STAT3 Regulates ABCA3 Expression and Influences Lamellar Body Formation in Alveolar Type II Cells

Yohei Matsuzaki¹, Valérie Besnard¹, Jean C. Clark¹, Yan Xu¹, Susan E. Wert¹, Machiko Ikegami¹, and Jeffrey A. Whitsett¹

¹Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, and Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio

ATP-Binding Cassette A3 (ABCA3) is a lamellar body associated lipid transport protein required for normal synthesis and storage of pulmonary surfactant in type II cells in the alveoli. In this study, we demonstrate that STAT3, activated by IL-6, regulates ABCA3 expression *in vivo* and *in vitro*. ABCA3 mRNA and immunostaining were decreased in adult mouse lungs in which STAT3 was deleted from the respiratory epithelium (*Stat3*^{Δ/Δ} mice). Consistent with the role of STAT3, intratracheal IL-6 induced ABCA3 expression *in vivo*. Decreased ABCA3 and abnormalities in the formation of lamellar bodies, the intracellular site of surfactant lipid storage, were observed in *Stat3*^{Δ/Δ} mice. Expression of SREBP1a and 1c, SCAP, ABCA3, and AKT mRNAs was inhibited by deletion of *Stat3* in type II cells isolated from *Stat3*^{Δ/Δ} mice. The activities of PI3K and AKT were required for normal *Abca3* gene expression *in vitro*. AKT activation induced SREBP expression and increased the activity of the *Abca3* promoter *in vitro*, consistent with the role of STAT3 signaling, at least in part via SREBP, in the regulation of ABCA3. ABCA3 expression is regulated by IL-6 in a pathway that includes STAT3, PI3K, AKT, SCAP, and SREBP. Activation of STAT3 after exposure to IL-6 enhances ABCA3 expression, which, in turn, influences pulmonary surfactant homeostasis.

Keywords: STAT3; ABCA3; IL-6; gene regulation; surfactant; hyperoxia

Pulmonary surfactant is a complex mixture of phospholipids and proteins required for postnatal adaptation to air breathing. Surfactant reduces surface tension at the air-liquid interface in the alveoli (1–3). Surfactant is synthesized and secreted into the airspace by alveolar type II cells (4). Defects in the synthesis, packaging, secretion, and function of pulmonary surfactant are associated with respiratory distress syndrome (RDS) in preterm infants and acute respiratory distress syndrome (ARDS) in older individuals (5).

ATP-binding Cassette A3 (ABCA3) protein is a member of a large family of ATP-dependent transport proteins that are involved in the transport of ions and a broad range of molecules, including phospholipids and sterols (6). ABCA3 is highly expressed in alveolar type II cells, where it is detected in the limiting membrane of lamellar bodies (7, 8). Pulmonary surfactant lipids and proteins are packaged in intracellular inclusions termed lamellar bodies. Surfactant protein B (SP-B) and ABCA3 are required for organization of normal lamellar bodies, and both are required for normal pulmonary function after birth. Mutations in *SFTPB* and *ABCA3* are associated with reduced pulmonary

CLINICAL RELEVANCE

This work provides a mechanism by which activation of IL-6/STAT3 regulates ABCA3 in type II cells in the alveoli. ABCA3 is required for lamellar body formation and surfactant homeostasis that maintains lung function at birth and after injury.

surfactant function and lethal respiratory distress in newborn infants (9, 10) and mice in which the *Abca3* gene is deleted (11–13). Autosomal recessive mutations in the *ABCA3* gene cause respiratory distress and, less frequently, interstitial lung disease. In the mouse and human lung, ABCA3 expression increases prior to birth and is enhanced by glucocorticoids (14, 15).

Signal transducer and activator of transcription 3 (STAT3) is a nuclear factor mediating IL-6-dependent signaling (16). STAT3 mediates cellular responses following stimulation by various cytokines and growth factors (16–18). STAT3 is activated by phosphorylation that is mediated by Janus kinase-1 (JAK-1). Phosphorylated STAT3 dimerizes, translocates to the nucleus, and regulates the expression of numerous transcriptional targets. STAT3 plays critical roles in various biological processes, including cell survival, migration, proliferation, metabolism, and inflammation (19). Deletion of the *Stat3* gene in transgenic mice demonstrated that it was essential for survival at embryonic day 6.5–7.5 (20). Therefore, many of its biological roles have been identified following its deletion in specific cell types *in vitro* and after cell conditional deletion in various organs *in vivo*. In the lung, mutation of *Stat3* in the respiratory epithelium did not alter lung morphogenesis or postnatal lung function; however, *Stat3*^{Δ/Δ} mice were highly susceptible to acute lung injury caused by hyperoxia and adenoviral infection (21, 22). STAT3 was required for the maintenance of surfactant homeostasis and surfactant protein B expression in the lung during hyperoxia-induced lung injury (22, 23). Since ABCA3 also plays a critical role in surfactant homeostasis, the present study was undertaken to assess the potential role of IL-6 and STAT3 in the regulation of ABCA3 in alveolar type II cells *in vivo*.

MATERIALS AND METHODS

Gene Construction and Doxycycline Administration

SP-C-rtTA/(tetO)-CMV-Cre/Stat3^{flx/flx} triple transgenic mice were generated as described previously (21, 22). *Stat3*^{flx/flx} mice were a kind gift of Dr. Takeda (Hyogo College of Medicine, Japan, 1998) (20, 24). In the presence of doxycycline, exon 21 of the *Stat3* gene is permanently deleted from respiratory epithelial cells before birth (*Stat3*^{Δ/Δ} mice). For genotyping, DNA was purified from the tails of experimental mice, and PCR was performed for *SP-C-rtTA* and *(tetO)-CMV-Cre* genes using the following primers: 5'-GAC ACA TAT AAG ACC CTG GCT A-3' and 5'-AAA ATC TTG CCA GCT TTC CC-3' for *SP-C-rtTA*; 5'-TGC CAC CAA GTG ACA GCA ATG-3' and 5'-AGA GAC GGA AAT CCA TCG CTC G-3' for *(tetO)-CMV-Cre*. *Stat3*-deleted

(Received in original form August 23, 2007 and in final form November 2, 2007)

This work was supported by National Institutes of Health grants HL61646, HL85610 (M.I., S.E.W., J.A.W.), and HL38859 (J.A.W.).

Correspondence and requests for reprints should be addressed to Jeffrey A. Whitsett, M.D., Cincinnati Children's Hospital Medical Center, Section of Neonatology, Perinatal and Pulmonary Biology, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. E-mail: jeff.whitsett@cchmc.org

Am J Respir Cell Mol Biol Vol 38, pp 551–558, 2008

Originally Published in Press as DOI: 10.1165/rcmb.2007-0311OC on December 20, 2007
Internet address: www.atsjournals.org

transgenic (*Stat3^{ΔΔ}*) and nondeleted littermates (*Stat3^{flx/flx}*, control mice) were used for the experiments. Doxycycline was administered to the dams in the food at a concentration of 625 mg/kg (Harlan Teklad, Madison, WI) from embryonic day 0 to postnatal day 21, resulting in extensive deletion of *Stat3* in respiratory epithelial cells (22). Mice were then provided normal food. Six- to eight-week-old *Stat3^{ΔΔ}* and control mice were used for this study. Survival and body weights of adult control and *Stat3^{ΔΔ}* mice were similar.

Tissue Preparation, Histology, and Immunohistochemistry

Lung tissue preparation and immunohistochemistry were performed essentially as previously described (21). 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); proSP-C, 1:4,000 (rabbit polyclonal; Chemicon); and ABCA3, 1:1,000 (rabbit polyclonal; Seven Hills Bioreagents, Cincinnati, OH). The secondary antibody was goat anti-rabbit IgG used at 1:200 (Vector, Burlingame, CA). All experiments shown are representative of findings from at least four *Stat3^{ΔΔ}* and control mice.

Quantification of ABCA3 Staining in Alveolar Type II Cells

To quantify the number of cells expressing ABCA3, 10 random fields per animal were photographed by optical microscopy. Fields were selected using a randomization function in Microsoft Excel. ABCA3-reactive cells and nuclear numbers were counted manually. The number of ABCA3-stained cells was normalized to total cell number.

Transmission Electron Microscopy

Mice were anesthetized with sodium pentobarbital (0.1 cc, intraperitoneally). Lungs were inflation-fixed at 25 cm of water pressure with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (SCB) and 0.1% calcium chloride (pH 7.3). Tissues were post-fixed in 1% osmium tetroxide; and stained *en bloc* overnight at 4°C in 4% aqueous uranyl acetate (25, 26). Tissues were dehydrated in a graded series of alcohol solutions and embedded in Embed 812 (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were cut at 100 nm of thickness using a Reichert Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany) and post-stained with or without 2% uranyl acetate and lead citrate. Digitized images were acquired with an AMT Advantage Plus 2K × 2K TEM CCD digital camera (AMT, Danvers, MA) attached to a Hitachi H-7600 transmission electron microscope (Hitachi Ltd., Tokyo, Japan). Lamellar body numbers were quantified by counting the total number of lamellar bodies in every type II cell found on sections cut from two to three randomly selected blocks from one control mouse and three *Stat3^{ΔΔ}* mice. Only cells with a visible nucleus and surface microvilli were used for the survey. The total number of type II cells examined was 43 in the control mice and 275 in the *Stat3^{ΔΔ}* mice, which represented a total of 271 and 2,368 lamellar bodies, respectively. The Mann Whitney test on the ranks/medians was used for statistical comparison, since the data for the *Stat3^{ΔΔ}* mice were not distributed normally.

Isolation of Alveolar Type II Epithelial Cells

Alveolar type II cells were isolated from 6-week-old control and *Stat3^{ΔΔ}* mice using collagenase and differential plating as described previously (27). Type II cells were used for protein and RNA analysis 2 hours after isolation.

RNA Extraction and RT-PCR Assay

RNA was extracted from alveolar type II epithelial cells isolated from *Stat3^{ΔΔ}* and control mice, using RNeasy Protect mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration was measured by spectrophotometer. cDNA was made with SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time RT-PCR was used to assess expression of genes known to influence surfactant lipid metabolism and surfactant function. *Abca3*, *Srebp* cleavage-activating protein (*Scap*), Sterol Regulatory Element-Binding Protein (*Srebp1a*, *Srebp1c*, *Srebp2*, and *Actin* mRNAs were detected using the following primers. *Abca3*: forward, GCA TTG CCC TCA TTG GAG AGC CTG and reverse, TCC GGC CAT CCT CAG

TGG TGG G; *Scap*: forward, TGA CCA CAA ACA AGG AGA GC and reverse, CAG GAA CAC CAA ACA GCA AG; *Srebp1a*: forward, AAG CCG GGT GGG CGC CGG CGC CAT and reverse, GTC GTT CAA AAC CGC TGT GTC CAG; *Srebp1c*: forward, ATC GGC GCG GAA GCT GTC GGG GTA G and reverse, ACT GTC TTG GTT GTT GAT GAG CTG G; *Srebp2*: forward, CAT CCA GCA GCC TTT GAT ATA CCA G and reverse, AGG ACC GGG ACC TGC TGC ACC TGT G; *Actin*: forward, GTG GGC CGC TCT AGG CAC CA and reverse, TGG CCT TAG GGT TCA GGG. Changes in mRNA were analyzed with Smart Cycler (Cepheid, Sunnyvale, CA) and normalized with *Actin*. Two group comparisons were performed by unpaired Student's *t* test and significance was accepted at the 5% level.

Intratracheal Administration of IL-6

Human IL-6 (B&D, Franklin Lakes, NJ) (5 μg) was diluted in 80 μl of PBS and administered intratracheally to 8-wk-old wild-type mice (FVB/N) by a 24-gauge animal feeding needle (Popper and Sons, New Hyde Park, NY) and laryngoscope (Bio Research Center, Nagoya, Japan) (21, 28). Mice were anesthetized with isoflurane during administration of IL-6 or saline. Twelve hours after instillation of IL-6 or saline, lungs were inflation-fixed at 25 cm H₂O and prepared for immunohistochemistry. The ABCA3 primary antibody was used at a concentration of 1:4,000.

Effect of IL-6 on ABCA3 mRNA in MLE-15 Cells *In Vitro*

Mouse lung epithelial cells (MLE-15), an immortalized mouse lung epithelial cell line, were cultured in HITES medium as previously described (29) in the presence or absence of IL-6 (20 ng/ml). MLE-15 cells were harvested 6 hours after the administration of IL-6 or saline. RNA was extracted with RNeasy Protect mini kit. Real-time RT-PCR was performed using the Smart Cycler with RT-PCR primer for mouse *Abca3* as described above.

Abca3 Gene Promoter Assays

The -2.591 to +0.011 kb mouse *Abca3* promoter was generated by PCR and cloned into KpnI/XhoI sites of the pGL3-basic vector (Promega, Madison, WI) as previously described (30). Cervical cancer cells HeLa (ATCC CCL-2, Rockville, MD) were grown in Dulbecco's modified Eagle's medium supplemented with 50 units of penicillin/μl, 50 μg of streptomycin/μl, and 10% fetal calf serum. MLE-15 or HeLa cells were transfected with 2.6-*Abca3*-luciferase reporter and pCMV β-galactosidase (Clontech, Mountain View, CA) vector using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. To assess the effect of AKT on ABCA3 expression, 2.6-*Abca3*-luciferase reporter construct was co-transfected with empty vector pcDNA3 (Invitrogen, Carlsbad, CA) or pcDNA3-Myr-Akt (pcDNA3-Myr-HA-Akt was a kind gift from Dr. William R. Sellers, Harvard Medical School, Boston, MA) (31). Transcriptional regulation of the *Abca3* gene promoter by SREBP was assessed by co-transfection of 2.6-*Abca3*-luciferase reporter with either pcDNA3 or pCMV-nSREBP1c plasmid (ATCC, Rockville, MD) encoding amino acid 1–436 of human nuclear SREBP1c. Forty-eight hours after transfection, cells were harvested and lysed with Protein Lysis Buffer (Promega, Madison, WI) as previously described (30). Luciferase activity was performed with Auto Lumat plus (Berthold Technologies, Oak Ridge, TN) and normalized for transfection efficiency by β-galactosidase activity. All transfections were performed in triplicate. pcDNA3 and pCMV β-galactosidase vectors were used to normalize DNA and transfection efficiency.

Phosphoinositide Kinase-3 (PI3K) inhibitor (20–40 μM, LY294002; Calbiochem, La Jolla, CA) or Protein kinase B (Akt) inhibitor (2.5–10 μM, Cat. #124005; Calbiochem) was added to MLE-15 cells 24 hours after transfection of 2.6-*Abca3*-luc reporter and pCMV β-galactosidase vector. Cells were then cultured for 24 hours in the presence of the inhibitor; diluents (DMSO or methanol) were used for controls. Survival rate, cell morphology, and transfection efficiency of pCMV-β-galactosidase vector were not influenced by the inhibitors. Luciferase activity was assessed as described above. Two group comparisons were performed by unpaired Student's *t* tests and significance was accepted at the 5% level.

Protein Preparation and Western Blot Analysis

MLE-15 cells were transfected with pcDNA3 or pcDNA3-Myr-HA-Akt vector and harvested 48 h after transfection. Protein was extracted using NE-PER Nuclear Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). SREBP1 protein was assessed by Western blot analysis. The following dilutions of antibody were used: SREBP1 at 1:1,000 (rabbit polyclonal; Santa Cruz, Santa Cruz, CA) and Actin at 1:1,000 (rabbit polyclonal; Santa Cruz). Peroxidase-conjugated secondary antibodies (Calbiochem, EMD Biosciences, San Diego, CA) were used at 1:5,000. Immunoreactive bands were detected with ECL reagents (Amersham Health, Chicago, IL). SREBP1 signals were normalized to Actin signals for quantification.

ABCA3 Expression during Hyperoxia

Stat3^{Δ/Δ} and control mice (*n* = 4 per group) were exposed either to 95% O₂ or to room air for 69 hours (22). Mouse lungs were homogenized and RNAs extracted with RNAeasy Protect mini kit. ABCA3 mRNA was assessed by RT-PCR. Each group comparison was performed by two-factor factorial ANOVA with Scheffe's test for *post hoc* analysis, and significance was accepted at the 5% level.

RESULTS

Decreased Expression of ABCA3 in Alveolar Type II Cells in *Stat3^{Δ/Δ}* Mice

Deletion of *Stat3* in respiratory epithelial cells in the mouse did not disrupt lung morphogenesis or function, but decreased surfactant lipid and surfactant proteins during exposure to hyperoxia, increasing susceptibility to lung injury (22, 32). Since ABCA3 plays a critical role in the regulation of surfactant homeostasis in type II epithelial cells, the expression of ABCA3 was assessed in adult *Stat3^{Δ/Δ}* mice by immunohistochemistry and mRNA analysis. Under normal vivarium conditions, ABCA3 mRNA was significantly reduced (2.4-fold, *P* < 0.05) in type II cells isolated from adult *Stat3^{Δ/Δ}* mice (Table 1). Immunostaining of ABCA3 was readily detected in type II epithelial cells in control *Stat3^{flx/flx}* mice. In contrast, both intensity and numbers of cells expressing ABCA3 were decreased in *Stat3^{Δ/Δ}* mice (Figure 1), indicating that STAT3 influenced ABCA3 expression in the adult mouse lung.

Deletion of Stat3 Influenced Lamellar Body Morphology in Type II Epithelial Cells

Because of the marked decrease in ABCA3 in type II cells in the *Stat3^{Δ/Δ}* mice and its known roles in surfactant homeostasis and lamellar body formation, ultrastructure of lamellar bodies was assessed. While lamellar bodies (LBs) in control mice (*Stat3^{flx/flx}/Cre*) were fairly uniform in size and in numbers (Figure 2A, mean = 6 LBs per cell, ranging from 1–19 LBs per cells), those observed in *Stat3^{Δ/Δ}* mice were variable in both size and number (mean = 9 LBs per cells, ranging from 0–116 LBs per cell). In the *Stat3^{Δ/Δ}* mice, 10% of the type II cells had no visible lamellar bodies (Figure 2B), while 8% of type II cells had more than 19 lamellar bodies per cells (Figure 2C). In addition, 15% of the type II cells were found to have lamellar-like bodies that were fusing or coalescing into larger structures, while 2% of the cells exhibited extremely large, or giant, single lamellar-like bodies (Figure 2D). These observations suggest that lamellar body formation and storage of intracellular lipids were disturbed in *Stat3^{Δ/Δ}* mice. Consistent with these morphologic findings, surfactant phosphatidylcholine content was decreased in bronchoalveolar lavage fluid from *Stat3^{Δ/Δ}* mice (32). SatPC content in BALF was 9.6 ± 0.6 in control, compared with 3.9 ± 0.7 μmol/kg body weight in *Stat3^{Δ/Δ}* lungs, mean ± SE, *n* = 6, *P* < 0.05 (32).

TABLE 1. RT-PCR QUANTITATION OF mRNAS

Gene	Ratio Stat3 D/D to Control	Value for RT-PCR
Abca3	–2.4	< 0.05
Scap	–1.4	< 0.05
Srebp1a	–1.4	< 0.05
Srebp1c	–1.9	< 0.05
Srebp2	–1.3	0.08
Akt1	–1.4	< 0.05
Akt2	–1.5	< 0.05

Quantitative RT-PCR was performed on cDNA prepared from mRNA isolated from lung tissue from *Stat3^{Δ/Δ}* and control mice as described in MATERIALS AND METHODS. Statistical differences were determined by unpaired Student's *t* test.

ABCA3 Expression Was Decreased during Hyperoxia

Adult *Stat3^{Δ/Δ}* mice are susceptible to lung injury induced by hyperoxia (22). Lung SatPC content was decreased and IL-6 was increased during oxygen induced injury in *Stat3^{Δ/Δ}* mice (22, 32). ABCA3 mRNA was decreased during hyperoxia and further reduced in *Stat3^{Δ/Δ}* mice when exposed to 95% oxygen (Figure 3), a finding that may contribute to the increased susceptibility of *Stat3^{Δ/Δ}* mice to lung injury as previously described (22).

IL-6 Induced ABCA3 Expression *In Vivo* and *In Vitro*

IL-6 and related cytokines bind gp130 to activate STAT3 transcription. IL-6 was administered intratracheally and STAT3 phosphorylation and ABCA3 expression were assessed. ABCA3 immunostaining was increased 12 hours after intratracheal IL-6 instillation (Figures 4A and 4B). ABCA3 mRNA was significantly increased 6 hours after exposure of MLE-15 cells to IL-6 *in vitro* (Figure 4C).

PI3K or Akt Inhibitors Inhibited Abca3 Gene Promoter Activity

STAT3 influences PI3K and AKT in other tissues (33, 34). AKT regulates SREBP in liver (35, 36). To assess the role of AKT in regulation of ABCA3, PI3K inhibitor LY2984002 and the AKT inhibitor (Protein Kinase B inhibitor) were added to MLE-15 cells that were transfected with the 2.6-*Abca3*-luciferase reporter construct. Both the LY298002 and AKT inhibitor decreased the activity of the *Abca3* gene promoter in the MLE-15 cells (Figures 5A and 5B). There was no evidence of cytotoxicity as indicated by cell survival, morphology, or β-galactosidase levels induced by the control plasmid.

SREBP Induced Abca3 Gene Expression *In Vitro*

The SREBP family of transcription factors is known to influence lipid metabolism in various tissues (37) including alveolar type II cells (38, 39). Two potential SRE regulatory sites were located in the 5' flanking region of the *Abca3* gene (40). To assess the effect of SREBP on *Abca3* gene expression, human SREBP1c was co-transfected with a 2.6-*Abca3*-luciferase reporter construct in HeLa cells. SREBP1c significantly increased *Abca3* gene promoter activity (Figure 6A). Consistent with the role of STAT3 in the SCAP/SREBP pathway, SREBP1a and SREBP1c, and SCAP mRNAs were significantly decreased in type II cells isolated from *Stat3^{Δ/Δ}* mice (Table 1).

AKT Induced SREBP1 and ABCA3

Both AKT1 and AKT2 mRNAs were modestly, but significantly, decreased in type II epithelial cells from *Stat3^{Δ/Δ}* mice (Table 1). Transfection with an activated Akt (Myr-HA-Akt) induced *Abca3* gene promoter activity and SREBP1 protein expression (Figures 6B–6D), consistent with their potential role in the regulation of ABCA3 expression. Taken together, we propose that IL-6 regulates ABCA3 expression, at least in part, through

Figure 1. ABCA3 staining was decreased in type II cells of *Stat3* Δ/Δ mice *in vivo*. Lung tissues from *Stat3* Δ/Δ and control mice (6–8 wk old) were immunostained with ABCA3 antibody as described in MATERIALS AND METHODS. (A) ABCA3 was detected in type II epithelial cells in lungs from control mice. (B) ABCA3 staining was markedly decreased in lungs from *Stat3* Δ/Δ mice. Photomicrographs are representative of $n \geq 4$; scale bar = 100 μ m. (C) The number of cells in which ABCA3 was detected, normalized to total cell number in *Stat3* Δ/Δ and control mice (* $P < 0.05$ as assessed by Student's *t* test; $n = 4$ –5).

the STAT3-dependent pathway that influences PI3K, AKT, and SREBP1c regulation of *Abca3* gene transcription in alveolar type II cells.

DISCUSSION

STAT3 is activated by members of the IL-6 family of cytokines and other growth factors to influence numerous cellular pro-

cesses, including survival, proliferation, metabolism, and migration (16–19). STAT3 plays critical roles during organogenesis, repair, and oncogenesis in various tissues. In the lung, IL-6 and other cytokines are rapidly expressed after injury and infection. IL-6 binds Gp130, activating STAT3 phosphorylation that is trafficked to the nucleus, where it regulates gene transcription. In transgenic mouse models, expression of either IL-6 or an activated STAT3 cDNA was cytoprotective during hyperoxic

Figure 2. Deletion of *Stat3* influences lamellar body formation in type II epithelial cells. (A) Sizes of lamellar bodies were consistent in type II cells from control mice. (B–D) Heterogeneity of lamellar body formation in type II cells from *Stat3* Δ/Δ mice is shown. Subsets of type II epithelial cells from *Stat3* Δ/Δ mice lacked lamellar bodies (B) or contained multiple lamellar bodies (C) or exhibited extremely large lamellar bodies (D). Original magnification: B, $\times 15,000$; A, C, and D, $\times 10,000$. Scale bars: 2 μ m.

Figure 3. ABCA3 expression is decreased during hyperoxia. Adult control (open bars) and *Stat3* Δ/Δ (solid bars) mice were killed 69 hours after exposure to 95% O₂. ABCA3 mRNA was assessed by RT-PCR. In control mice, ABCA3 mRNA was decreased to 30%. In *Stat3* Δ/Δ mice, ABCA3 mRNA was decreased to 15% of control activity (*P < 0.05 *Stat3* versus control; †*P* < 0.05 hyperoxia versus room air, as assessed by Two-factor factorial ANOVA with Scheffe's test for *post hoc* analysis; *n* = 3–5).

lung injury (23, 41, 42). Consistent with important roles of STAT3 signaling in the lung, the lack of STAT3 in respiratory epithelial cells enhanced toxicity of hyperoxia and pulmonary adenoviral infection (21, 22). The increased susceptibility of *Stat3* Δ/Δ mice to hyperoxia was associated with decreased surfactant function and decreased lung content of both SatPC and SP-B; treatment with surfactant containing SP-B protected the *Stat3* Δ/Δ mice during hyperoxia (22). Consistent with a role for STAT3 in surfactant homeostasis, the present studies demonstrated that IL-6 enhanced the expression of ABCA3, a lipid transport protein critical for formation of lamellar bodies. Cell-selective deletion of *Stat3* in respiratory epithelial cells resulted in decreased expression of ABCA3 that was mediated, in part,

Figure 4. IL-6 increased ABCA3 expression *in vivo* and *in vitro*. Lungs were inflation-fixed 12 hours after intratracheal administration of saline (A) or 5 μ g of IL-6 (B). Lung tissue was immunostained for ABCA3 at 1:4,000 dilution. At this dilution of antibody, ABCA3 was not detected in control tissue (A). Treatment (intratracheally) with IL-6 increased ABCA3 staining in type II cells (B). Scale bar = 100 μ m. MLE-15 cells (2×10^5 cells) were treated with saline or 20 ng/ml of IL-6 and ABCA3 mRNA assessed by RT-PCR. ABCA3 mRNA was significantly increased by IL-6 (C) (*P < 0.05 as assessed by Student's *t* test; *n* = 3).

Figure 5. PI3K or Akt inhibitors inhibited *Abca3* gene promoter activity. MLE-15 cells were transfected with 2.6-*Abca3*-luciferase reporter and treated with (A) LY294002 or (B) AKT inhibitor 24 hours after transfection. *Abca3* promoter activity was assessed 24 hours after treatment with the inhibitors. Luciferase activity, after normalization to β -galactosidase activity and to the control, was significantly inhibited by the PI3K or Akt inhibitors in a dose-dependent manner (*P < 0.05 as assessed by Student's *t* test; *n* = 3–5).

by the effects of STAT3 on SCAP/SREBP, the latter directly activating transcription of the *Abca3* gene. The present studies demonstrate that the IL-6/Gp130/Stat3 signaling pathway regulates ABCA3 via a mechanism that includes the activities of PI3K, AKT, and SREBP. These studies provide a potential mechanism by which surfactant homeostasis can be maintained during lung injury.

While IL-6/STAT3 enhanced ABCA3 expression *in vivo* and *in vitro*, we were unable to demonstrate a direct effect of activated STAT3 on the *Abca3* gene promoter in MLE-15 cells *in vitro*. SREBP1c, also decreased in *Stat3* Δ/Δ mice, was found to directly activate the *Abca3* promoter *in vitro*. Consistent with our previous microarray prediction, AKT is the potential direct transcriptional target of STAT3, mediating a number of STAT3 effects in alveolar type II cells, including phospholipid biosynthesis and surfactant homeostasis (43). Inhibition of AKT or PI3K also regulated ABCA3, presumably, in part, via the activation of SREBP. Taken together, these data support a pathway in which STAT3 regulates ABCA3 primarily through AKT-mediated activation of SREBP1c (Figure 7). The finding that STAT3 did not directly activate ABCA3 transcription suggests that it is not a direct transcriptional target of STAT3. It remains possible, however, that the lack of a direct effect of activated STAT3 on ABCA3 transcription is an experimental finding that is dependent upon cell type or the lack of appropriate STAT3 regulatory elements in the *Abca3* gene promoter construct used in the assay.

Hyperoxia is a cause of acute lung injury in both clinical and experimental conditions, and is associated with the disruption of

Figure 6. Myr-Akt activates SREBP1c and ABCA3 expression. (A) The effect of SREBP1c on the *Abca3* promoter was assessed after co-transfection of 2.6-*Abca3*-luciferase reporter with either pcDNA3 or pCMV-SREBP1c in HeLa cells. Activities are shown as fold activation compared with controls. SREBP1c markedly increased *Abca3* gene promoter activity (* $P < 0.05$ as assessed by Student's *t* test; $n = 3$). (B) The 2.6-*Abca3*-luciferase reporter construct was co-transfected with pcDNA3 or pcDNA3-Myr-HA-Akt in MLE-15 cells. Luciferase activity was assessed 48 hours after transfection and normalized to controls. Activated Akt significantly induced *Abca3* gene promoter activity (* $P < 0.05$ as assessed by Student's *t* test; $n = 3-5$). (C) MLE-15 cells were harvested 48 h after transfection of the pcDNA3 or pcDNA3-Myr-HA-Akt. SREBP1c, phospho-AKT, and AKT were assessed by Western blot. SREBP1 protein was increased by transfection with activated AKT. (D) Quantitative representation of SREBP1 protein levels, obtained by image analysis. SREBP1 was normalized to Actin. Results were expressed in arbitrary units. SREBP1 was normalized to Actin (* $P < 0.05$ as assessed by Student's *t* test; $n = 3$).

surfactant homeostasis (44-47). Hyperoxia induces the expression of inflammatory cytokines including IL-6, causes diffuse alveolar capillary injury, inhibiting the function and synthesis of components of the pulmonary surfactant system. The present studies are consistent with a role for STAT3 in the regulation of

various aspects of surfactant homeostasis, and are consistent with previous findings that IL-6/Stat3 influences surfactant protein B and lipid content *in vivo* (22, 23).

While SP-B expression and surfactant function are normal in *Stat3*^{Δ/Δ} mice at baseline (22), the increased susceptibility of these mice to hyperoxia is in part related to the ability of the mice to maintain surfactant activity during the injury and repair. In the present study, expression of ABCA3 was significantly decreased in *Stat3*^{Δ/Δ} mice at baseline, being reduced approximately 50%. In spite of the reduction of ABCA3, lung histology and function was normal in adult *Stat3*^{Δ/Δ} mice. These findings are consistent with the lack of pulmonary abnormalities in heterozygous *Abca3*^{+/-} mice, as well as in humans bearing single and inactivating mutations in the *ABCA3* gene (12, 13). In the present studies, ABCA3 expression was decreased during hyperoxia both in *Stat3*^{flx/flx} and was further reduced in *Stat3*^{Δ/Δ} mice. Thus the reduction of ABCA3 in *Stat3*^{Δ/Δ} mice may contribute to their susceptibility to hyperoxia.

Deletion of ABCA3 leads to disruption of normal lamellar body formation (11-13). Small, lamellar-like bodies with tightly packed lamellae and electron dense cores were observed in patients with *ABCA3* mutations (10). Abnormalities in lamellar body formation observed in the *Stat3*^{Δ/Δ} mice varied greatly. Some type II epithelial cells lacked lamellar bodies, while others had increased numbers and sizes of lamellar body-like organelles. The heterogeneity of lamellar body formation in the *Stat3*^{Δ/Δ} mice may be related to variability in STAT3 targeting, variability in the levels of ABCA3, as well as to differences in the compensation by type II epithelial cells of various *Stat3* genotypes. The finding that lung SatPC content was decreased in *Stat3*^{Δ/Δ} mice (22) suggests that SatPC levels influence lamellar body numbers and structure.

SCAP/SREBP activity regulates lipid synthesis in many tissues (48-50). Since STAT3 is known to regulate lipid homeostasis in the lung, it is likely that the loss of STAT3 influences the expression of many genes involved in surfactant lipid homeostasis in type II cells, whether directly or indirectly, via SREBP. SCAP, also decreased in the *Stat3*^{Δ/Δ} mice, activates SREBP through proteolytic cleavage, directing nuclear trans-

Figure 7. A model of the role of STAT3 in the regulation of *Abca3*. A model is proposed by which STAT3 regulates ABCA3 expression. Lung injury and inflammation induce IL-6, which binds and activates gp130/JAK signaling to enhance STAT3 phosphorylation. Phospho-Stat3 activates PI3K, inducing AKT, which in turn, enhances SREBP1c to activate *Abca3* gene expression. ABCA3 is required for normal lamellar body synthesis and surfactant homeostasis.

location and activation of transcriptional targets involved in lipid synthesis (51).

The present study identifies a mechanism by which the stimulation of innate host defense systems during lung injury, via IL-6/Gp130/Stat3, regulates ABCA3, thereby enhancing surfactant homeostasis that, in turn, may play an important role in maintaining lung function during infection and injury.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Ann Maher for secretarial assistance. They also thank K. Takeda for the gift of *Stat3^{flx/flx}* mice.

References

- Klaus MH, Clements JA, Havel RJ. Composition of surface-active material isolated from beef lung. *Proc Natl Acad Sci USA* 1961;47:1858–1859.
- Rossdale PD, Pattle RE, Mahaffey LW. Respiratory distress in a newborn foal with failure to form lung lining film. *Nature* 1967;215:1498–1499.
- Farrell PM, Avery ME. Hyaline membrane disease. *Am Rev Respir Dis* 1975;111:657–688.
- Harwood JL. Lung surfactant. *Prog Lipid Res* 1987;26:211–256.
- Whitsett JA. Genetic disorders of surfactant homeostasis. *Paediatr Respir Rev* 2006;7:S240–S242.
- Takahashi K, Kimura Y, Nagata K, Yamamoto A, Matsuo M, Ueda K. ABC proteins: key molecules for lipid homeostasis. *Med Mol Morphol* 2005;38:2–12.
- Yamano G, Funahashi H, Kawanami O, Zhao LX, Ban N, Uchida Y, Morohoshi T, Ogawa J, Shioda S, Inagaki N. Abca3 is a lamellar body membrane protein in human lung alveolar type II cells. *FEBS Lett* 2001;508:221–225.
- Mulgeta S, Gray JM, Notarfrancesco KL, Gonzales LW, Koval M, Feinstein SI, Ballard PL, Fisher AB, Shuman H. Identification of LBM180, a lamellar body limiting membrane protein of alveolar type II cells, as the ABC transporter protein ABCA3. *J Biol Chem* 2002;277:22147–22155.
- Bullard JE, Wert SE, Noguee LM. ABCA3 deficiency: neonatal respiratory failure and interstitial lung disease. *Semin Perinatol* 2006;30:327–334.
- Shulenin S, Noguee LM, Annilo T, Wert SE, Whitsett JA, Dean M. ABCA3 gene mutations in newborns with fatal surfactant deficiency. *N Engl J Med* 2004;350:1296–1303.
- Ban N, Matsumura Y, Sakai H, Takanezawa Y, Sasaki M, Arai H, Inagaki N. ABCA3 as a lipid transporter in pulmonary surfactant biogenesis. *J Biol Chem* 2007;282:9628–9634.
- Fitzgerald ML, Xavier R, Haley KJ, Welti R, Goss JL, Brown CE, Zhuang DZ, Bell SA, Lu N, McKee M, *et al.* ABCA3 inactivation in mice causes respiratory failure, loss of pulmonary surfactant, and depletion of lung phosphatidylglycerol. *J Lipid Res* 2007;48:621–632.
- Cheong N, Zhang H, Madesh M, Zhao M, Yu K, Dodia C, Fisher AB, Savani RC, Shuman H. ABCA3 is critical for lamellar body biogenesis in vivo. *J Biol Chem* 2007;282:23811–23817.
- Stahlman MT, Besnard V, Wert SE, Weaver TE, Dingle S, Xu Y, von Zychlin K, Olson SJ, Whitsett JA. Expression of ABCA3 in developing lung and other tissues. *J Histochem Cytochem* 2007;55:71–83.
- Yoshida I, Ban N, Inagaki N. Expression of ABCA3, a causative gene for fatal surfactant deficiency, is up-regulated by glucocorticoids in lung alveolar type II cells. *Biochem Biophys Res Commun* 2004;323:547–555.
- Akira S. IL-6-regulated transcription factors. *Int J Biochem Cell Biol* 1997;29:1401–1418.
- Darnell JE Jr. STATs and gene regulation. *Science* 1997;277:1630–1635.
- Boccaccio C, Ando M, Tamagnone L, Bardelli A, Micheli P, Battistini C, Comoglio PM. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. *Nature* 1998;391:285–288.
- Bromberg J, Darnell JE Jr. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000;19:2468–2473.
- Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA* 1997;94:3801–3804.
- Matsuzaki Y, Xu Y, Ikegami M, Besnard V, Park KS, Hull WM, Wert SE, Whitsett JA. Stat3 is required for cytoprotection of the respiratory epithelium during adenoviral infection. *J Immunol* 2006;177:527–537.
- Hokuto I, Ikegami M, Yoshida M, Takeda K, Akira S, Perl AK, Hull WM, Wert SE, Whitsett JA. Stat-3 is required for pulmonary homeostasis during hyperoxia. *J Clin Invest* 2004;113:28–37.
- Yan C, Naltner A, Martin M, Naltner M, Fangman JM, Gurel O. Transcriptional stimulation of the surfactant protein B gene by STAT3 in respiratory epithelial cells. *J Biol Chem* 2002;277:10967–10972.
- Takeda K, Kaisho T, Yoshida N, Takeda J, Kishimoto T, Akira S. Stat3 activation is responsible for IL-6-dependent T cell proliferation through preventing apoptosis: Generation and characterization of T cell-specific Stat3-deficient mice. *J Immunol* 1998;161:4652–4660.
- Na CL, Beck DC, Breslin JS, Wert SE, Weaver TE. An en bloc staining protocol improves the preservation of lamellar bodies in alveolar type II epithelial cells for transgenic mice expressing modified pulmonary surfactant B. *Microsc Microanal* 1998;4:852–853.
- Ikegami M, Na CL, Korfhagen TR, Whitsett JA. Surfactant protein D influences surfactant ultrastructure and uptake by alveolar type II cells. *Am J Physiol* 2005;288:L552–L561.
- Rice WR, Konkright JJ, Na CL, Ikegami M, Shannon JM, Weaver TE. Maintenance of the mouse type II cell phenotype in vitro. *Am J Physiol* 2002;283:L256–L264.
- Epaud R, Ikegami M, Whitsett JA, Jobe AH, Weaver TE, Akinbi HT. Surfactant protein B inhibits endotoxin-induced lung inflammation. *Am J Respir Cell Mol Biol* 2003;28:373–378.
- Wikenheiser KA, Vorbroke DK, Rice WR, Clark JC, Bachurski CJ, Oie HK, Whitsett JA. Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc Natl Acad Sci USA* 1993;90:11029–11033.
- Dave V, Childs T, Xu Y, Ikegami M, Besnard V, Maeda Y, Wert SE, Neilson JR, Crabtree GR, Whitsett JA. Calcineurin/Nfat signaling is required for perinatal lung maturation and function. *J Clin Invest* 2006;116:2597–2609.
- Ramaswamy S, Nakamura N, Vazquez F, Batt DB, Perera S, Roberts TM, Sellers WR. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci USA* 1999;96:2110–2115.
- Martis PC, Whitsett JA, Xu Y, Ikegami M. Stat-3 is required for surfactant homeostasis following LPS induced lung injury. *Proc Am Thorac Soc* 2005;2:A87.
- Abell K, Watson CJ. The Jak/Stat pathway: a novel way to regulate PI3K activity. *Cell Cycle* 2005;4:897–900.
- Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Acts. *Genes Dev* 1999;13:2905–2927.
- Du X, Kristiana I, Wong J, Brown AJ. Involvement of Akt in ER-to-Golgi transport of SCAP/SREBP: a link between a key cell proliferative pathway and membrane synthesis. *Mol Biol Cell* 2006;17:2735–2745.
- Portmann T, Griffiths B, Chung YL, Delpuech O, Griffiths JR, Downward J, Schulze A. PKB/Akt induces transcription of enzymes involved in cholesterol and fatty acid biosynthesis via activation of SREBP. *Oncogene* 2005;24:6465–6481.
- Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125–1131.
- Mason RJ, Gao B, Pan T, Jiang X, Eckart M, Neben S. Role of keratinocyte growth factor in regulating lipogenesis in alveolar type II cells: a gene-profiling approach. *Chest* 2002;121:77S.
- Mason RJ, Pan T, Edeen KE, Nielsen LD, Zhang F, Longphre M, Eckart MR, Neben S. Keratinocyte growth factor and the transcription factors C/EBP alpha, C/EBP delta, and SREBP-1c regulate fatty acid synthesis in alveolar type II cells. *J Clin Invest* 2003;112:244–255.
- Besnard VN, Dingle S, Clark JC, Whitsett JA. Regulation of ABCA3 by TTF-1 and SREBPs in the alveolar epithelium. *Am J Respir Crit Care Med* 2007;175:A512.
- Ward NS, Waxman AB, Einarsson O, Elias JA. Interleukin-6 protection in hyperoxic lung injury. *Chest* 1999;116:26S.
- Ward NS, Waxman AB, Homer RJ, Mantell LL, Einarsson O, Du Y, Elias JA. Interleukin-6-induced protection in hyperoxic acute lung injury. *Am J Respir Cell Mol Biol* 2000;22:535–542.
- Xu Y, Ikegami M, Wang Y, Matsuzaki Y, Whitsett JA. Gene expression and biological processes influenced by deletion of *Stat3* in pulmonary type II epithelial cells. *BMC Genomics* (in press).

44. Smith LJ. Hyperoxic lung injury: biochemical, cellular, and morphologic characterization in the mouse. *J Lab Clin Med* 1985;106:269–278.
45. Horowitz S, Shapiro DL, Finkelstein JN, Notter RH, Johnston CJ, Quible DJ. Changes in gene expression in hyperoxia-induced neonatal lung injury. *Am J Physiol* 1990;258:L107–L111.
46. Nogee LM, Wispé JR, Clark JC, Weaver TE, Whitsett JA. Increased expression of pulmonary surfactant proteins in oxygen-exposed rats. *Am J Respir Cell Mol Biol* 1991;4:102–107.
47. Tokieda K, Iwamoto HS, Bachurski C, Wert SE, Hull WM, Ikeda K, Whitsett JA. Surfactant protein-B-deficient mice are susceptible to hyperoxic lung injury. *Am J Respir Cell Mol Biol* 1999;21:463–472.
48. Wang X, Sato R, Brown MS, Hua X, Goldstein JL. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell* 1994;77:53–62.
49. Hua X, Nohturfft A, Goldstein JL, Brown MS. Sterol resistance in CHO cells traced to point mutation in SREBP cleavage-activating protein. *Cell* 1996;87:415–426.
50. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 1997;89:331–340.
51. Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. *Proc Natl Acad Sci USA* 1999;96:11041–11048.