA. Nkx2.1, Foxa2, and Cebpa mRNAs were significantly reduced in NR- cells compared with control and NR+ cells. Foxa2 and Cebpa mRNAs were significantly decreased in NR+ cells compared with control cells. In contrast, Nkx2.1 was significantly increased in NR++ and NR+ cells compared with control cells. Results are representative of 4 animals per group. *P < 0.05 and **P < 0.01 vs. NR++ cells. §P < 0.05 vs. control cells. Lung tissue from adult control (B and C) and Abca3 Δ/Δ (D and E) mice was immunostained for FOXA2 at 1:4,000 (B and D) and 1:8,000 (C and E) dilutions. FOXA2 was detected in control lungs at both dilutions (B and C). In Abca3 Δ/Δ mice, while FOXA2 was detected in the bronchial epithelium (Br) at both dilutions, FOXA2 was detected in few type II cells (arrows) at 1:4,000 (D) and absent at 1: 8,000 (E). Scale bars, 100 μm.

Deletion of Abca3 altered Sat PC levels and phospholipid composition in newborn and adult Abca3 Δ/Δ mice. The decreased PC and PG content in newborn Abca3 Δ/Δ lungs is consistent with previous reports in Abca3 Δ/Δ mice (2, 13, 19). PC and PG contribute to surfactant function, and thus their dramatic reduction in newborn Abca3 Δ/Δ mice is likely directly correlated with respiratory distress and death at birth. Quantitative RT-PCR was performed to estimate gene mRNA levels for transcription factors known to play a critical role in lung homeostasis (Nkx2.1, Foxa2, and Cebpa). Results were normalized to 18S rRNA. Nkx2.1, Foxa2, and Cebpa mRNAs were significantly reduced in NR- cells compared with control and NR+ cells. Foxa2 and Cebpa mRNAs were significantly decreased in NR+ cells compared with control cells. In contrast, Nkx2.1 was significantly increased in NR++ and NR+ cells compared with control cells. Results are representative of 4 animals per group. *P < 0.05 and **P < 0.01 vs. NR++ cells. §P < 0.05 vs. control cells. Lung tissue from adult control (B and C) and Abca3 Δ/Δ (D and E) mice was immunostained for FOXA2 at 1:4,000 (B and D) and 1:8,000 (C and E) dilutions. FOXA2 was detected in control lungs at both dilutions (B and C). In Abca3 Δ/Δ mice, while FOXA2 was detected in the bronchial epithelium (Br) at both dilutions, FOXA2 was detected in few type II cells (arrows) at 1:4,000 (D) and absent at 1: 8,000 (E). Scale bars, 100 μm.
label into BALF PC in the two groups of animals indicates little change in the kinetics of surfactant PC secretion from functional type II cells in Abca3Δ/Δ mice. PG was markedly reduced in LB and BALF fractions, but not in the lung tissue, indicating that PG transport and packaging in LBs and secretion are altered after deletion of Abca3. Analysis of the minor surfactant phospholipids demonstrated significant increases in PI (lung tissue after lavage and LBs) in Abca3Δ/Δ mice. Similar alterations in PC, PG, and PI were shown in “at-risk” or acute respiratory distress syndrome patients (22). Whether ABCA3 deficiency enhances susceptibility to lung injury is unclear. Adult Stat3Δ mice, in which levels of Abca3 mRNA and protein and lung Sat PC are reduced, are susceptible to lung injury induced by hyperoxia (31).

Stard2 mRNA was increased in ABCA3-deficient type II cells. The steroiodogenic acute regulatory protein (StAR)-related lipid transfer (START) domain proteins bind lipids (1). The START domain is thought to be a lipid-exchange and/or a lipid sensing domain that mediates lipid transfer between intracellular compartments. PC and PE are known ligands for Stard2 and STARD10. STARD2 may play a role in the maintenance of lipid homeostasis in Abca3Δ/Δ mice. Since START domain proteins frequently contain homeodomains, they may function as lipid-responsive transcription factors or coactivators (41). Kanno et al. (27) showed that STARD2 coactivates transcriptional activity of paired box protein (PAX3) in vitro, providing a potential mechanism by which lipid metabolism is linked to transcriptional programs.

Previous studies established that Nkx2.1 (15), Foxa2 (47, 48), CeBpαx (30), and Nfatc3 (14) are critical for perinatal lung function, directly regulating surfactant protein, and influencing surfactant lipid synthesis. Comparison of lung mRNAs from CebpaΔ/Δ (30), Cnb1Δ/Δ (14), Foxa2Δ/Δ (47), and Tiff1m/m mice (15) demonstrated that a number of mRNAs were similarly affected in each model. Genes involved in the regulation of lung lipid homeostasis were decreased after deletion or mutation of each of these transcription factors. In the present study, Foxa2 and Cebpa mRNAs were markedly reduced in Abca3Δ/Δ type II cells, suggesting their potential role in the downregulation of genes regulating lung lipid homeostasis. Susceptibility of adult CebpaΔ/Δ mice to lung injury induced by hyperoxia was associated with the decrease of genes regulating surfactant lipid homeostasis and surfactant protein biosynthesis, processing, and transport (51). Mechanisms by which ABCA3 deficiency influences transcriptional pathways to modulate lipid homeostasis in type II cells remain unknown.

The present study demonstrates that Abca3 is required not only for respiration at birth, but also for maintaining lung phospholipid homeostasis after birth, influencing lipid biosynthesis and trafficking. Present findings support the presence of a sensing pathway within and between ABCA3-sufficient and -deficient alveolar type II cells that initiates compensation at transcriptional and metabolic levels. Maintenance of respiratory function, despite alterations in lipid synthesis and secretion by a subset of Abca3 gene-deleted type II epithelial cells, indicates compensatory mechanisms by which type II cells maintain surfactant lipid homeostasis in the alveolar epithelium. Alterations of ABCA3 expression may render individuals, newborns, infants, and older individuals more susceptible to acute and chronic lung diseases.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. Alpy F, Tomasetto C. Give lipids a START: the StAR-related lipid transfer (START) domain proteins bind lipids. The present study demonstrates that Abca3 is required not only for respiration at birth, but also for maintaining lung phospholipid homeostasis after birth, influencing lipid biosynthesis and trafficking. Present findings support the presence of a sensing pathway within and between ABCA3-sufficient and -deficient alveolar type II cells that initiates compensation at transcriptional and metabolic levels. Maintenance of respiratory function, despite alterations in lipid synthesis and secretion by a subset of Abca3 gene-deleted type II epithelial cells, indicates compensatory mechanisms by which type II cells maintain surfactant lipid homeostasis in the alveolar epithelium. Alterations of ABCA3 expression may render individuals, newborns, infants, and older individuals more susceptible to acute and chronic lung diseases.

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