ABCA3 Is Critical for Lamellar Body Biogenesis in Vivo*

Naeun Cheong§*, Huayan Zhang*, Muniswamy Madesh§, Ming Zhao†, Kevin Yu§, Chandra Dodia§, Aron B. Fisher§, Rashmin C. Savani**, and Henry Shuman§†

From the *Department of Physiology, †Institute for Environmental Medicine, and **Division of Cancer Biology, University of Pennsylvania School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the †Department of Pediatrics, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, and the **Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9063

Mutations in ATP-binding cassette transporter A3 (human ABCA3) protein are associated with fatal respiratory distress syndrome in newborns. We therefore characterized mice with targeted disruption of the ABCA3 gene. Homozygous Abca3−/− knock-out mice died soon after birth, whereas most of the wild type, Abca3+/+, and heterozygous, Abca3+/−, neonates survived. The lungs from E18.5 and E19.5 Abca3−/− mice were less mature than wild type. Alveolar type 2 cells from Abca3−/− embryos contained no lamellar bodies, and expression of mature SP-B protein was disrupted when compared with the normal lung surfactant system of wild type embryos. Small structural and functional differences in the surfactant system were seen in adult Abca3−/− compared with Abca3+/+ mice. The heterozygotes had fewer lamellar bodies, and the incorporation of radiolabeled substrates into newly synthesized disaturated phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidylserine in both lamellar bodies and surfactant was lower than in Abca3+/+ mice. In addition, since the fraction of near term Abca3−/− embryos was significantly lower than expected from Mendelian inheritance ABCA3 probably plays roles in development unrelated to surfactant. Collectively, these findings strongly suggest that ABCA3 is necessary for lamellar body biogenesis, surfactant protein-B processing, and lung development late in gestation.

Lung surfactant is a complex mixture of 90% lipids, predominantly dipalmitoylphosphatidylcholine, and 10% proteins (SP-A, SP-B, and SP-C) that functions to lower surface tension at the interface between air and the surface lining layer of the alveolar epithelium and prevents lung collapse on end expiration (1–3). The surfactant proteins SP-B and SP-C and lipids are transported to and stored in lamellar bodies, the intracellular secretory organelles of the alveolar epithelial type 2 (AT2) cell through which pulmonary surfactant homeostasis is regulated (4–6). The final steps in post-translational modification of both SP-B and SP-C occur in lamellar bodies (7–11). One of the striking abnormalities observed in hereditary SP-B deficiency, which is characterized by deficiencies of surfactant quantity and function, is derangement of lamellar body morphology (12), abnormalities that are replicated in the SP-B knock-out mouse (13, 14).

Fatal deficiencies of lung surfactant in the neonate and interstitial lung disease have recently been associated with mutations in the ATP-binding cassette protein A3 gene (ABCA3) (15, 16). ABCA3 is required for normal lamellar body formation in AT2 cells (17). Similarly, the lamellar bodies in electron micrographs from lung tissue of infants with surfactant deficiency and ABCA3 mutations have dense inclusions and are structurally immature (15, 18, 19). ABCA3 expressed exogenously in cultured cells, in the absence of surfactant proteins, facilitates the transfer of phosphatidylcholine and cholesterol into the lysosomes, the likely progenitor of lamellar bodies, whereas the surfactant in infants with ABCA3 mutations has decreased phosphatidylcholine content (17, 20). The surfactant phosphatidylcholine (PC) and phosphatidylglycerol (PG) content is decreased in Abca3−/− mice (21, 22). Decreased expression of ABCA3 in both isolated AT2 cells and in lungs alters the expression, localization, and processing of SP-B protein (16, 17, 19). Together, these findings are suggestive that ABCA3 is responsible for converting lysosomes or similar vesicular bodies into phospholipid storage organelles, which are then transformed into lamellar bodies by the subsequent trafficking and processing of SP-B and SP-C.

To further explore the role of ABCA3 expression, an in vivo mouse model of Abca3 gene disruption was generated. Overall, the Abca3 knock-out mouse phenotype was similar to the clinical findings in patients with the most severe forms of surfactant deficiency associated with ABCA3 mutations. Abca3 null mice were underrepresented late in gestation, and those that reached term died soon after birth, establishing an unambiguous causal link between ABCA3 and fatal surfactant deficiency. As in the infants, lung architecture, AT2 cell morphology, surfactant protein-B processing, and lipid synthesis were all disrupted. Both the Abca3−/− and Abca3+/− mice will be useful models for providing insights into surfactant metabolism in normal and disease states.

MATERIALS AND METHODS

Generation of Abca3−/− Mice—Heterozygous Abca3+/− founder mice were obtained from Deltagen (San Carlos, CA). A
6.93-kb IRES-LacZ reporter and neomycin resistance cassette (IRES-LacZ-neo) was subcloned into a 5.4-kb fragment isolated from a mouse genomic phage library, such that 70 base pairs coding for the protein were replaced by IRES-LacZ-neo. The IRES-LacZ-neo cassette was flanked by 3.9 kb of mouse genomic DNA at its 5' aspect and 1.5 kb of mouse genomic DNA at its 3' aspect. The targeting vector was linearized and electroporated into 129/OlaHsd mouse embryonic stem cells. Embryonic stem cells were selected for G418 resistance, and colonies carrying the homologously integrated neomycin DNA were identified by PCR amplification using a 5' neomycin-specific primer (5'-GGATCTTGCCATGTAGCTGAT-3') paired with a primer located outside the targeting homology arms on the 5' side (GS1; 5'-AGTGAAATCCAAAGATGGC-3'). The homologous recombination event was confirmed on the 3' side using a 3' neomycin-specific primer (5'-GAAGAACGAGATCAGCCTCTGTGCC-3') paired with a primer located outside the targeting homology arm on the 3' side (GS2; 5'-TGGTGGAGTTGTTGACCATCTGCC-3'). Colonies that gave rise to the correct size PCR product were confirmed by Southern blot analysis using a probe adjacent to the 3' region of homology. The presence of a single neomycin cassette was confirmed by Southern blot analysis using a neomycin gene fragment as a probe. Male chimeric mice were generated by injection of the targeted embryonic stem cells into C57Bl/6J blastocysts. Chimeric mice were bred with C57Bl/6J mice to produce F1 heterozygotes. Germ line transmission was confirmed by PCR analysis. Initial germ line heterozygotes were also tested for the homologous recombination event using the primers described above (located outside of the targeting construct). Following confirmation of the targeting event in animals, subsequent genotyping tracked transmission of the targeting construct. F1 heterozygous males and females were mated to produce F2 wild-type, heterozygous, and homozygous null mutant animals. Mice were backcrossed with C57Bl/6J mice to produce F1 heterozygotes. Germ line transmission was confirmed by PCR analysis. Initial germ line heterozygotes were also tested for the homologous recombination event using the primers described above (located outside of the targeting construct). Following confirmation of the targeting event in animals, subsequent genotyping tracked transmission of the targeting construct. F1 heterozygous males and females were mated to produce F2 wild-type, heterozygous, and homozygous null mutant animals. Mice were backcrossed with C57Bl/6J mice, and all phenotypic analysis was performed in a hybrid C57Bl/6/129 background (75%/25%, respectively).

PCR Genotyping—PCR primers were as follows: reverse, 5'-TGAGCTGGAATCCACTTGAGTC-3' (specific for both wild type and targeted alleles); forward 1, 5'-CGTCTGTCATACACCTGTAAATGC-3' (specific to wild-type allele); forward 2, 5'-GGGGATCAGATTCAATGCTCTTC-3' (specific for targeted allele only, neomycin specific), generating 274-bp (wild type) and 537-bp (targeted) PCR products, respectively. PCR conditions were as follows: 95 °C for 10 min (one cycle); 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min 30 s (25 cycles); 72 °C 10 min.

Histology Tissue Preparation—Lungs were inflated to a constant pressure of 25 cm of H2O with 4% paraformaldehyde in PBS and immersed in the same fixative for 24 h. Lungs were extensively washed with PBS, dehydrated in a series of increasing alcohol concentrations, embedded in paraffin, and cut into 5-μm-thick sections. Paraffin sections were stained with hematoxylin and eosin.

EM Tissue Preparation—E18.5 embryo lungs were inflated to a pressure of 25 cm of H2O with 2.5% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer through the trachea, removed, and immersed in the same fixative at 4 °C. For 6-month-old adult mice, lungs were perfused through the pulmonary artery with 2.5% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer for 15 min while the lungs were artificially ventilated. The lungs were then removed and immersed in the same fixative at 4 °C. After 30 min, both embryonic and adult mouse lungs were cut into 1 × 1-mm pieces and continuously fixed for another 6 h and washed with 0.1 M cacodylate buffer (pH 7.4) three times, followed by two steps of postfixation: 1% OsO4 in cacodylate buffer and then 2% uranyl acetate in distilled water (1 h for each step). Tissue pieces were embedded in EM-bed 812 resin and polymerized at 60 °C for 48 h. Ultrathin sections (70 nm) were prepared on a Leica Ultracut UCT (Vienna, Austria) with a diamond knife, counterstained with uranyl acetate and lead citrate, and imaged with a JEOL electron microscope model 100 CX (Tokyo, Japan).

Immunohistochemistry—Lungs embedded in cryomatrix were cut into 7-μm sections and then mounted on glass slides. Sections were washed with PBS buffer to remove cryomatrix and treated with blocking buffer containing 5% bovine serum albumin, 10% normal goat serum in PBS (pH 7.4) for 1 h. Sections were incubated with primary antibody, either anti-SP-B antibody (Chemicon, Temecula, CA) at 1:250 dilution or anti-3C9 monoclonal antibody (23) (1:250 dilution) in blocking buffer for 1 h. After washing with T-PBS (five times for 5 min each), the sections were incubated for 1 h at room temperature with Texas Red-conjugated goat anti-rabbit IgG (1:250 dilution) in blocking buffer. After a final washing (five times for 5 min each with T-PBS and twice for 5 min each with distilled H2O), the sections were mounted with Vectashield mounting medium (Vector Laboratories) and observed with a confocal microscope (Radiance 2000; Bio-Rad).

Western Blot Analysis of Surfactant Protein Expression in Mouse Fetal Lung Tissue—Lungs were immersed in lysis buffer (20 mM Heps, 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA) containing 1% Nonidet P-40 and homogenized with Pellet Pestle (Kontes). Subsequently, the samples were centrifuged for 15 min at 22,000 × g, and protein in the supernatant was quantified by the Bradford method (Bio-Rad). Separation of surfactant proteins was carried out under nonreducing conditions followed by Western blotting with 1:5000 dilution of rabbit anti-sheep SP-B (mature SP-B) or rabbit anti-human (prepro-SP-B) (Chemicon, Temecula, CA) or under reducing conditions followed by Western blotting with a 1:1000 dilution of rabbit anti-SP-A polyclonal antibody (Chemicon, Temecula, CA), rabbit anti-SP-D polyclonal antibody (Chemicon), or mouse anti-actin monoclonal antibody (Chemicon). Peroxidase-conjugated secondary antibody was used at a 1:5,000 dilution. Immunoreactive bands were detected with ECL reagents (Amersham Biosciences). Protein bands were quantified by densitometric analyses with Fluor-S Multimager (Bio-Rad) and Quantity One analysis software.

Measurement of Radiolabeled Substrate [3H]Choline or [14C]Palmitate Incorporation into Phospholipids—Briefly, anesthetized mice were injected in the tail vein with a mixture of [methyl-3H]choline (10 μCi; PerkinElmer Life Sciences) and [1-14C]palmitate (1 μCi; PerkinElmer Life Sciences). After 24 h, the mice were killed by exsanguination under anesthesia. To obtain bronchoalveolar lavage fluid and to isolate subcellular
results

Lethality of Abca3−/− at Birth

All of the newborn mice were genotyped with PCR, simultaneously detecting the endogenous and targeted alleles (Fig. 1). We examined 167 newborns in 25 litters from eight separate breeding pairs of heterozygous Abca3+/− mice (Table 1). Of these, only 13 were homozygous null Abca3−/− neonates, and none of those survived more than 16 h past birth, whereas 43 of the 50 wild type Abca3+/+ and 92 of the 104 heterozygous Abca3+/− neonates survived for at least 16 h, and most survived far longer. Because Abca3−/− is neonatal lethal, a count of the precise number of Abca3−/− mice was complicated by maternal cannibalism. To obtain a more accurate distribution of genotypes, mice were delivered by cesarean section at embryonic days 18.5 (E18.5) and 19.5 (E19.5). The cumulative distribution of the genotypes of late stage mouse embryos from heterozygote Abca3+/− crosses was 24:44:12 (wild type/heterozygote/null), as shown in Table 2. The frequency of the homozygote null allele was found to be approximately half the expected frequency and differs significantly from purely Mendelian ratios of inheritance, with a 95% confidence level from a χ² test > 5.99. These data suggest that Abca3−/− embryos are only partially viable or that Abca3 is required for appropriate fertilization. Since lung surfactant is not essential until after birth, ABCA3 may have multiple functions other than in surfactant maturation.

Morphology of Abca3−/− Embryo Lungs

Light Microscopy Analysis—To determine the effect of differential expression of ABCA3 on mouse lung morphology, the lungs of 36 pups from three litters of E18.5 embryos were excised, inflated with 25 cm of H₂O of appropriate buffer, and fixed for either light (Fig. 2) or electron microscopy (Fig. 3). We focused on this stage of development, since, like surfactant proteins, ABCA3 is expressed late in gestation (27, 28). The morphology of the embryonic lungs differed among the genotypes. Although the morphology of the lungs from wild type Abca3+/+ mice was comparatively normal and the lungs appeared well developed (Fig. 2a), lungs from Abca3−/− embryos had thick interalveolar septa and small air spaces compared with Abca3+/+ embryonic lungs (Fig. 2c). The morphology of lungs from the Abca3−/− mice was more variable than either Abca3+/+ or Abca3−/− mice, with some areas of the heterozygote lungs appearing well developed, whereas other areas did not (Fig. 2b). These data suggest that a deficiency or insufficiency of ABCA3 protein leads to abnormal lung development late in gestation.

EM Analysis—There were also clear differences at the ultrastructural level among the genotypes. In E18.5 lungs, abundant AT2 cells with lamellar bodies were seen in lungs of Abca3+/+ (Fig. 3a) and Abca3+/− (Fig. 3b) mice. Little or no difference in the peripheral epithelial cells of Abca3+/+ and Abca3+/− mouse lungs were observed at this stage. Although, cuboidal AT2 cells were readily observed throughout the peripheral lung epithelium in Abca3−/− embryo lung, no lamellar bodies were detected after extensive searching even in the lungs of animals from the same litter (Fig. 3c), corroborating the earlier in vitro observation that ABCA3 protein expression is necessary for lamellar body genesis (17).
Peripheral lung tissues and the surfactant system continue to develop after birth. Although there were no clear differences between Abca3+/+ and Abca3+/– embryonic lungs, qualitative differences in their ultrastructures were apparent 6 months after birth (Fig. 4). Heterozygous mice (Fig. 4, c and d) appeared to have fewer lamellar bodies than wild type mice (Fig. 4, a and b).

Effects of Abca3 Deletion on Surfactant Protein Expression and Processing

The expression of SP-B in the absence of ABCA3 was determined in late gestation wild type, heterozygote, and null mouse embryos using immunofluorescence staining (Fig. 5) and Western blot analysis (Fig. 6). Staining for ABCA3 with monoclonal antibody 3C9 was not detected in immunofluorescence images of lungs from E18.5 embryonic Abca3−/− mice (Fig. 5c), but ABCA3 staining was readily observed in lungs from Abca3+/+ (Fig. 5a) and Abca3+/– (Fig. 5b) E18.5 mouse embryos. SP-B staining was decreased in Abca3+/– (Fig. 5e) and Abca3−/− (Fig. 5f) mice compared with Abca3+/+ (Fig. 5d) mice. The level of SP-B in its mature, nonreduced, 16-kDa form was significantly decreased, as demonstrated by Western blot of lung homogenates from Abca3−/− mice compared with Abca3+/+ and Abca3+/– mice (Fig. 6, a and b). However, similar levels of 42-kDa pro-SP-B precursor were found in wild type, heterozygote, and null mice, suggesting that depletion of ABCA3 affects SP-B processing rather than its expression (Fig. 6c). The final step in the processing of SP-B from its 42-kDa proprotein precursor to its mature form occurs in the lamellar bodies of AT2 cells (9, 29). Changes in lamellar body biogenesis caused by lowered ABCA3 expression in patients and isolated human AT2 cells are associated with altered processing of SP-B protein (16, 17, 19) and confirmed here in Abca3 mutant mice. Trafficking of all forms of SP-B to lamellar bodies is decreased in the Abca3−/− mice (Fig. 5). However, the decrease, apparent in the
Incorporation of radiolabeled choline and palmitate into surfactant phospholipids and cholesterol was measured after 24 h. Incorporation of substrates into phospholipids in both fractions was expressed as specific activity (dpm/nmol DSPC).

DISCUSSION

The phenotype of mice with targeted disruption of the Abca3 gene was similar to that of human patients with functional or trafficking mutations in the ABCA3 gene (15). Homozygous Abca3+/− mice were of normal size and weight at birth but died soon afterward. Heterozygous Abca3+/− mice were indistinguishable from wild type mice, developing and growing normally. However, the distribution of genotypes from crosses of heterozygous Abca3+/− mice does not follow the pattern expected from Mendelian inheritance, since it yielded the Abca3 null genotype occurring at half the expected frequency (Table 1). We suspect that ABCA3 plays important roles at an earlier stage of embryonic development. This embryonic lethality probably is not related to the role of ABCA3 in surfactant homeostasis, where it is expressed late in development (27) and is required primarily for postnatal air breathing. Indeed, the Abca3 gene transcript is detected in other organs, where its absence may have additional consequences (27).

Previous in vitro studies suggested that transport of phospholipids and cholesterol across lamellar body membrane by ABCA3 is a key step in assembly of lung surfactant in primary cultures of human AT2 cells and the human carcinoma A549 cell line (17, 30). Garmany et al. (20) reported that in human patients, mutations in the ABCA3 gene are associated with decreased surfactant phosphatidylcholine content and increased surface tension of the lung lavage fluid, further sug-
gesting that ABCA3 plays an important role in pulmonary surfactant phospholipid homeostasis. Recently, Fitzgerald et al. (21) and Ban et al. (22) showed that short acyl chain PG and PC are significantly reduced in Abca3+/−/− mice compared with wild type mice. We observed reduced incorporation of radiolabeled lipid precursors into dipalmitoylphosphatidylcholine, PC, and PG as well as minor surfactant phospholipids (PE and PS) into adult Abca3+/−/− mouse lamellar bodies and surfactant. Clearly, at least one pathway in surfactant lipid processing or trafficking is disrupted in both the Abca3+/−/− and Abca3+/+/− mouse model.

Defects in lamellar body genesis and SP-B processing that were observed in human patients with ABCA3 mutations (16, 19, 25, 31) and in human AT2 cells with siRNA-mediated knockdown of ABCA3 expression (17) were recapitulated in the Abca3 null mouse. Instead of normal lamellar bodies, lysosome-like electron-dense vesicles were found in Abca3−/− mouse AT2 cells (Fig. 3c). A simple explanation for the SP-B processing defect is that the final step in SP-B processing occurs in lamellar bodies, which are absent in the Abca3 null mice (9, 23, 26, 29, 32). It is less likely that the causal relationship is reversed (i.e. an absence of processed SP-B leads to the absence of lamellar bodies), since lamellar body-like organelles were observed in HEK 293 cells expressing exogenous Abca3 but not expressing SP-B.

Single gene defects of the hydrophobic surfactant proteins, SP-B and SP-C, cause severe neonatal and chronic interstitial lung diseases (27, 28, 33, 34), and recently ABCA3 mutations were also associated with pediatric interstitial lung disease associated with abnormal surfactant function (16, 29, 35). Although 6-month-old heterozygous Abca3+/−/− mice had morphologically (Fig. 4) and functionally (Fig. 7) different lamellar bodies compared with wild type mice, they had no respiratory difficulties. It remains possible that under normal circumstances, heterozygous Abca3+/−/− mice had morphologically (Fig. 4) and functionally (Fig. 7) different lamellar bodies compared with wild type mice, they had no respiratory difficulties. It remains possible that under normal circumstances, heterozygous Abca3+/−/− mice survive without difficulty but that following lung injury or stress, there is an increased susceptibility to RDS or interstitial lung disease. Overall, disruption of the Abca3 gene in this mouse model demonstrates a clear causal link between ABCA3 expression and lamellar body biogenesis, SP-B processing, and lipid synthesis and trafficking in the lungs of mice.

REFERENCES
1. Wright, J. R., and Dobbs, L. G. (1991). Annu. Rev. Physiol. 53, 395–414
2. Rooney, S. A., Young, S. L., and Mendelson, C. R. (1994). FASEB J. 8, 957–967
3. Johansson, J., and Curstedt, T. (1997) Eur. J. Biochem. 244, 675–693

FIGURE 7. Incorporation of radiolabeled substrate, [3H]choline, and [14C]palmitate into surfactant phospholipids for adult Abca3+/+/− and Abca3−/− littermate mice. Shown is [3H]choline (A) and [14C]palmitate incorporation (B) into newly synthesized DSPC measured in the lamellar bodies and surfactant of adult Abca3−/− (black bar) and Abca3+/−/− mice (white bar). C, [14C]palmitate incorporation into newly synthesized DSPC, PC, PG, PE, and PS was measured in the surfactant of adult Abca3−/− and Abca3+/−/− mice. D, [14C]palmitate incorporation into newly synthesized DSPC, PC, PG, PE, and PS were measured in the lamellar bodies of adult Abca3−/− and Abca3+/−/− mice. Results are mean ± S.E. for n = 3. *, p < 0.05 versus wild type (WT).
