Surfactant-Associated Protein B Is Critical to Survival in Nickel-Induced Injury in Mice

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The etiology of acute lung injury is complex and associated with numerous, chemically diverse precipitating factors. During acute lung injury in mice, one key event is epithelial cell injury that leads to reduced surfactant biosynthesis. We have previously reported that transgenic mice that express transforming growth factor α (TGFA) in the lung were protected during nickel-induced lung injury. Here, we find that the mechanism by which TGFA imparts protection includes maintenance of surfactant-associated protein B (SFTPB) transcript levels and epidermal growth factor receptor–dependent signaling in distal pulmonary epithelial cells. This protection is complex and not accompanied by a diminution in inflammatory mediator transcripts or additional stimulation of antioxidant transcripts. In mouse lung epithelial (MLE-15) cells, microarray analysis demonstrated that nickel increased transcripts of genes enriched in MTF1, E2F-1, and AP-2 transcription factor–binding sites and decreased transcripts of genes enriched in AP-1-binding sites. Nickel also increased Jun transcript and DNA-binding activity, but decreased SFTPB transcript. Expression of SFTPB under the control of a doxycycline-sensitive promoter increased survival during nickel-induced injury as compared with control mice. Together, these findings support the idea that maintenance of SFTPB expression is critical to survival during acute lung injury.

Keywords: adult respiratory distress syndrome; innate immunity; chemokine; surfactant

Acute lung injury is clinically manifested by a low arterial oxygen, protein-rich edema, capillary injury, and diffuse alveolar epithelial cell damage (1). The incidence of acute lung injury in the United States has been estimated at 194,000 cases/year, and although many clinical trials have been attempted, survival remains low and unpredictable (74,000 deaths/yr) (2). The etiology of acute lung injury is complex and can be induced by numerous causes including sepsis, trauma, pneumonia, and inhaled irritants (3, 4). Several laboratory animal models have been developed to investigate the clinical features of lung injury *in vivo*. To begin to identify the genetic determinants and

CLINICAL RELEVANCE

Surfactant-associated protein B biosynthesis was found to be critical to survival during lung injury in mice. Future clinical strategies could be developed to maintain differentiated function of type II alveolar cells.

understand the molecular basis of gene–environment interactions that control survival during irritant-induced lung injury, mice were exposed to nickel, which produced lethal lung injury, marked by alveolar epithelial damage, edema, perivascular distention, hemorrhage, and neutrophil infiltration (5, 6).

To map the genetic loci controlling survival, we previously performed a quantitative trait locus (QTL) analysis and found that survival during lung injury is a complex trait and dependent on interactions among multiple chromosomal regions (6, 7). Candidate genes that map to a significant QTL interval on chromosome 6 included transforming growth factor α (*Tgfa*), which previously had been noted to increase in the bronchoalveolar lavage (BAL) fluid of patients with acute lung injury (8). While the precise physiologic role of TGFA in lung injury is not completely understood, overexpression of human TGFA in the distal lung under the control of a human surfactant-associated protein C (*SFTPC*) promoter protected transgenic mice against nickel-induced lung injury, markedly increasing survival (9). However, the molecular determinants of TGFA-mediated protection have not been clearly defined. The purpose of this study was to determine whether TGFA contributes to survival during nickel-induced injury by maintaining the expression of critical genes, including surfactant-associated proteins. To examine the mechanism of TGFA protection, we assessed transcripts altered during nickel-induced lung injury, how nickel alters SFTPB expression *in vitro*, and whether maintenance of SFTPB could increase survival.

MATERIALS AND METHODS

Experimental Design

All studies performed were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. Previously we determined that transgenic mice with a constitutive human *TGFA* transgene directed to the respiratory epithelium under the control of human *SFTPC* promoter were protected from nickel-induced lung injury (9). Our *in vivo* and *in vitro* studies here were aimed at understanding the molecular basis of nickel-induced injury survival. First, we measured survival in TGFA transgenic mice exposed to nickel. To determine whether epidermal growth factor receptor (EGFR) expressed on alveolar epithelial cells plays a role in survival, we exposed transgenic mice constitutively expressing human TGFA and bitransgenic mice constitutively expressing TGFA and dominant-negative EGFR to nickel. Both transgenes were under the control of

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human SFTPC promoter. Second, because these mice display varied survival, we contrasted the lung mRNA profiles of these mice during nickel-induced injury using quantitative real-time polymerase chain reaction (qRT-PCR) assays. Third, mouse lung epithelial (MLE-15) cells (10) were used to investigate alteration in the transcriptional profile and *Sftpb* regulation. Fourth, to determine if maintenance of SFTPB expression can improve survival, we exposed transgenic mice that have *SFTPB* expression directed to lung epithelial cells under the control of a dox-sensitive promoter to nickel.

Transgenic Mice

The generation and characterization of the transgenic mice used in this study have been described previously (11–15). All mice were derived from the FVB/NJ strain and housed in a pathogen-free environment. Transgenic mice expressing human TGFA in the pulmonary epithelium can be protected against nickel-induced lung injury (11).

The mouse lines we used included: (i) constitutive TGFA transgenic mice (Line 28), in which the human *TGFA* transgene is under the control of the human *SFTPC* promoter; (ii) TGFA Line 28 × dn mutEGFR mice, generated by crossing TGFA transgenic mice with mice that expressed dominant-negative mutant EGFR under the control of the human *SFTPC* promoter (13, 14); and (iii) SFTPB conditional mice (Scgbl1a1-rtTA/TetO₇ *SFTPB/Sftpb*^{−/−}), in which the human *SFTPB* gene is expressed under dox control in type II cells in mice lacking the native mouse *Sftpb* gene (15). The Scgbl1a1-rtTA/TetO₇ *SFTPB/Sftpb*^{−/−} mice are offspring of *Sftpb*^{+/−} dams given dox in the drinking water from Day 0 of gestation to stimulate transcription of the *SFTPB* transgene during fetal lung development, and can survive when maintained on dox. These mice were compared with Scgbl1a1-rtTA/TetO₇ *SFTPB/Sftpb*^{+/+} littermates treated with doxycycline.

Mouse Exposure and Nickel Aerosol Generation

Mice (6–9 wk old) were placed in a 0.32 m³ stainless steel inhalation chamber as described previously (6). Nickel aerosol (150 µg Ni/m³, 0.5 µm mass median aerodynamic diameter) was generated from 50 mM NiSO₄ · 6H₂O (Sigma Chemical Co, St. Louis, MO). The nickel concentration in the chamber was determined using the methylglyoxime method (16). Mouse survival time was recorded hourly for the first 72 hours and then once every 3 hours thereafter.

Quantitative Real-Time Polymerase Chain Reaction

Eighteen transcripts selected on the basis of previous microarray analysis of mice during nickel-induced lung injury were measured by quantitative (q)RT-PCR (17). After 72 hours, exposed mice (*n* = 6 mice/group) were anesthetized with sodium pentobarbital (150–200 mg/kg, intraperitoneally; Henry Schein, Indianapolis, IN), followed by exsanguination via severing of the posterior abdominal aorta. All the lung samples were harvested at a terminal anesthesia point and snap-frozen in liquid nitrogen. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and quantity was assessed by absorbance (SmartSpec 3000; Bio-Rad, Hercules, CA). Lung RNA quality was assessed by separation with a denaturing formaldehyde/agarose/ethidium bromide gel and an Agilent Bioanalyzer (Quantum Analytics, Inc., Foster City, CA). RNA (100 ng) was reverse transcribed (High Capacity cDNA Archive Kit; Applied Biosystems, Inc., Foster City, CA) and cDNA was PCR amplified with primers and TaqMan Universal PCR Master Mix (Applied Biosystems). Arranged into functional groups, primers (Applied Biosystems Catalog No. presented in parentheses) were selected on the basis of previous microarray experiments examining mouse lung transcript levels during nickel-induced lung injury and included the following. Innate Immunity Group: CD14 antigen (Cd14, Mm00438094_g1), chemokine (C-X-C motif) ligand 2 (Cxcl2, a.k.a. Mip2, Mm 00436450_m1), interleukin 6 (Il6, Mm00446191_m1), lipocalin 2 (Lcn2, Mm00809552_s1); Antioxidant Group: glutamate-cysteine ligase, catalytic subunit (Gclc, Mm0802655_m1), heme oxygenase (decycling) 1 (Hmox1, Mm00516004_m1), metallothionein 2 (Mt2, Mm00809556_1), thioredoxin reductase 1 (Txnrd1, Mm0043675_m1); Surfactant Lipid Transport and Biosynthesis Group: ATP-binding cassette, sub-family A (ABC1), member 3 (Abca3, Mm00550501_m1), fatty acid binding protein 5, epidermal (Fabp5, Mm00783731_s1), fatty acid synthase (Fasn, Mm00662319_m1), phospholipid transfer protein (Pltp, Mm00448202_m1),

solute carrier family 34 (sodium phosphate), member 2 (Slc34a2, Mm00448749_m1); and the Surfactant Protein Biosynthesis Group: surfactant-associated protein A1 (Sftpa1, Mm00499170_m1), surfactant-associated protein B (Sftpb, Mm 00455681_m1), surfactant-associated protein C (Sftpc, Mm00488144_m1), surfactant-associated protein D (Sftpd, Mm00486060_m1), and napsin A aspartic peptidase (Napsa, Mm00492829_m1). Analysis was performed with an Applied Biosystems 7900HT System (95°C, 10 min; 40 cycles 95°C, 15 s; 60°C, 1 min). The expression of each transcript relative to ribosomal protein L32 (Rpl32, Mm02538467_s1) transcript level was determined using the 2^{−ΔΔCT} method (18) and normalized to the corresponding inbred strain- or transgenic line-matched mice exposed to filtered air. The expression of each transcript is expressed as fold change relative to filtered air exposed mice (untreated control).

Mouse Lung Epithelial Cell Culture

Mouse lung epithelial (MLE)-15 cells (passages 22–28) were grown in RPMI 1640 medium (Cat. No. 31800–022, GIBCO, Grand Island, NY) containing HITES (10 nM hydrocortisone Cat. No. H-0888, 5 µg/ml insulin Cat. No. I-1884, 10 µg/ml transferrin, bovine Cat. No. T-1248, 10 nM β-estradiol Cat. No. E-2758, and 30 nM sodium selenite Cat. No. 26970-82-1; Sigma), L-Glutamine (Cat. No. G-7513, Sigma), 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES Cat. No. H-0887; Sigma), and 4% fetal bovine serum (Gibco, Grand Island, NY) (10). All cells were maintained at 37°C in a 5% CO₂/95% air humidified incubator. For RNA or nuclear protein extract preparations after NiSO₄ treatments, MLE-15 cells were grown in 6-well plates or 100-mm dishes, and experiments were performed using cells at approximately 80% confluence.

Cytotoxicity Assay

Cell viability was assessed using MLE-15 cells. Cells were grown in 75 cm² culture flask in 5% CO₂, 37°C, placed in RPMI 1640 plus 1% serum for 24 hours, and then exposed to 100 to 600 µM NiSO₄. After 24 hours, the supernatant was collected and lactate dehydrogenase (LDH) content measured (Ct. No. G1780; Promega, Madison, WI). The cell layer was lysed (4°C, 24 h) and analyzed for LDH content. The results are expressed as percent LDH released.

RNA Expression Analysis by Oligonucleotide Microarray

For oligonucleotide microarray studies, MLE-15 cells were treated with 0 (untreated control) or 600 µM NiSO₄ (24 h, 37°C). Cells were then washed with 1 ml ice-cold 1× phosphate-buffered saline (PBS). Total RNA was isolated from cells with 800 µl TRIzol reagent/well (Invitrogen) and quantified using an Agilent Bioanalyzer (Quantum Analytics, Inc.). RNA quality was assessed by separation with a denaturing formaldehyde/agarose/ethidium bromide gel. To examine differential gene expression of 31,775 70-mer oligonucleotides, a microarray was fabricated by the Genomic and Microarray Laboratory, Center for Environmental Genetics, University of Cincinnati (<http://microarray.uc.edu/>) using a commercial library (v. 3.0.1; Qiagen, Alameda, CA) as described previously (17). RNA samples from untreated control and NiSO₄-treated MLE-15 cells were compared using 20 µg total RNA/array. Each sample of mRNA was reverse transcribed and randomly reciprocal tagged with fluorescent Cyanine 3 (Cy3) or Cyanine 5 (Cy5) (e.g., Cy3 for untreated control and Cy5 for NiSO₄-treated). Cy3 and Cy5 samples were co-hybridized with the printed 70-mers. After hybridization, slides were washed and scanned at 635 (Cy5) and 532 (Cy3) nm (GenePix 4000B; Axon Instruments, Inc., Union City, CA).

Transcription Factor Activity Assay

We determined the binding activities of 50 transcription factors in MLE-15 cells treated with saline (vehicle) or 600 µM NiSO₄ for up to 24 hours (xMAP Luminex; Marligen Biosciences, Ijamsville, MD). The assay is based on a specific binding of transcription factors to labeled cognate nucleotide probes. Nuclear extracts from vehicle and NiSO₄-treated MLE-15 cells (10 µg) were incubated with a mixture of PE-conjugate oligonucleotides containing appropriate cognate DNA binding sequences. This mixture was then incubated with a digestion reagent. In the presence of active transcription factors, label remains

associated with the probes, whereas it is removed in the absence of transcription factor binding. Finally, the oligonucleotides were captured onto distinctly colored agarose microspheres that allow each reaction to be quantified using a Bio-Plex system (Bio-Rad, Hercules, CA) reader. The amount of label remaining correlates with the amount of active transcription factor derived from the nuclear extract. The following 50 binding motifs were tested: AML1, AP2, AP4, AR, CEBPa, CEBPd, CEBPg, C-MYB, CRE-ATF1, CREB, E2F1-5, E2F6, EGR, ER, ETS, GATA, HIF3, HIC1, HIF, HNF1, HNF3, HNF4, HSF1, ISRE, LEF1, MEF2, MTF1, Myc-Max, NF1, NFAT, NF- κ B, NFY1, OCTAMER, P53, PAX6, PBX, PLZF, PPAR, SAF1, SMAD 1-5, SMAD 2-3, SOX9, SP1, SREBP, STAT, GRR, TAL1, TGIF, TRE-AP1, and YY1. Data were analyzed using least median squares regression analysis to distinguish differences between untreated control and NiSO₄-treated samples.

Promoter Analysis for Transcription Factor-Binding Site Enrichment

To identify putative acute lung injury-related transcriptional factors critical to nickel-induced injury susceptibility, we acquired promoter sequences (−2,000 bp upstream from the tentative transcription start site) of genes whose mRNA levels were increased or decreased after NiSO₄ treatment and searched for over-represented transcription factor-binding sites in these sequences using MatInspector (Genomatix, Munich, Germany) as described previously (19).

S1 Nuclease and RNase Protection Assays

For studies on dose-response of SFTPB mRNA levels, MLE-15 cells were treated with 0 (untreated control) or 10 to 1,000 μ M NiSO₄ for 24 hours. Time course experiments on SFTPB mRNA levels were conducted by treating MLE-15 cells with 300 μ M NiSO₄ for 0 (untreated control), 4, 8, 12, 18, and 24 hours. After nickel treatment, cells were washed with 1 ml ice-cold 1 $\times$ PBS, and total RNA was isolated from cells with TRIzol as described above. To examine mRNA levels of activator protein 1 (AP-1) family members, transcript levels for Jun oncogene (Jun, a.k.a. c-Jun), FBJ osteosarcoma oncogene (Fos, a.k.a. c-Fos), Jun proto-oncogene-related gene d (Jund, a.k.a. JunD), and Jun-B oncogene (Junb, a.k.a. JunB) were determined in cells treated with 0 (untreated control), 100, 300, or 600 μ M NiSO₄ for 24 hours.

After treatment of MLE-15 cells with nickel, SFTPB mRNA levels were measured by S1 nuclease protection assays as described previously (7, 20). RNase protection assays were performed to assess mRNA levels of AP-1 family members. Riboprobes specific for mouse Jun, Fos, Jund, and Junb, and Rpl32 were radiolabeled with [α -³²P] uridine 5'-triphosphate (UTP, 3000Ci/mM), combined, and hybridized (16 h, 56°C) with 10 μ g of total RNA from each sample. RNase digestion was performed with RNase A + T1 mix (PharMingen, San Diego, CA) at 33°C for 1 hour. For both S1 nuclease and RNase protection assays, the protected fragments were electrophoresed through 6% acrylamide gels containing 8 M urea. The gels were dried and band intensities were analyzed with a Typhoon 8600 imager and ImageQuant software (Amersham Biosciences, Piscataway, NJ). All samples were normalized to their respective Rpl32 band intensities.

Electrophoretic Mobility Shift and Supershift Assays

Nuclear protein extracts were prepared (NE-PER 78833; Pierce Biotechnology, Inc., Rockford, IL) from control and NiSO₄-treated MLE-15 cells (600 μ M, 24 h) in the presence of ethylenediaminetetraacetic acid (EDTA)-free protease inhibitors (Roche Applied Science, Indianapolis, IN) and aliquots were stored at −80°C until processed. Total protein concentration of nuclear extracts was measured using a bicinchoninic acid (BCA) assay with bovine serum albumin as a standard (Pierce Biotechnology).

A biotinylated probe containing a binding site for AP-1 Jun homodimer and Jun/Fos heterodimeric complexes (5'-CGCTTGATGACTCA GCGGAA-3' annealed with 3'-GCGAACTACTGAGTCGGC CTT-5') was used for EMSA. Nuclear extracts (1.5 μ g) were incubated in EMSA binding buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 25 mM KCl, 50 mM MgCl₂, 1 mM EDTA, 4 mM dithiothreitol, 0.2 mM phenylmethanesulphonyl fluoride, 33 ng/ μ l poly-[dI-dC], and 0.05% IGEPAL CA-630 with 2.5 ng of biotinylated probe [30 min, 15°C]). For

competition assays, a 100-fold excess of unbiotinylated AP-1 oligonucleotides was added to reaction mixtures before probe addition. To detect Jun protein in the shifted complexes, control mouse IgG or anti-Jun antibodies (sc-822; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the mixture. The samples were pre-incubated (30 min, 23°C) before probe addition. The incubation mixture was fractionated on a 6% nondenaturing polyacrylamide gel in 0.5 $\times$ TBE (0.045 M Tris-borate, 0.001 M EDTA, pH 8.3) and transferred to Hybond-N⁺ membrane (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The blot was subjected to streptavidin horseradish peroxidase conjugate-based detection method (Panomics, Inc., Fremont, CA) and the chemiluminescence signal was detected by exposing to film.

Promoter Reporter Plasmid Constructs and Transient Transfection Assays

To determine the *Sftpb* promoter region responsive to Jun inhibition, luciferase reporter constructs were generated. The −653/+35 *Sftpb* promoter region was used as a template and proximal −100/+35 and distal −397/−272 portions were PCR-amplified using oligonucleotide primers containing 5' Bgl II or 3' Hind III sites. The resulting amplified PCR products were cloned in pGL4.10 (Promega). The identity of the inserts was verified by sequencing. To perform promoter reporter transient transfection assays, MLE-15 cells were seeded in 24-well plates (24–48 h, 37°C) and transfected using Eugene 6 (Roche Applied Science). The DNA transfection mix contained 180 ng pGL3 −653/+35 reporter, 0 to 45 ng of Jun expression construct (pJun), and 20 ng pCMV- β -gal. The vector pcDNA3 was used to adjust the total amount of DNA to 245 ng per well. To assay the proximal and distal portions, the DNA transfection mix contained 300 ng *Sftpb* promoter fragment driven reporter plasmid (pGL4 −100/+35 or pGL4 −397/−272), and 90 ng pCMV-XL4 control vector or 90 ng pCMV-Jun (pJun) (Origene Technologies, Inc., Rockville, MD). Transfection efficiency was corrected by co-transfection of 40 ng pCMV- β -Gal. The day after transfection, cells were PBS washed twice and incubated in serum-free RPMI (24 h, 37°C). To determine promoter reporter activity, cells were lysed using GloLysis buffer, assayed in 96-well plates using the Bright-Glo luciferase or Beta-Glo systems (Promega), and luminescence was measured (Fusion α plate reader; Packard Bioscience/Perkin Elmer Life and Analytical Science, Waltham, MA).

Statistical Analysis

Survival curves obtained for transgenic TGFA Line 28, Line 28 $\times$ dn mutEGFR, transgenic Scgbl1a1-rtTA/TetO₇ *SFTPB/Sftpb*^{−/−} mice, and corresponding control mice were compared using Kaplan-Meier log-rank survival analysis. For microarray analysis, three biological replicates were used. Data were normalized and *P* values for identifying differentially expressed genes were calculated as described previously (21). Resulting *t*-statistics were modified using an intensity-based empirical Bayes method (22), and *P* values were adjusted using the false discovery rate (FDR) method (23). Using MatInspector (Genomatix, Munich, Germany), promoter sequences (−2,000 bp upstream from the tentative transcription start site) of genes whose mRNA levels were increased or decreased after NiSO₄ treatment were searched for overrepresented transcription factor-binding sites as described previously (19). Using the complete Vertebrate Matrix Library 6.2, the *P* value was calculated with binomial distribution probability by comparing the matrix match of the promoter regions of differentially expressed genes with the promoters from random mouse genes sets (−2 kb for all the promoters). The single-step Bonferroni adjustment was used to control for the multiple comparisons effect (i.e., multiplication of *P* value by the number of transcription factor binding sites in Genomatix Vertebrate Matrix Library). Lung transcript levels were expressed relative to Rpl32 transcript level using the 2^{− $\Delta\Delta$ CT} method (18), and significance differences from untreated strain-matched control were determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure. SFTPB mRNA levels in MLE-15 cells exposed to nickel were compared with vehicle control, and significant differences were assessed by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure. Transcription factor DNA binding in nuclear extracts of MLE-15 cells was assessed using least median squares (LMS) regression analysis and presented as percent different from control.

Significant difference in transcript levels of Jun, Fos, Jund, and Junb (each normalized to Rpl32) or *Sftpb* promoter reporter activity (assessed by luciferase) in MLE-15 cells were determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

RESULTS

EGFR Signaling in Bronchiolar and Type II Epithelium Contributes to TGFA-Dependent Survival during Nickel-Induced Injury

Previously, we generated transgenic mice expressing a mutant EGFR lacking a portion of the intracytoplasmic domain (dn mutEGFR) (13). Because the mutant EGFR is under control of the human *SFTPC* promoter, these mice express the transgene selectively in distal bronchiolar and type II epithelial cells. Although mice lacking EGFR are not viable, selective expression of mutant EGFR in the presence of normal EGFR has a dominant-negative effect, and the mice are viable and without abnormalities in lung morphology. When these mice were crossed with Line 28 transgenic mice, lung morphology was normal in the offspring, although TGFA mRNA levels remained elevated.

The only known TGFA receptor is EGFR. To determine whether EGFR signaling contributes to survival during nickel-induced lung injury, Line 28 mice, Line 28 mice crossed with the dn mutEGFR mice, and the strain-matched FVB/NJ mice were exposed to nickel and survival monitored for 14 days. Survival increased in TGFA mice compared with the strain-matched FVB/NJ control mice (83% versus 0%, respectively; Figure 1). The FVB/NJ exhibited a median survival time of 69 hours. The TGFA Line 28 $\times$ dn mutEGFR had an intermediate response of 17% survival, with median survival time of 120 hours. These findings indicate that EGFR signaling in the distal lung epithelium contributes to survival during nickel-induced lung injury.

Surfactant-Associated Proteins Transcripts Are Maintained in TGFA Transgenic Mice

The growth factor TGFA is pleiotropic and expected to have multiple downstream targets that lead to protection during acute lung injury. Previous experiments using microarray analysis have identified several transcriptional changes in critical pathways that are altered during nickel-induced lung injury (17, 24). To further understand the transcriptional signature of nickel-induced injury altered during TGFA-induced protection, we analyzed the transcript levels of 18 genes representing four functional groups important in stress response and lung injury. We used qRT-PCR analysis of lung RNA samples of transgenic mice with varied survival response. The functional categories analyzed included innate immunity (Figure 2A), antioxidant defense (Figure 2B), surfactant lipid biosynthesis (Figure 2C), and surfactant-associated protein biosynthesis (Figure 2D). We reasoned that the transcripts most critical in nickel-induced injury would exhibit contrasting expression patterns in protected versus sensitive mice.

The transcript levels analyzed for the innate immunity and antioxidant categories increased similarly in all groups of mice (Figures 2A and 2B). In contrast, transcripts for surfactant lipid and surfactant-associated proteins (Figures 2C and 2D) decreased in the sensitive mice but not in protected mice. This difference in transcript levels suggests that maintenance of surfactant synthesis plays a critical role in determining the outcome.

In addition, the reversal of diminished surfactant synthesis appears to be critical to survival during nickel-induced injury.

One possible outcome that could lead to protection would be improved capacity to handle oxidative stress. However, antioxidant transcripts (e.g., *Gclc*) were similar among groups. In addition, another possible outcome that would lead to protection would be a decrease in inflammation. However, innate immunity transcripts (e.g., *Cxcl2*) remain similar among groups. Because decreases in transcript levels do not always reflect functional differences, these changes should be viewed with caution. The lack of change in transcript levels alone does not rule out that differences in inflammation occurred.

Nickel Decreased SFTPB mRNA in MLE-15 Cells

Of the multiple surfactant-associated proteins (SFTPA, B, C, and D), maintenance of SFTPB expression has been found to be the most important in determining survival (15, 25). To further understand the mechanism by which nickel inhibits SFTPB expression, SFTPB mRNA levels in mouse lung epithelial (MLE-15) cells were assessed by S1 nuclease protection assay. SFTPB transcript levels were decreased in a dose- and time-dependent manner. Levels of SFTPB mRNA were unchanged at 100 μ M, but decreased in MLE-15 cells after treatment with 300, 600, and 1,000 μ M NiSO₄ for 24 hours ($38 \pm 3\%$, $11 \pm 1\%$, and $10 \pm 1\%$ of control, respectively, mean $\pm$ SE, $P < 0.05$) (Figure 3A). Significant inhibition of SFTPB mRNA was delayed, being observable 18 and 24 hours after initiation of treatment with 300 μ M NiSO₄ ($65 \pm 8\%$ and $38 \pm 3\%$ of control, respectively, mean $\pm$ SE, $P < 0.05$) (Figure 3B). To assess whether these doses were cytotoxic, we tested MLE-15 cells with nickel concentrations of 100, 300, and 600 μ M and found that these doses produced minimal cytotoxicity ($0.1 \pm 2.0\%$, $0.3 \pm 3.0\%$, and $5.8 \pm 4.3\%$ LDH release/24 h, respectively, mean $\pm$ SE, each value is not significantly different than vehicle-treated control, ANOVA).

Analysis of Transcriptional Changes in Nickel-Treated MLE-15 Cells

To discern putative transcription factors that may be regulated by nickel, we used several approaches. We first performed an assay of DNA binding activity with nuclear protein extracts from nickel-treated MLE-15 cells to assess 50 common transcription factors in MLE-15 cells (600 μ M NiSO₄ $\leq$ 24 h). Nickel increased (Figure 4) binding in target domains for metal response element-binding transcription factor 1 (MTF1), AP-1 (a.k.a. TPA responsive element-AP1, TRE-AP1), E2F transcription factor 1–5 (E2F-1–5), and activator protein-2 (AP-2). Modest increases were noted in E twenty-six domain (ETS) and nuclear factor of activated T cells (NFAT), with a slight decrease or no change noted in nuclear factor I (NFI). Binding activity of the remaining 43 DNA binding motifs after nickel exposure was not different than that for control cells.

After identification of altered transcription factors binding activity, we performed an additional computational promoter analysis using genome-wide oligonucleotide microarray data of RNA isolated from MLE-15 cells. For the purpose of Gene Ontology (GO) analysis, a stringent threshold of significance ($P < 0.7 \times 10^{-6}$, false discovery rate [FDR] < 0.01 , and average intensity > 300) of difference between treated (600 μ M NiSO₄, 24 h) and control was selected to ensure confidence in the identified genes. Microarray analysis revealed 1,019 transcripts (522 increased and 497 decreased) that were significantly different from control (FDR ≤ 0.05). Transcripts with the greatest increases (Table E1) and decreases (Table E2) are presented in the online supplement.

To ascertain molecular pathways altered in MLE-15 cells after treatment, computational analysis was used to identify

Figure 1. Epidermal growth factor receptor (EGFR) signaling in the respiratory epithelium contributes to survival during nickel-induced injury. Survival of transgenic transforming growth factor- α (TGFA) Line 28 mice was greater than that in strain-matched FVB/NJ control mice or TGFA Line 28 $\times$ dn mutEGFR mice (that have dominant mutant EGFR expression directed to the respiratory epithelium), as determined by Kaplan-Meier log-rank survival ($P < 0.05$; $n = 5$ mice/group). Mice were exposed to NiSO_4 aerosol ($150 \mu\text{g Ni/m}^3$, $0.5 \mu\text{m MMAD}$, continuous exposure) and survival monitored for up to 2 weeks.

overrepresented GO categories among genes with significantly altered expression. Among 522 significantly increased transcripts ($\text{FDR} \leq 0.05$), GO categories determined to be overrepresented ($\text{FDR} \leq 0.01$) are summarized in Table 1. Particularly, several GO categories related to glucose processing (e.g., glycolysis, glucose metabolism and catabolism), which is critical to improved cell survival during stress (26), were significantly overrepresented. In addition, among 497 significantly decreased genes ($\text{FDR} \leq 0.05$), GO categories determined to be significantly overrepresented ($\text{FDR} \leq 0.01$) are

summarized in Table 2. Notably, the prevalence of GO categories related to nucleotide biosynthesis and metabolism were significantly enriched in transcripts that decreased.

To discern putative regulatory mechanisms that may underlie the lung epithelium's differential transcriptional response to nickel treatment, we performed computational promoter analysis (25) with this microarray dataset. Transcription factors were selected to determine the recognition elements that were enriched in the promoters of genes with significantly increased and decreased expression (Table 3). Among increased transcripts, E2F-1, AP-2, sterol regulatory element-binding protein (SREBP), trans-acting transcription factor 1 (SP1), peroxisome proliferator-activated receptor (PPAR), nuclear factor kappa B (NF- κ B), and hepatocyte nuclear factor-4 (HNF-4) were determined to be significantly enriched in the promoters ($P < 0.05$) (Table 3). Among decreased transcripts, only AP-1 was determined to be significantly enriched ($P < 0.01$). The recognition site for cyclic AMP response elements (CRE-ATF and CREB), Nuclear factor of activated T cells (NFAT), and ETS were tested but were not enriched significantly in either group of transcripts.

Together with the DNA-binding analysis, the computational analysis suggests that the transcription factors most consistently altered by nickel included AP-1, AP-2, and E2F-1. The microarray analysis was not used to test for MTF1 because this motif is relatively uncommon. However, MTF1 is clearly associated with increased Mt1/Mt2 expression (27, 28), and Mt2 transcripts are among the greatest increases noted in mouse lung after nickel exposure (Figure 2B and Ref. 24) and were increased in these cells. Finally, the microarray analysis suggested that SREBP, SP1, PPAR, NF- κ B, and HNF-4 were significantly enriched in the promoters, but these transcription factors were not identified by the DNA-binding analysis. Although additional studies are needed to fully interpret these results, we

Figure 2. Lung transcriptional profile of nickel-induced injury altered during TGFA-mediated protection. Selected transcripts were measured based on previous microarray experiments examining mouse lung transcript levels during lung injury and included the following. (A) Innate Immunity Group: interleukin 6 (IL6), chemokine (C-X-C motif), ligand 2 (Cxcl2, a.k.a. Mip2), lipocalin 2 (Lcn2), and CD14 antigen (Cd14). (B) Antioxidant Group: metallothionein 2 (Mt2), glutamate-cysteine ligase, catalytic subunit (Gclc), thioredoxin reductase 1 (Txnrd1), and heme oxygenase (decycling) 1 (Hmox1). (C) Surfactant Lipid Transport and Biosynthesis Group: solute carrier family 34 (sodium phosphate), member 2 (Slc34a2), fatty acid-binding protein 5, epidermal (Fabp5), phospholipid transfer protein (Pltp), fatty acid synthase (Fasn), and ATP-binding cassette, sub-family A (ABC1), member 3 (Abca3). (D) Surfactant Protein Biosynthesis group: surfactant-associated protein C (Sftpc), surfactant-associated protein A (Sftpa1), surfactant-associated protein B (Sftpb) and surfactant-associated protein D (Sftpd), and napsin A aspartic peptidase

(Napsa). Mice were exposed to NiSO_4 aerosol ($150 \mu\text{g Ni/m}^3$, $0.5 \mu\text{m MMAD}$, 72 h) and lung mRNA isolated and analyzed using quantitative real-time polymerase chain reaction. The expression of each transcript relative to ribosomal protein L32 (Rpl32) transcript level was determined using the $2^{-\Delta\Delta\text{CT}}$ method (18). Values are mean $\pm$ SE ($n = 6$ mice/group). All values were significantly different from untreated strain-matched control ($P < 0.05$) determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure, except those values indicated by NS (not significantly different than control).

chose to focus on whether AP-1 could contribute to the nickel-induced decreased *Sftpb* expression.

AP-1 Family Member Transcripts and DNA-Binding Activity Increase with Nickel Treatment

The mRNA levels of the AP-1 family members (Jun, Fos, JunD, and JunB assessed by RNase protection assay) increased in a dose-dependent fashion after NiSO_4 treatment of MLE-15 cells as compared with untreated control cells. Of the AP-1 family members examined, Jun mRNA increased the greatest after treatment with 100 to 600 μM nickel for 24 hours, exhibiting a greater than 9-fold increase over control after the 600 μM treatment (Figure 5A). Likewise, Fos mRNA levels increased after treatment with 100, 300, or 600 μM nickel for 24 hours (1.9 ± 0.1 -fold, 2.8 ± 0.3 -fold, and 4.3 ± 0.2 -fold, respectively, $P < 0.05$) (Figure 5A), albeit not to the same magnitude as Jun. JunD mRNA also increased after treatment with 300 and 600 μM NiSO_4 (1.9 ± 0.1 -fold and 1.8 ± 0.1 -fold, respectively, $P < 0.05$), but not with 100 μM (Figure 5A). Similarly, JunB mRNA increased after treatment with 600 μM NiSO_4 (1.6 ± 0.1 -fold, $P < 0.05$), but not 100 or 300 μM (Figure

Figure 3. Surfactant-associated protein B (SFTPb) mRNA levels decreased in mouse lung epithelial (MLE-15) cells exposed to nickel. (A) Dose-response relationship of SFTPb mRNA inhibition as measured by S1-nuclease protection assay in MLE-15 cells exposed to 0 (untreated control), or 10 to 1,000 μM NiSO_4 (37°C , 24 h). (B) Time course of SFTPb mRNA inhibition in MLE-15 cells after exposure to 0 (untreated control) or 300 μM NiSO_4 . All data are normalized to ribosomal protein L32 expression and are presented as percent of untreated control values (mean $\pm$ SE, $n = 3$ –8/group). * Significantly different from untreated control ($P < 0.05$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

5A). To determine whether the increased levels of AP-1 family members led to increased AP-1 DNA-binding activity, an EMSA assay was performed with nuclear extracts of MLE-15 cells treated with 600 μM NiSO_4 for 24 hours, and untreated controls. The DNA-binding activity of AP-1 increased in NiSO_4 -treated MLE-15 cells compared with untreated controls (Figure 5B). The specificity of the AP-1-binding activity is supported by competition with excess of unbiotinylated AP1 consensus oligonucleotides (Figure 5B, lanes 3 and 6). In addition, anti-Jun antibodies supershifted the AP-1 complex detected in nuclear extracts of both control (Figure 5C, lane 2) and nickel-treated (Figure 5C, lane 4) MLE-15 cells.

Jun Inhibition of *Sftpb* Promoter Activity Is Dependent on the Distal Region

Previously, Jun was demonstrated to inhibit $-1797/+42$ (with respect to the transcription initiation site) *Sftpb* promoter-CAT reporter and its deletion variants (20). To examine c-Jun-dependent inhibition, we used a luciferase reporter under control of the $-653/+35$ promoter and observed that Jun inhibited reporter activity in a dose-dependent manner (Figure 6A). This promoter contains proximal (nucleotide $-18/-12$) and distal (nucleotide $-370/-364$) AP1 recognition elements. To further dissect the relative influence of the proximal and distal regions on Jun-dependent inhibition of *Sftpb* expression, promoter constructs encompassing nucleotides $-100/+35$ and nucleotides $-397/-272$, respectively, were used to drive luciferase reporter activity. Co-expression of Jun inhibited the pGL4 $-397/-272$, but not pGL4 $-100/+35$, *Sftpb* promoter reporter activity (Figure 6B). These findings demonstrate that the distal,

Figure 4. Transcription factor DNA-binding activity in nuclear extracts of mouse lung epithelial (MLE-15) cells exposed to nickel. Nuclear protein extract was obtained from MLE-15 cells treated with 0 (vehicle control) or 600 μM NiSO_4 (37°C , 24 h). Nickel increased target binding by metal response element binding transcription factor 1 (MTF1), activator protein 1 (AP-1 a.k.a. TPA responsive element-AP1, TRE-AP1), E2F transcription factor 1–5 (E2F-1–5), and activator protein-2 (AP-2). Modest increases were noted in E twenty six-domain (ETS), nuclear factor of activated T cells (NFAT), and a slight decrease or no change was noted in nuclear factor I (NFI). Data were analyzed using least median squares (LMS) regression analysis comparing nickel-treated samples to untreated control and are presented as percent different from control (values are means of samples tested on two occasions).

TABLE 1. OVERREPRESENTED GENE ONTOLOGY CATEGORIES AMONG INCREASED TRANSCRIPTS DETERMINED IN MLE-15 CELLS TREATED WITH 600 μ M NiSO₄ FOR 24 H

GO ID	GO Category	FDR
GO:0015980	Energy derivation by oxidation of organic compounds	0.003
GO:0006066	Alcohol metabolism	0.004
GO:0031497	Chromatin assembly	0.005
GO:0009987	Cellular process	0.005
GO:0007582	Physiologic process	0.007
GO:0006334	Nucleosome assembly	0.007
GO:0006096	Glycolysis	0.007
GO:0007606	Sensory perception of chemical stimulus	0.007
GO:0007608	Sensory perception of smell	0.007
GO:0007166	Cell surface receptor-linked signal transduction	0.007
GO:0006092	Main pathways of carbohydrate metabolism	0.008
GO:0046365	Monosaccharide catabolism	0.009
GO:0019320	Hexose catabolism	0.009
GO:0006006	Glucose metabolism	0.009
GO:0016126	Sterol biosynthesis	0.009
GO:0046164	Alcohol catabolism	0.010
GO:0007186	G-protein-coupled receptor protein signaling pathway	0.010
GO:0006007	Glucose catabolism	0.010
GO:0006333	Chromatin assembly or disassembly	0.010
GO:0016052	Carbohydrate catabolism	0.010
GO:0007600	Sensory perception	0.010

Definition of abbreviations: FDR, false discovery rate; GO, gene ontology; MLE-15 cells, mouse lung epithelial cells; NiSO₄, nickel sulfate.

For the analysis, gene changes satisfying the FDR $\leq$ 0.05 level cutoff were considered significantly increased, and GO categories were considered significant if FDR $\leq$ 0.01.

but not the proximal, *Sftpb* promoter region mediates Jun-dependent inhibition of SFTPB expression.

The Protective Role of SFTPB Expression against Nickel-Induced Injury

Having demonstrated that nickel decreased SFTPB mRNA *in vivo* and *in vitro*, we sought to investigate the contribution of SFTPB to protection against nickel-induced injury *in vivo*. To circumvent the fatal effect of *Sftpb* loss, nickel-induced injury was induced in transgenic Scgblal-rtTA/TetO₇ *SFTPB/Sftpb*^{-/-} mice that have conditional SFTPB expression under the control of a dox-sensitive promoter (29). Because *Sftpb*^{-/-} mice die perinatally unless the *Sftpb* transgene is expressed, Scgblal-rtTA/TetO₇ *SFTPB/Sftpb*^{-/-} mice must be maintained on dox-containing food from Day 0 of gestation. We reasoned that maintaining these mice on dox during nickel exposure would increase survival because unlike the nascent promoter, the inducible transgene could be insensitive to the nickel-induced Jun inhibition. Maintenance of SFTPB under the control of a dox-sensitive promoter system increased survival in mice during nickel-induced injury as compared with control mice (Figure 7), albeit not as long as TGFA transgenic mice (Figure 1). These results demonstrate that SFTPB expression contributes to the protection against nickel-induced injury.

DISCUSSION

Previously, we reported that transgenic mouse lines with varying human TGFA expressed in the lung were protected during nickel-induced lung injury in a concentration-dependent manner (11). Transgenic TGFA mice with the dominant-negative mutant EGFR (TGFA Line 28 $\times$ dn-mutEGFR) survived less than TGFA transgenic mice (Figure 1). These mice lacked a portion of the EGFR intracytoplasmic domain, but contained the EGFR transmembrane and extracellular ligand-binding domains (13). The reversal was partial possibly because the dn-mutEGFR produced partial inhibition of EGFR

TABLE 2. OVERREPRESENTED GENE ONTOLOGY CATEGORIES AMONG SIGNIFICANTLY DECREASED GENES DETERMINED IN MLE-15 CELLS TREATED WITH 600 μ M NiSO₄ FOR 24 H

GO ID	GO Category	FDR
GO:0050875	Cellular physiologic process	< 0.001
GO:0008152	Metabolism	< 0.001
GO:0009117	Nucleotide metabolism	< 0.001
GO:0006091	Generation of precursor metabolites and energy	< 0.001
GO:0044237	Cellular metabolism	< 0.001
GO:0051186	Cofactor metabolism	< 0.001
GO:0009165	Nucleotide biosynthesis	< 0.001
GO:0051179	Localization	< 0.001
GO:0051234	Establishment of localization	< 0.001
GO:0006732	Coenzyme metabolism	< 0.001
GO:0006163	Purine nucleotide metabolism	< 0.001
GO:0006164	Purine nucleotide biosynthesis	< 0.001
GO:0009205	Purine ribonucleoside triphosphate metabolism	< 0.001
GO:0009199	Ribonucleoside triphosphate metabolism	< 0.001
GO:0006118	Electron transport	< 0.001
GO:0009144	Purine nucleoside triphosphate metabolism	< 0.001
GO:0044238	Primary metabolism	< 0.001
GO:0009145	Purine nucleoside triphosphate biosynthesis	< 0.001
GO:0009141	Nucleoside triphosphate metabolism	< 0.001
GO:0009259	Ribonucleotide metabolism	< 0.001
GO:0009206	Purine ribonucleoside triphosphate biosynthesis	< 0.001
GO:0044248	Cellular catabolism	0.001
GO:0006810	Transport	0.001
GO:0009201	Ribonucleoside triphosphate biosynthesis	0.001
GO:0044249	Cellular biosynthesis	0.001
GO:0009260	Ribonucleotide biosynthesis	0.001
GO:0009142	Nucleoside triphosphate biosynthesis	0.001
GO:0009150	Purine ribonucleotide metabolism	0.001
GO:0009056	Catabolism	0.001
GO:0009058	Biosynthesis	0.001
GO:0006221	Pyrimidine nucleotide biosynthesis	0.001
GO:0006220	Pyrimidine nucleotide metabolism	0.002
GO:0009152	Purine ribonucleotide biosynthesis	0.002
GO:0007582	Physiologic process	0.004
GO:0009987	Cellular process	0.005
GO:0006752	Group transfer coenzyme metabolism	0.005
GO:0051188	Cofactor biosynthesis	0.008
GO:0046034	ATP metabolism	0.008

For definition of abbreviations, see Table 1.

For the analysis, gene changes satisfying the FDR $\leq$ 0.05 level cutoff were considered significantly decreased, and GO categories were considered significant if FDR $\leq$ 0.01.

signaling (complete inhibition would be embryonic lethal). Because the dn-mutEGFR was constructed under control of the human *SFTPC* promoter, the transgene is expressed principally in type II alveolar and distal bronchiolar epithelial cells. This observation suggests that respiratory epithelial cells may play a critical role in TGFA-mediated protection during lung injury. Overall, these findings support the idea that EGFR signaling in the respiratory epithelium contributes to protection against nickel-induced lung injury, and are consistent with clinical reports of acute lung injury as an adverse event associated with an EGFR inhibition by gefitinib (30).

To identify critical determinants of susceptibility, mRNA isolated from sensitive (FVB/NJ), intermediate (TGFA $\times$ dn-mutEGFR), and resistant (constitutive TGFA transgenic) mouse lungs was analyzed for expression levels of four groups of genes (Figure 2). The genes selected are associated with innate immunity, antioxidant defense, and surfactant production. Most notable was the contrasting pattern of surfactant protein and lipid biosynthesis transcript levels between sensitive and resistant mouse strains. The clearest difference between the resistant and sensitive mice was the restoration of transcripts coding for surfactant-associated proteins, especially SFTPB and NAPSA, an enzyme critical in SFTPB proprotein processing

TABLE 3. TRANSCRIPTION FACTORS ENRICHED IN THE PROMOTERS OF GENES WITH INCREASED OR DECREASED TRANSCRIPT LEVELS IN MOUSE LUNG EPITHELIAL CELLS

Increased Transcripts		Decreased Transcripts	
Transcription Factor	P Value	Transcription Factor	P Value
E2F-1	0.0001	AP-1	0.0097
AP-2	0.0005		
SREBP	0.0006		
SP1	0.0113		
PPAR	0.0370		
NF-κB	0.0398		
HNF-4	0.0476		

MLE-15 cells were treated (37°C, 24 h) with 600 μM NiSO₄ and mRNA analyzed by oligonucleotide microarray. Using the complete Genomatix Vertebrate Matrix Library 6.2, the *P* value was calculated using binomial distribution probability by comparing the matrix match of the promoter regions of differentially expressed genes with the promoters from random mouse genes sets (−2 kb for all the promoters). The single-step Bonferroni adjustment is used to control for the multiple comparisons effect (i.e., multiplication of *P* value by the number of transcription factor-binding sites in Genomatix Vertebrate Matrix Library). For the analysis, transcription factors were considered significantly enriched in the gene promoters (*P* ≤ 0.05).

(31). These findings suggested that regulation of surfactant production, which is clearly necessary for normal pulmonary function in humans and mice (15, 29, 32), played an important role in determining survival during nickel-induced injury.

In contrast to surfactant-associated transcripts, innate immunity and antioxidant transcripts increased in resistant strains nearly as much as in the sensitive strains. These increased transcripts therefore could be viewed as compensatory processes. Inflammation is likely to have a complex role in nickel-induced injury, and because we studied only one irritant, inflammation may have differing roles for different etiologic agents. Neutrophils and neutrophil chemokines (e.g., Cxcl2 or IL-8) are elevated in patients with acute lung injury (33). However, acute lung injury can occur in neutropenic patients, suggesting that neutrophils might augment macrophage function that could improve survival (34–36). At a minimum, the lack of difference between resistant and sensitive mice implies that survival in nickel-induced lung injury is not accompanied by a diminution of inflammatory transcripts.

The increases in antioxidant transcripts (i.e., Mt2, Txnrd1, and Hmox1) are somewhat easier to interpret because the translated proteins have demonstrated protective effects in our and other mouse models (24, 37, 38) of lung injury. Thus increased transcript levels can be viewed as a protective mechanism. In other cell types, Jun activation is associated with increased transcription of Mt2 (39), Gclc (40), Txnrd1 (likely through interactions with SP-1) (41), and Hmox1 (42). However, our findings also suggest that increased levels of these transcripts may be independent of TGFA-EGFR signaling *in vivo*. For example, DNA binding of MTF1, the transcription factor most likely to be responsible for increases in Mt1 and

Figure 5. Activator protein 1 (AP-1) mRNA levels and DNA-binding activity increased in mouse lung epithelial (MLE-15) cells exposed to nickel. (A) Transcript levels of Jun, Fos, Jund, and Junb increased in MLE-15 cells after exposure to 0 (untreated control) or 100 to 600 μM NiSO₄ (37°C, 24 h). Data are normalized to ribosomal protein L32 expression and presented as fold changes as compared with untreated control, as measured by RNase protection assay (mean ± SE, *n* = 4–5/group). * Significantly different from untreated control (*P* < 0.05) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure. (B) AP-1 DNA-binding activity increased in nuclear extracts of MLE-15 cells treated with 600 μM NiSO₄ (37°C, 24 h) and binding was competed by unbiotinylated AP-1 oligonucleotides, as determined by electrophoretic mobility shift assay (EMSA). (C) Jun-binding activity increased in MLE-15 cells treated with 600 μM NiSO₄ (37°C, 24 h). Pre-incubation with anti-Jun antibodies, but not normal mouse IgG, supershifted AP-1 bands, as determined by EMSA. The blot is representative of tests performed on three occasions.

Figure 6. Distal *Sftpb* promoter region mediates Jun inhibitory function in MLE-15 cells. (A) Dose-response relationship of Jun-dependent inhibition of pGL3 -653/+35 *Sftpb* promoter reporter activity ($n = 4$). (B) Expression of Jun inhibited distal (pGL4 -397/-272) but not proximal (pGL4 -100/+35) *Sftpb* promoter reporter activity ($n = 7$). * Decreased compared with control with no pJun ($P < 0.01$) as determined by one-way analysis of variance with Holm-Sidak all pairwise multiple comparison procedure.

Mt2 transcripts (43), was increased in nuclear protein extract of MLE-15 after nickel treatment. The exact role of antioxidant gene products and the possible interactions are likely to be complex during acute lung injury.

To better understand the molecular links between nickel and diminished SFTPB mRNA, we analyzed the regulation of the *Sftpb* promoter in MLE-15 cells. Nickel decreased SFTPB mRNA levels in a dose- and time-dependent manner (Figure 3). These results, along with the transgenic mice studies that demonstrated decreased SFTPB mRNA and protein (11), suggested that nickel acted directly on type II epithelial cells and decreases SFTPB at the transcriptional (or post-transcriptional) level. To discern putative transcription factors that can be activated during nickel exposure, we used several approaches, including assays of nuclear protein extracts and a computational promoter analysis of our microarray dataset. The DNA-binding activity (Figure 4) and EMSA (Figure 5) assays revealed that nickel increased DNA binding to AP-1 target elements. In addition, MTF1, E2F-1-5, and AP-2 DNA binding also increased (Figure 4). Microarray analysis implicated E2F-1, AP-2, SREBP, SP1, PPAR, NF- κ B, and HNF-4 as enriched in the promoters ($P < 0.05$) among increased transcripts (Table 3). Together with the DNA binding analyses, the microarray

Figure 7. Maintenance of SFTPB expression protects during nickel-induced injury: Injury was induced in transgenic Scgb1a1-rtTA/tetO₂ SFTPB/Sftpb^{-/-} mice that have conditional *Sftpb* expression under the control of a dox-sensitive promoter or control (Scgb1a1-rtTA /tetO₂ SFTPB/Sftpb^{+/+} littermates) mice by exposure to nickel aerosol. Mice were treated with doxycycline in food and water, exposed to NiSO₄ aerosol (150 μ g Ni/m³ 0.5 μ m MMAD for 24 h), and survival monitored for up to 2 weeks. Maintenance of SFTPB under the control of a dox-sensitive promoter system increased survival in mice during lung injury as compared with control mice as determined by Kaplan-Meier log-rank survival analysis ($P < 0.05$; $n = 10$ mice/group).

analysis suggests that the predominant transcription factors altered by nickel include AP-1, AP-2, MTF1, and E2F-1. Of these, AP-1 has previously been found to mediate increased SERPINE1 expression after nickel treatment of BEAS-2B human airway epithelial cells (44). Importantly, AP-1-binding sites were significantly enriched in the promoter regions of genes with decreased transcripts in our microarray analysis.

Various environmental toxicants differentially regulate Jun and Fos expression in lung cells both *in vivo* and *in vitro* (45). In mouse lung epithelial cells, nickel increased AP-1 family members (Jun, Junb, Jund, Fos) mRNA levels (Figure 5A) and AP-1 DNA-binding activity (Figure 5B). Previous studies have identified two distinct (proximal and distal) regions of the mouse *Sftpb* promoter bearing AP-1-binding sites (20). We found that Jun co-expression inhibited the distal (-397/-272), but not the proximal promoter region (Figure 6). Previously, mutation of the distal AP-1-binding site increased basal promoter activity 5-fold in MLE-15 cells (20). Because these were conducted in an immortalized cell line, further studies with primary alveolar type II cells are needed to confirm these findings.

Another limitation of this study is that only a few transcription factors were examined. Other transcription factors may also contribute to SFTPB inhibition *in vitro*. In our mouse model of nickel-induced injury, TGFB1 protein levels increase in the BAL fluid (17). The TGFB1 response involves Smad signaling. Smad3 can prevent binding of transcription factors (NKX2.1 and FOXA1) to SFTPB promoter (46). Thus, it is likely that in addition to Jun, Smad signaling could contribute to the inhibition of SFTPB. Because the inhibition appears to involve protein-protein interactions between transcription factors rather than direct binding of Smad to the promoter (46), the DNA binding assay used in this study was unlikely to detect secondary protein complex binding (thus demonstrating one limitation of this technique). Nonetheless, the implication of our findings is that during lung injury aberrant Jun induction and diminished *Sftpb* expression may contribute to lung pathogenesis.

Surfactant-associated proteins, especially SFTPB, are critical to adult respiratory function. Gene-targeted mice lacking SFTPB (25), but not SFTPC (47), succumb to respiratory failure

shortly after birth. SFTPB deficiency also causes respiratory failure in adult mice (15). Therapeutic application of surfactant preparations to premature infants with acute respiratory distress syndrome reduces mortality and has become routine clinical practice (48, 49). However, whether surfactant therapy can be improved to reduce mortality in adult acute lung injury remains a major therapeutic challenge (50, 51). One possible corollary to our findings of the critical role for SFTPB is the support for future therapy directed at maintaining differentiated function of type II alveolar cells and SFTPB biosynthesis during acute lung injury (1).

If inhibition of *Sftpb* expression contributes to nickel-induced injury, then maintenance of SFTPB should protect mice and improve survival. In support of this hypothesis, the survival of conditional transgenic mice in which dox was used to control SFTPB expression instead of the native promoter was greater than control FVB/NJ mice after nickel exposure (Figure 7). However, it is also clear that, unlike TGFA expression, SFTPB alone may not be sufficient to obtain the level of protection imparted by TGFA during nickel-induced injury. As noted above, several other lung transcripts involved in surfactant synthesis were decreased during exposure and could also contribute to the increased survival in resistant mice. Of the transcripts that are restored in the resistant mouse line, NAPSA and SFTPC are particularly noteworthy. NAPSA is necessary for protein processing of SFTPB (31), and SFTPC is critical to rapid and efficient adsorption of surfactant phospholipids into the air-liquid interface (52).

In summary, SFTPB production capacity is reduced during nickel-induced injury in mice and survival is enhanced when SFTPB is maintained. TGFA-EGFR signaling in the pulmonary epithelium contributes to survival. However, the molecular mechanism for this protection is complex. Although our findings suggest increased survival is not associated with a diminution in inflammatory mediator transcripts or additional stimulation of antioxidant transcripts, several targets are likely to be involved. *In vitro* microarray analysis demonstrates that nickel can alter numerous pathways in epithelial cells. Genes with increased transcripts were enriched in promoter binding sites for E2F-1, AP-2, SREBP, SP1, PPAR, NF- κ B, and HNF-4, while genes with decreased transcripts were enriched only for AP-1. Nickel diminished SFTPB transcripts in MLE-15 cells, which is, in part, due to Jun activation and diminished transcription mediated through a distal binding site in the promoter. Maintenance of SFTPB under the control of a dox-sensitive promoter system increased survival during nickel-induced injury as compared with control mice, but not as well as TGFA protection. This supports the role for maintenance of SFTPB expression in protection against acute lung 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.

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