GP130-STAT3 Regulates Epithelial Cell Migration and Is Required for Repair of the Bronchiolar Epithelium

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Following injury, bronchiolar cells undergo rapid squamous metaplasia, followed by proliferation and re-establishment of the complex columnar epithelium that is characteristic of the normal airway. Mechanisms that regulate the repair of bronchiolar epithelium are of considerable relevance for understanding the pathogenesis of both acute and chronic lung diseases associated with airway remodeling. This study was designed to identify the role of the GP130-STAT3 signaling pathway during repair of the bronchiolar epithelium. STAT3 (signal transducer and activator of transcription 3) and GP130 (glycoprotein 130) were each selectively deleted from the pulmonary epithelial cells of transgenic mice *in vivo*, producing *Stat3*^{Δ/Δ} and *Gp130*^{Δ/Δ} mice, respectively. Airway injury was induced in adult mice by administration of naphthalene, a toxicant of nonciliated respiratory epithelial cells (Clara cells). Nuclear STAT3 staining was induced in bronchiolar epithelial cells following naphthalene-mediated injury in control (*Stat3*^{fl^{ox}/fl^{ox}) mice. Whereas nearly complete repair of the bronchiolar epithelium was observed in control mice within 13 days, restoration of cell shape, cell density, and the pattern of ciliated and nonciliated cells did not occur in the peripheral bronchioles of either *Stat3*^{Δ/Δ} or *Gp130*^{Δ/Δ} mice. Expression of dominant-negative STAT3 inhibited airway epithelial cell migration during repair *in vitro*; wild-type STAT3 expression activated such migration. In the present study, we show that GP130-STAT3 signaling functions in a cell-autonomous manner to restore cell shape and numbers}

required for repair of the bronchiolar epithelium following injury. (*Am J Pathol* 2008, 172:1542–1554; DOI: 10.2353/ajpath.2008.071052)

The respiratory epithelium is recurrently subject to injury by pathogens, particles, and toxicants. Following extensive bronchiolar injury, remaining cells undergo squamous metaplasia to maintain the epithelial barrier. Thereafter, epithelial cell proliferation and differentiation restore the normal populations of ciliated and nonciliated cells lining the bronchioles. Since acute and chronic airway injuries are associated with many pulmonary diseases, the mechanisms and cellular processes regulating repair of the respiratory epithelium are of considerable interest. Repair of the bronchiolar epithelium has been studied using various agents to cause epithelial cell injury, including naphthalene, respiratory pathogens, and inhaled toxicants (see review¹). Naphthalene has been used to selectively kill nonciliated bronchiolar cells in the mouse lung *in vivo*.^{2–4} Within 24 hours after naphthalene exposure, Clara cells are sloughed from the bronchiolar surface. Remaining cells, consisting primarily of ciliated cells and nonciliated, naphthalene-resistant cells, undergo squamous metaplasia and spread to maintain the epithelial lining. Dynamic changes in cell shape are accompanied by the expression of a number of transcription factors and cellular markers that are also associated with the differentiation of respiratory epithelial cells during

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lung morphogenesis.⁵ In the normal mouse lung, proliferation of respiratory epithelial cells is maximally increased 2 to 3 days after naphthalene-induced injury.^{2,5,6} Recent lineage analysis demonstrated that proliferating cells were derived from nonciliated progenitors.⁷ In the naphthalene-treated mouse model, repair of the bronchiolar epithelium, with re-establishment of columnar, ciliated and nonciliated cells is substantially complete within 10 to 14 days.^{2,4}

Interleukin 6 (IL-6), various cytokines, and growth factors regulate cellular processes by activating STAT3 (signal transducer and activator of transcription 3) phosphorylation (see review⁸). Phosphorylation of STAT3 causes dimerization and nuclear translocation that mediates transcriptional responses to many extracellular signals. IL-6 and other IL-6-related polypeptides bind to the transmembrane receptor GP130 (glycoprotein 130), activating Janus-associated kinase, and enhancing STAT3 phosphorylation. STAT3 plays diverse roles in cellular processes and is required for normal embryogenesis in the mouse.^{8,9} The functions of STAT3 have been studied *in vitro* and after cell-specific deletion *in vivo*. STAT3 influences cell survival, metabolism, growth, differentiation, and migration in multiple organs.^{10–12} In the lung, IL-6 and STAT3 enhanced cell survival and pulmonary surfactant lipid homeostasis following injury.^{13–15} Whereas pulmonary epithelial deletion of *Stat3* did not perturb lung morphogenesis or postnatal lung function in the mouse, STAT3 was required for maintenance of alveolar function, surfactant homeostasis, and cell survival following hyperoxic and adenoviral infection-related alveolar injury.^{16,17} To assess the potential role of STAT3 and GP130 in repair of the bronchiolar epithelium, we produced mice in which the genes were selectively deleted from respiratory epithelial cells *in vivo*. Cell migration and restoration of cuboidal-columnar cell shape following injury were dependent on STAT3 signaling in respiratory epithelial cells.

Materials and Methods

Transgenic Mice and Animal Husbandry

SP-C-rtTA/(tetO)₇CMV-Cre/*Stat3*^{flox/flox} triple-transgenic mice were generated as described previously.¹⁶ *Gp130*^{flox/flox} mice were generated by Dr. Werner Müller (University of Manchester, Manchester, UK).¹⁸ SP-C-rtTA/(tetO)₇CMV-Cre/*Gp130*^{flox/flox} triple-transgenic mice were generated essentially as previously described.¹⁹ *Gp130*^{flox/flox} mice were mated with SP-C-rtTA^{tg/-} transgenic mice and (tetO)₇CMV-Cre^{tg/tg} or (tetO)₇CMV-Cre^{tg/-} transgenic mice to generate double-transgenic mice that are SP-C-rtTA^{tg/-}/*Gp130*^{flox/wild} or (tetO)₇CMV-Cre^{tg/-}/*Gp130*^{flox/wild}. Mice were then mated to generate SP-C-rtTA^{tg/-}/*Gp130*^{flox/flox}, (tetO)₇CMV-Cre^{tg/tg}/*Gp130*^{flox/flox}, or (tetO)₇CMV-Cre^{tg/-}/*Gp130*^{flox/flox} mice. These mice were mated to generate SP-C-rtTA^{tg/-}/(tetO)₇CMV-Cre^{tg/-}/*Gp130*^{flox/flox} triple-transgenic mice in which *Gp130* is deleted (*Gp130*^{Δ/Δ}) in respiratory epithelial cells. The PCR primers used for genotyping were 5'-GACACA-TATAAGACCCTGGTCA-3' and 5'-AAAATCTTGCCAGC-

TTTCCCC-3' for SP-C-rtTA; 5'-TGCCACGACCAAGTG-ACAGCAATG-3' and 5'-AGAGACGGAAATCCATCGCTCG-3' for (tetO)₇CMV-Cre; and 5'-ACGTCACAGAGCTGAGT-GATGCAC-3' and 5'-GGCTTTTCCTCTGGTCTTG-3' for *Gp130*^{flox}. PCR for SP-C-rtTA and (tetO)₇CMV-Cre was performed as follows: denaturation at 94°C for 5 minutes; 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a 5-minute extension at 72°C. PCR for *Gp130*^{flox} was performed as follows: denaturation at 94°C for 5 minutes; 5 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 1 minute, and extension at 72°C for 30 seconds; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 30 seconds; followed by a 10-minute extension at 72°C.²⁰

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 postembryonic day 25. Mice were then provided normal mouse chow. Mice were kept in a pathogen-free vivarium according to the institutional guidelines. All protocols were approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Research Foundation.

Naphthalene Treatment

At 3 to 4 months of age, *Stat3*^{Δ/Δ} or *Gp130*^{Δ/Δ} and control mice were anesthetized in a 2.5% isoflurane/O₂ chamber to facilitate weighing and injection. Naphthalene (Sigma Chemical Co., St Louis, MO) was dissolved in corn oil at a concentration of 30 mg/ml and administered to mice (275 mg/kg) via intraperitoneal injection. After injection, mice were maintained in pathogen-free conditions.

Intratracheal Injection of IL-6

Intratracheal injection was performed as previously described.¹⁷ Briefly, mice were anesthetized in a 2.5% isoflurane/O₂ chamber and intubated via the mouth with 24-gauge animal feeding needles (Popper & Sons, New Hyde Park, NY) under direct laryngoscopy. IL-6 (5 μg) (R&D Systems, Minneapolis, MN) was diluted in a total volume of 80 μl of PBS and injected intratracheally. Lungs were inflation-fixed at 25 cm of water pressure with 4% paraformaldehyde/PBS 15 minutes after intratracheal injection of IL-6 for immunohistochemical analysis.

Surgical Procedure for Left Pneumonectomy

For the pneumonectomy (PNX) studies, adult 10-week-old *Stat3*^{Δ/Δ} and control mice were used. The operative procedure for left PNX was performed essentially as previously described.²¹ Briefly, following tracheal intubation under isoflurane anesthesia, the animals were ventilated with 2.5% isoflurane/O₂ at a rate of 200 strokes/minute with a tidal volume of 250 μl/stroke using a MiniVent 845 (Hugo Sachs Elektronik, March-Hugstetten, Germany). A left thoracotomy was performed and the bronchovascular bundle was ligated at the left hilum using a small titanium

ligating chip (Teleflex Medical, Research Triangle Park, NC). The left lung was excised and the thoracic incision was closed with suture. The skin was closed using surgical glue (Nexaband; Closure Medical Corp., Raleigh, NC). The anesthesia was discontinued and the mouse was extubated when spontaneous breathing was resumed. Sham-operated mice were given anesthesia, ventilated, and underwent left thoracotomy. After 7 days, the mice were anesthetized with pentobarbital and the final body weight of each mouse was recorded. The right lungs of pneumonectomized mice or both lungs of sham-operated mice were removed, dried with gauze, weighed, frozen in liquid nitrogen, and stored at -80°C for protein analysis. The lung weight was expressed as a ratio to the final body weight (lung weight index or LWI). The percentage of recovery was calculated as LWI of PNx-treated control and *Stat3* $^{\Delta/\Delta}$ mice divided by that of sham-treated control and *Stat3* $^{\Delta/\Delta}$ mice, respectively. Male and female mice were analyzed separately.

Immunohistochemistry

Mice were euthanized by an injection of pentobarbital. Lungs were inflation-fixed at 25 cm of water pressure with 4% paraformaldehyde/PBS for 1 minute and immersed in the same fixative. Tissue was fixed for 15 to 24 hours at 4°C and processed according to standard methods for paraffin-embedded blocks. Immunohistochemistry was performed on 5- μm -thick sections using antibodies generated to FOXJ1 (1:8000, rabbit polyclonal, kindly provided by Dr. Robert Costa, University of Illinois, Chicago), CCSP or Clara cell secretory protein (1:1000, rabbit polyclonal, kindly provided by Dr. Barry Stripp, Duke University, Raleigh, NC), STAT3 (1:50, rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT3 (1:50, rabbit polyclonal, Cell Signaling Technology Inc., Danvers, MA) and phosphohistone-3 (1:500, rabbit polyclonal, US Biological, Swampscott, MA) as described previously.

Immunofluorescence

Dual labeling immunofluorescence was performed on paraformaldehyde-fixed tissue using 5- μm -thick sections. FOXJ1 (1:1000, rabbit polyclonal), CCSP (1:10,000, guinea pig polyclonal, generated internally), phosphohistone-3 (Ser10) (1:500, sc-8656-R, rabbit polyclonal, Santa Cruz Biotechnology), Ki-67 (1:500, M7249, rat monoclonal, DAKO, Carpinteria, CA) and β -catenin (1:1000, sc-7963, mouse monoclonal, Santa Cruz Biotechnology) were used as primary antibodies. Fluorescence was developed with secondary antibodies conjugated with Alexa Fluor 594 (red) or Alexa Fluor 488 (green) fluorochromes (Molecular Probes, Invitrogen Corp., Carlsbad, CA) at a dilution of 1:200. All imaging was performed on a Zeiss Axioplan2 Imaging microscope and optical sectioning with the Zeiss Apotome.

Lung Morphometry

Slides were viewed by using a 20 $\times$ objective, and the images (fields) were transferred by video camera to a computer screen using METAMORPH imaging software (Universal Imaging, West Chester, PA). Cells within 500 pixel length (219 μm) from the broncho-alveolar duct junction (BADJ) from approximately 60 BADJs per mouse lung were counted manually.

Adenovirus Vectors

An E1A-region deleted adenoviral vector expressing the wild-type Stat3 cDNA (AD/WT) and dominant-negative Stat3 cDNA (AD/DN) were kindly provided by Dr. Yasushi Fujio (Osaka University, Suita, Japan).²² A control adenoviral vector expressing GFP (AD/GFP) was kindly provided by Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center, Cincinnati, OH). Adenoviruses were purified and concentrated by BD Adeno-X Virus Purification Kit (BD Bioscience, San Jose, CA) and Centriprep (Millipore, Bedford, MA). Viral titers were determined by Adeno-XTM Rapid Titer Kit (Clontech, Mountain View, CA).

Migration Assay

Human bronchial epithelial cells (HBECs) were kindly provided by Drs. Adi Gazdar and John Minna (University of Texas Southwest, Dallas, TX).²³ The migration assay was performed essentially as previously described.¹² Cells were cultured in keratinocyte serum-free medium (Invitrogen) supplemented with bovine pituitary extract and recombinant human epidermal growth factor (5 ng/ml). Cells were cultured in 24-well plates until confluent and infected at a multiplicity of infection of 10:1 and incubated for 2 hours. The viral suspension was removed and cells were cultured for an additional 2 days. Cells were starved for 24 hours and treated with 10 $\mu\text{g}/\text{ml}$ mitomycin C for 2 hours to arrest cell proliferation. A wound track was made by scraping the cell monolayer with a pipette tip. Cell migration into the cell-free area was evaluated 48 hours later in the absence or presence of IL-6 (20 ng/ml) (R&D Systems).

Western Blot Analyses

Whole cell lysates from the lungs of pneumonectomized or sham-operated/treated mice or adenovirus-transfected cells were prepared with radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology). Protein (100 μg) was mixed with lane marker reducing sample buffer (Pierce, Rockford, IL), separated by electrophoresis on Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes. Antibodies for immunodetection of each protein were STAT3 (1:1000, rabbit polyclonal, Cell Signaling Technology Inc.), phospho-STAT3 (1:1000, rabbit polyclonal, Cell Signaling Technology Inc.), actin (I-19HRP) (1:400, goat polyclonal, Santa Cruz Biotechnology), and proliferation cell nuclear antigen (PCNA) (1:2000, mouse monoclonal, Cell Signaling

Technology Inc.). Peroxidase-conjugated anti-rabbit IgG or anti-mouse secondary antibody (Calbiochem, Darmstadt, Germany) was used at 1:5000. Immunoreactive bands were visualized with ECL reagents (GE Healthcare, Chicago, IL).

RNA Extraction and RT-PCR

RNA was extracted from lung tissue or isolated type II cells using TRIzol reagent (Invitrogen Corp.). After DNase treatment, cDNA was synthesized using Superscript II. Reverse transcriptase (RT)-PCR was performed using primers 5'-TCAGCGAGAGCAGCAAAGAAGG-3' and 5'-GCATCAATGAATCTAAAGTGC GG-3' for Stat3. Quantitative RT-PCR for GP130 and L32 mRNA was performed using Smart Cycler (Cepheid, Sunnyvale, CA). Primers were 5'-CGTGGGAAAGGAGATGGTTGTG-3' and 5'-AGGGTTGTCAGGAGGAAGGCTAAG-3' for GP130; and 5'-GTGAAGCCCAAGATCGTC-3' and 5'-AGCAATCTCAGCACAGTAAG-3' for L32.

Isolation of Alveolar Type II Epithelial Cells

Alveolar type II epithelial cells were isolated from *Stat3 Δ/Δ* , *Stat3 $\Delta/wild$* , and *Stat3 $^{flox/flox}$* mice or *Gp130 Δ/Δ* and *Gp130 $^{flox/flox}$* mice at 5 weeks of age by collagenase digestion and differential plating as described by Rice et al.²⁴

Statistical Analysis

Values were expressed as means $\pm$ SE. Two group comparisons were carried out by unpaired Student's *t*-tests. Comparisons among groups were done by analysis of variance with Bonferroni/Dunn used for post hoc analysis. Significance was accepted at the 5% level.

Results

Deletion of Stat3 in Respiratory Epithelial Cells in Vivo

Two lox-P sites were inserted into introns on both sides of exon 21 to produce the *Stat3 flox* allele.¹¹ Exon 21 is 43 bp in size and contains tyrosine⁷⁰⁵, which is indispensable for STAT3 activation. RNA was extracted from the lung of *Stat3 $^{flox/flox}$* or *Stat3 Δ/Δ* mice and RT-PCR was performed using the primers flanking both sides of exon 21 (Figure 1A). In *Stat3 Δ/Δ* mice, two products appeared, those corresponding to the product from wild-type *Stat3* mRNA (378 bp) and *Stat3 Δ* mRNA (335 bp) respectively (Figure 1B). A single *Stat3* mRNA was detected in lungs from the *Stat3 $^{flox/flox}$* mice. After deletion, a second, smaller *Stat3* mRNA was also present at very low levels in lung tissue from *Stat3 Δ/Δ* mice, indicating that a truncated mRNA was produced after gene recombination. Western blotting for Stat3 from cell lysates from type II cells demonstrated the marked reduction in STAT3 protein after recombination in *Stat3 $\Delta/wild$* and *Stat3 Δ/Δ* mice (Figure 1C), but did not detect the predicted truncated STAT3 protein. To assess whether STAT3 phosphorylation was altered after recombination, the mice were treated with an intratracheal injection of IL-6 followed by staining for phospho-STAT3. IL-6-induced STAT3 phosphorylation was blocked in the bronchiolar epithelium of *Stat3 Δ/Δ* mice consistent with the loss of STAT3 after recombination (Figure 1D).

STAT3 is Activated in Airway Epithelial Cells after Naphthalene Injury

At steady state, STAT3 immunostaining was detected primarily in the apical cytoplasm of ciliated airway epithelial cells in the mouse lung (Figure 2A). After naphthalene exposure, STAT3 staining was detected in the residual squamous epithelial cells that line the injured airway epithelium. STAT3 staining was increased 2 days after

Figure 1. Conditional, cell-selective deletion of *Stat3* in the bronchiolar epithelium. **A:** In the *Stat3 flox* allele, the exon 21 (shaded rectangle) of *Stat3* is flanked by two lox-P sites (triangles). The primers for RT-PCR were designed on both sides of exon 21 in *Stat3* mRNA. **B:** RNA was extracted from whole lungs from *Stat3 Δ/Δ* or control (*Stat3 $^{flox/flox}$*) mice. RT-PCR produced two bands corresponding to the products from wild-type *Stat3* mRNA (378 bp) and *Stat3 Δ* mRNA (335 bp), respectively. **C:** Western blot analysis for STAT3 and β -actin using cell lysates from isolated type II alveolar epithelial cells, representative of three separate experiments. **D:** Immunohistochemistry for phospho-STAT3 15 minutes after intratracheal injection of IL-6. Airway epithelial cells in control (*Stat3 $^{flox/flox}$*) mice (*flox/flox*) had nuclear STAT3 staining. No or little staining was observed in the bronchial epithelium of *Stat3 Δ/Δ* mice (Δ/Δ). The figure represents *n* = 4 mice per group. Scale bars: 50 μ m.

Figure 2. STAT3 and phospho-STAT3 expression after naphthalene injury. Immunohistochemistry was performed with polyclonal antibody versus Stat3 (Santa Cruz Biotechnology) before (A), 1 day (B), 2 days (C), and 2 weeks (D) after naphthalene-induced injury. The inset in A shows STAT3 staining in ciliated cells at higher power. Phospho-STAT3 was detected with phospho-specific antibody (Cell Signaling Technology Inc.). Although not detectable before naphthalene injury (E), phospho-STAT3 was readily detected 2 days after naphthalene, indicating activation of the pathway (F). The figure is representative of three separate experiments. Scale bars: 50 μ m.

naphthalene injury and was most prominent as the epithelial cells become more cuboidal (Figure 2, B and C). The normal pattern of staining in the bronchioles was restored 2 weeks after exposure to naphthalene (Figure 2D). In normal lung, phospho-STAT3-positive epithelial cells were readily observed 2 days after naphthalene exposure, but were not detected earlier (Figure 2, E and F), indicating that squamous metaplasia of the remaining FOXJ1-positive cells occurs via a non-Stat3-dependent mechanism. The temporal changes in STAT3 phosphorylation are consistent with its potential role in the restoration of cuboidal/columnar cell morphology that occurs several days after injury in this model.

STAT3 Is Required for Normal Airway Epithelial Cell Repair after Naphthalene Injury

Since the cell types lining the respiratory tract differ from proximal to distal regions of the lung, the numbers and morphology of ciliated cells (FOXJ1-positive) and Clara cells (CCSP-positive) were counted in a defined length of airway from the end of the broncho-alveolar duct junction. Several groups of control mice were also studied: SP-C-rtTA/Stat3^{fllox/flox}, (tetO)₇-Cre/Stat3^{fllox/flox}, and Stat3^{fllox/flox} mice, to control for variability that might be related to rtTA/Cre²⁵ rather than to recombination of the Stat3 gene.

Total cell numbers and cellular compositions did not differ between Stat3^{Δ/Δ} and two control groups at baseline. On day 2 after naphthalene injury, the mean number of cells lining this length of each bronchiole (cell density) decreased to 40% of steady-state levels in all groups, consistent with the known abundance and expected loss of most of the CCSP-positive Clara cells that are present in terminal bronchioles. Thirteen days after injury, cell density had returned to the steady-state levels in both control groups. In contrast, the number of total cells present in the bronchioles of Stat3^{Δ/Δ} mice was significantly decreased (65%) compared to steady state (Figure 3A, a–c). On day 2 after injury, the number of epithelial cells (cell density) was decreased similarly in all groups of mice, likely reflecting the extensive squamous metaplasia seen at this time (Figure 3). Consistent with the squamous metaplasia and decreased numbers of cell percent area, cell length, indicated by β -catenin staining of cell membranes, was increased in proportion to the surface area altered by the squamous cells (Figure 4). By day 13, the density of ciliated cells had returned to steady-state numbers in both control groups. In contrast, the numbers of ciliated cells per unit length did not increase in Stat3^{Δ/Δ} mice during this time period (Figure 3A, d–f). A statistically significant decrease in ciliated cells was observed in Stat3^{Δ/Δ} compared to both control groups on both day 5 and day 13. Clara cells, indicated by CCSP staining, were virtually absent on day 2 (Figure 3A, g–i). CCSP staining increased thereafter, nearing pre-injury levels at 13 days in controls (Figure 3A, g and h). In contrast, cell numbers of both ciliated (FOXJ1-stained) and nonciliated (Clara) cells (CCSP-stained) remained decreased, and the distribution of cell remained abnormal in Stat3^{Δ/Δ} mice (Figure 3A, f and i).

In control mice, the epithelial cells lining terminal bronchioles had completely regained their columnar shape and were normally distributed along the BADJ region, ciliated and Clara cells being arranged alternately in most regions along the airway (Figure 3B, a, c, e, and g). In Stat3^{Δ/Δ} mice, epithelial cells remained highly squamous on day 13; cell numbers per unit length (density) remained significantly decreased from baseline. At this time, the pattern of distribution of ciliated and Clara cells was abnormal in Stat3^{Δ/Δ} mice, being detected primarily in distinct clusters of squamous (FOXJ1-positive) and cuboidal (CCSP-positive) cells in the Stat3^{Δ/Δ} mice, rather than in dispersed patterns typical of the controls (Figure 3B, b, d, f, and h).

Proliferation of the Bronchiolar Epithelium after Naphthalene Injury

Epithelial repair following naphthalene injury occurs in several distinct phases that include squamation, proliferation, and redifferentiation. The extent of injury and squamous metaplasia was similar in control and Stat3^{Δ/Δ} mice as assessed by cell counts and lung morphology on day 2 after injury (Figure 3A). In general, proliferation of the airway epithelium is most active 2 to 3 days after injury in

Figure 3. Delayed repair of the terminal bronchiolar epithelium in *Stat3*^{ΔΔ} mice after naphthalene injury. **A:** Terminal bronchiolar epithelial cells (total, ciliated, and Clara cell numbers) were counted after hematoxylin-eosin staining and immunohistochemistry for FOXJ1 and CCSP, respectively. Data are expressed as mean $\pm$ SE, $n = 3-7$ for each group. * $P < 0.05$. **B:** Representative histology 13 days after naphthalene injury after FOXJ1 staining (a–d) and CCSP staining (e–h) is shown. Scale bar: 50 μ m.

the naphthalene model.^{2,5,6} After naphthalene injury, cell proliferation in the bronchiolar epithelium was assessed by immunohistochemistry using phosphohistone-3 and Ki-67 as markers. The numbers of phosphohistone-3-stained cells per unit length were not significantly different in the control and *Stat3*^{ΔΔ} mice 2 days after naphthalene injury (Figure 5A). On day 3, CCSP expression was again detected and the bronchiolar epithelium was primarily cuboidal. At this time, Ki-67 and phosphohistone-3 was detected primarily in CCSP- but not in FOXJ1-stained cells in control and *Stat3*^{ΔΔ} mice, consistent with previous lineage tracing data.⁷ The respiratory epithelium remained squamous in *Stat3*^{ΔΔ} mice (Figure 6). FOXJ1, CCSP (double positive cells) were occasionally detected from 2 to 3 days after injury, likely representing the transitional redifferentiation of previously squamous cells, and/or the differentiation of residual Clara or proliferative “repopulating” cells. Taken together, the results show that the acute proliferative response induced by naphthalene was not significantly altered by deletion of STAT3; however, the reduction in cell numbers seen in *Stat3*^{ΔΔ} mice indicates that subtle changes in cell proliferation or survival throughout the repair process may contribute to

the failure to fully restore the bronchiolar epithelium. Double-positive proSP-C/CCSP cells were almost never detected during the injury (days 0 to 14) despite careful evaluation of more than 500 BADJs by co-immunofluorescence (Supplemental Figure 1S, see <http://ajp.amjpathol.org>). Of $n = 22$ mice examined, only 2 CCSP/proSP-C double-stained cells were identified, indicating the rarity of this cell.

Lung Regeneration after Pneumonectomy Is Not Stat3 Dependent

To independently determine whether STAT3 is required for proliferation of the airway epithelium during repair, left-pneumonectomy was performed on *Stat3*^{ΔΔ} and control mice, and compensatory growth of the remaining lung was assessed. In this model, pneumonectomy triggers the proliferation of resident lung cells, including airway epithelial cells, that is maximal at approximately 7 days.²⁶ Neither the recovery of LWI (Figure 5B) nor the increase of the expression of PCNA as assessed by Western blot after PNX (Figure 5C) differed in *Stat3*^{ΔΔ}

Figure 4. Squamous metaplasia repair following naphthalene injury. On day 2 after naphthalene injury, squamous metaplasia of remaining cells is observed lining the bronchioles of *Stat3^{lox/lox}* mice. FOXJ1-positive cells (green) (A and C) line the airway, CCSP (red) staining is absent. On day 4 following naphthalene injury, the transition to cuboidal cells is shown (B and D). FOXJ1 is expressed and weak CCSP staining (B and D) is present in the more cuboidal cells lining the airway. β -Catenin staining (E and F) of the basal cell membranes provides an estimate of cell length (red) in relationship to FOXJ1 (green) in the nuclei. Blue staining is from DAPI. **Scale bars:** A and B, 50 μ m; C-F, 10 μ m. White vertical bars delineate cell boundaries at sites of increased β -catenin staining.

and control mice, demonstrating that STAT3 was not required for cell proliferation and regeneration in the adult mouse lung. LWI of sham-treated *Stat3^{Δ/Δ}* mice was slightly, but not significantly, increased compared to that of control mice (male, 116%; female, 114%). Similarly, expression of PCNA was not increased in sham-treated

Stat3^{Δ/Δ} mice compared to sham-treated control mice at baseline.

STAT3 Influences Migration of Bronchial Epithelial Cells in Vitro

The persistence of clustering of CCSP-stained versus FOXJ1-stained squamous cells and the failure of FOXJ1-stained cells to recover their normal columnar shape indicated that abnormalities in cell migration might contribute to the failure of bronchiolar epithelium to repair in *Stat3^{Δ/Δ}* mice. Therefore, the role of the STAT3 pathway on migration of bronchial epithelial cells was examined using HBECs (a CDK-4/hTERT-immortalized cell line expressing cyclin-dependent kinase 4 and human telomerase) with proximal pulmonary epithelial cell characteristics.²³

Transfection of HBECs with an adenoviral vector expressing STAT3 enhanced the levels of phospho-STAT3 in the presence of IL-6. Transfection with a dominant inhibitory STAT3 construct blocked STAT3 phosphorylation (Figure 7A). IL-6 stimulated the motility of HBECs after scratch-induced injury *in vitro*, an effect significantly enhanced by expression of STAT3. Conversely, migration was inhibited by the dominant-negative STAT3 adenovirus (Figure 7, B and C). Taken together, these data demonstrate that IL-6 stimulates the migration of pulmonary epithelial cells (HBECs) via the STAT3 pathway.

Conditional Deletion of Gp130 (Gp130^{Δ/Δ}) in the Respiratory Epithelium

GP130 is the receptor mediating activation of STAT3 phosphorylation following stimulation by various IL-6-type cytokines.²⁷ To test whether the effects of STAT3 on airway epithelial repair were mediated by GP130, transgenic mice were produced in which GP130 was conditionally deleted from respiratory epithelial cells *in vivo*. In *Gp130^{lox/lox}* mice, lox-P sites are inserted into the introns

Figure 5. Stat3 independence of airway epithelial cell proliferation. **A:** On day 2 after naphthalene injury, immunohistochemistry for phosphohistone-3 (PH-3) was performed in control ($n = 6$ male, 3 female) and *Stat3^{Δ/Δ}* ($n = 4$ male, 3 female) adult mice. Positive epithelial cells per unit length (1 mm) were counted. **B:** Left PNx was performed on control ($n = 5$ male, 4 female) and *Stat3^{Δ/Δ}* ($n = 3$ male, 3 female) mice. Recovery (%) was determined as described in Materials and Methods. **C:** Western blot for PCNA was performed to assess proliferation. Lanes were normalized to total protein content. The intensity of the band from PNx-treated mice was divided by the average of intensity of the bands of sham-treated mice, $n = 4$ for each group, to provide quantitation. P, positive control. Data were expressed as means $\pm$ SE.

Figure 6. Ki-67 and phosphohistone-3 staining of bronchiolar epithelial cells after naphthalene injury. On day 5 after naphthalene, FOXJ1 (green) and weakly stained CCSP (red) cells are observed in squamous cells lining the bronchioles in control and *Stat3*^{ΔΔ} mice (A–C). CCSP (green) and phosphohistone-3 (red) are co-localized in the more cuboidal Clara cells lining the bronchioles of *Stat3*^{flox/flox} mice (D and F), circled in white to mark cell boundaries; cells expressing CCSP are generally lacking in the squamous regions lining the bronchioles of *Stat3*^{ΔΔ} mice (E). Ki-67 (red)- and FOXJ1 (green)-stained cells are not co-localized in cells lining the airways of either *Stat3*^{flox/flox} (G and I) or *Stat3*^{ΔΔ} mice (arrow in H). Z-stack images of the cells lining the bronchioles of *Stat3*^{flox/flox} mice show co-localization of both proliferation markers with CCSP-stained cells (F and I). Neither Ki-67 nor phosphohistone-3 co-localized with FOXJ1 (I). In *Stat3*^{flox/flox} mice at day 5 after naphthalene injury, CCSP-stained cells were generally distinguished from FOXJ1-stained cells in cuboidal cells lining the bronchioles (C). At this time, double-positive (CCSP, FOXJ1) cells were occasionally observed (not shown). Scale bar is 50 μ m. The scale bar in the inset is 10 μ m.

of both sides of exon 16, the region encoding the transmembrane domain of GP130 (Figure 8A).¹⁹ Consistent with observations with *Stat3*^{ΔΔ} mice, *Gp130*^{ΔΔ} mice survived normally and were without apparent abnormalities in lung morphogenesis, function, or morphology under normal conditions in the vivarium. RNA was extracted from type II alveolar epithelial cells isolated from adult *Gp130*^{ΔΔ} and control (*Gp130*^{flox/flox}) mice. Quantitative RT-PCR performed with primer sets within the deleted exon 16 was used to demonstrate that GP130 mRNA was reduced to 20% of that in control type II cells (Figure 8B). To document the loss of GP130 signaling in bronchiolar epithelial cells, IL-6 was injected intratracheally into *Gp130*^{ΔΔ} and control mice. In control mice, bronchiolar epithelial cells stained for phospho-STAT3. Phospho-STAT3 staining was markedly reduced in bronchiolar and alveolar epithelial cells but was readily detected in inflammatory cells in *Gp130*^{ΔΔ} mice after IL-6 exposure (Figure 8C), consistent with epithelial cell-specific targeting of the receptor.

Effect of Naphthalene Injury in *Gp130*^{ΔΔ} Mice

The effects of GP130 on airway epithelial repair after naphthalene injury in *Gp130*^{ΔΔ} mice were examined (Figure 9). At baseline, total cell numbers and cellular composition in the peripheral bronchioles did not differ in *Gp130*^{ΔΔ} and control mice. The extent of naphthalene injury was similar in *Gp130*^{ΔΔ} and control mice with extensive loss of Clara cells. Thirteen days after naphthalene injury, the recovery of the bronchiolar epithelium was markedly delayed in *Gp130*^{ΔΔ} mice. In control mice, the columnar shape and distribution of ciliated and Clara cells lining terminal bronchioles were substantially restored. In contrast, clusters of epithelial cells remained squamous and the distribution of CCSP versus FOXJ1 staining remained abnormal, with each cell type often found in clusters rather than well dispersed along the bronchioles of *Gp130*^{ΔΔ} mice. The phenotype seen in *Gp130*^{ΔΔ} mice on day 13 after naphthalene injury was indistinguishable from that of *Stat3*^{ΔΔ} mice, suggesting

Figure 7. Stat3 influences migration of bronchial epithelial cells *in vitro*. **A:** Human bronchial epithelial cells were transfected with adenovirus expressing wild-type STAT3 (AD/WT) or dominant-negative STAT3 (AD/DN) and cultured for 2 days. Cells were treated with or without IL-6 (15 minutes) and harvested. Western blots for STAT3 (**top**), phospho-STAT3 (**middle**), and actin (**bottom**) were performed and are representative of three independent experiments. **B** and **C:** Human bronchial epithelial cells transfected with AD/GFP (control), AD/WT, or AD/DN were subjected to the *in vitro* migration assay in the absence or presence of IL-6 stimulation. A wound track was made by scraping. Forty-eight hours later, the numbers of migrating cells within the wound track were counted in five non-overlapping fields, $n = 4$ for each treatment. The figure is representative of three independent experiments. **Scale bar:** 50 μm . Data were expressed as means $\pm$ SE. * $P < 0.05$.

that signaling via GP130 was critical for the activation of STAT3 during repair of the bronchioles following naphthalene injury.

Discussion

In this study, the role of GP130-STAT3 signaling in airway epithelial repair following naphthalene-induced injury was addressed. Epithelial cell STAT3 phosphorylation was enhanced during the early phase of airway epithelial repair. After cell-selective deletion of *Stat3* or *Gp130*

Figure 8. Conditional deletion of *Gp130* in the airway epithelium. **A:** In the *Gp130*^{lox} allele, the exon 16 (shaded rectangle) of *Gp130* is flanked by two lox-P sites (triangles). The primers for RT-PCR (arrows) were designed on both sides of exon 16 in GP130 mRNA. **B:** RNA was extracted from whole lung of *Gp130*^{Δ/Δ} ($n = 3$) and control (*Gp130*^{lox/lox}) ($n = 4$) mice. Quantitative RT-PCR using the primers (**A**) was done as described in Materials and Methods. Data were expressed as means $\pm$ SE, * $P < 0.05$. **C:** Immunohistochemistry for phospho-STAT3 (p-STAT3) 15 minutes after intratracheal injection of IL-6. Airway epithelial cells in control (*Gp130*^{lox/lox}) mice were strongly stained for p-STAT3. p-STAT3 staining was absent in *Gp130*^{Δ/Δ} mice. The figure is representative of 4 mice in each group. **Scale bars:** 100 μm .

genes in airway epithelial cells, recovery was incomplete, with persistent areas of squamous metaplasia and failure to restore cell numbers and normal morphology of ciliated and nonciliated bronchiolar epithelial cells along the terminal bronchioles. STAT3 activation enhanced the migration of airway epithelial cells *in vitro* and, conversely, inhibition of STAT3 blocked cell migration during wound repair. Taken together, the results show that the GP130-STAT3 pathway is required for normal repair of the bronchiolar epithelium following extensive injury to Clara cells, at least in part, by influencing epithelial cell migration and cell shape.

GP130-STAT3 Signaling Promotes Cell Migration

The migration of airway epithelial cells is recognized as an important behavior during wound repair in tissues, including the lung.^{12,28–30} Deletion of GP130 and STAT3

Figure 9. Delayed repair of the terminal bronchiolar epithelium in *Gp130*^{Δ/Δ} after naphthalene injury. **A:** Thirteen days after naphthalene treatment, terminal bronchiolar epithelial cell, ciliated cell, and Clara cell numbers were counted after hematoxylin-eosin and immunohistochemistry for FOXJ1 and CCSP, respectively. Data were expressed as mean ± SE, *n* = 3 to 7 for each group, **P* < 0.05. **B:** Representative histology 13 days after naphthalene injury of FOXJ1-stained (a–d) or CCSP-stained (e–h) lung tissue is shown. Scale bar: 50 μm.

did not influence the extent of injury, the initial spreading of remaining ciliated cells, or early proliferative responses in the epithelium. Restoration of normal cell shape and numbers that occurs later in the repair process was blocked in both *Gp130*^{Δ/Δ} and *Stat3*^{Δ/Δ} mice. Restoration of the normal columnar epithelium is likely dependent on the migration of cells produced both from dividing progenitor cells and from the restoration of cell size and shape of residual epithelial cells that must migrate and recover their columnar shape during repair. Whereas no defects were observed in the initial squamous metaplasia in *Gp130*/*Stat3*-deleted mice, defects in repair and migration caused by disruption of STAT3 signaling were observed both *in vivo* and *in vitro*, indicating that cell migration is likely to play an important role in the repair process. After injury, remaining epithelial cells migrate and differentiate/redifferentiate to restore the normal pattern of ciliated and nonciliated cells lining the airways. The role of STAT3 in cell migration has been demon-

strated in a number of experimental models (see reviews^{12,31–34,35}). STAT3 affects cell motility through both transcriptional and non-transcriptional pathways. Recent studies support a non-transcriptional mechanism by which STAT3 influences cell migration that is both tyrosine phosphorylation dependent and independent.^{34,35} The finding that the effects of *Stat3* and *Gp130* were similar and that STAT3 phosphorylation was inhibited in both models supports the likelihood that phosphorylation of STAT3 mediates cell processes required for repair of the airway epithelium.

Proliferation after Naphthalene Injury

Staining for phosphohistone-3 or Ki-67 was not altered in *Stat3*^{Δ/Δ} mice 2 days after naphthalene injury, indicating that initial proliferation was not perturbed by the lack of STAT3. However, decreases in the number of epithelial

cells lining the bronchioles of *Stat3^{Δ/Δ}* mice were observed 13 days after naphthalene injury. Whether more subtle changes in proliferation, continuing later in the repair process, occur is difficult to assess, since cell division proceeds at very low levels and small changes in the rate of cell proliferation are not readily determined in this model. We were unable to detect ongoing apoptosis after naphthalene injury by either caspase-3 or terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining (data not shown). Whereas acute proliferative response to the injury was not inhibited by deletion of STAT3, the decrease in cell numbers and persistent squamous metaplasia indicate a failure of normal migration, proliferation, and/or cell survival in the absence of STAT3.

Compensatory Growth following Pneumonectomy Is Not Stat3 Dependent

Dynamic changes in expression of various transcription factors regulating epithelial differentiation occur following both pneumonectomy and naphthalene injury.⁵ Increased cell proliferation occurs approximately 3 to 7 days after pneumonectomy and occurs throughout the conducting airways and alveolar regions of the lung. Growth factors, including epidermal growth factor, hepatocyte growth factor (HGF), and insulin-like growth factor (IGF-I) stimulate cell proliferation and growth in this model.^{36–38} For example, anti-HGF antibody blocked cell proliferation and lung weight after pneumonectomy, indicating that endogenous HGF plays an important role in compensatory lung growth.³⁷ Although STAT3 is involved as a possible effector in some of these signaling pathways, the lack of STAT3 in airway and alveolar epithelial cells in *Stat3^{Δ/Δ}* mice did not inhibit regeneration after PNX, suggesting that these growth factors exert their effects on cell proliferation and repair independently of epithelial STAT3.

The Role of GP130-STAT3 Signaling in Wound Repair

IL-6-family cytokines (IL-6, IL-11, leukemia inhibitory factor, ciliary neurotrophic factor, oncostatin M, and cardiotropin 1) bind to GP130, which activates two main signaling pathways, the Janus-associated kinase-STAT3 pathway and the RAS-MAPK pathway. On the other hand, STAT3 is also activated by non-IL-6-family cytokines, including granulocyte-colony-stimulating factor, leptin, and growth factors such as epidermal growth factor, platelet-derived growth factor, and HGF.⁸ GP130-STAT3 pathway has been reported to influence epithelial repair in some organs. In skin, a similar defect in wound healing was observed after deletion of IL-6 or cell-specific deletion of STAT3.^{12,39} In gastrointestinal tract, *Gp130^{ΔSTAT3}* mice harboring a “knock-in” mutation that deleted all STAT-binding sites in GP130 displayed spontaneous intestinal ulceration and impaired wound healing after sodium dextran sulfate-induced epithelial damage.⁴⁰ In the biliary

tract, IL-6 knockout mice were sensitive to biliary cirrhosis after bile duct ligation.^{41,42} Consistent with the role of GP130/STAT3 in tissue repair, the present study demonstrated that the activation of STAT3 in airway epithelial cells following naphthalene injury was blocked in *Gp130^{Δ/Δ}* mice. The observation that abnormalities in bronchiolar repair were similar in *Stat3^{Δ/Δ}* and *Gp130^{Δ/Δ}* mice indicates a shared signaling pathway, which is required for full restoration of the bronchiolar epithelium.

The Role of GP130-STAT3 Signaling in the Airway Epithelium

After naphthalene exposure, most Clara cells are killed. Remaining cells consist primarily of FOXJ1-positive squamous cells and/or other naphthalene-resistant cells. Several studies support the presence of different “naphthalene-resistant” cells, which have the capacity of self-renewal and differentiation into both Clara cells and ciliated cells, depending on the location along the airway.^{43–45} In the present study, proliferating epithelial cells detected after naphthalene injury by phosphohistone-3 or Ki-67 expressed CCSP and were often localized

Figure 10. Model of airway epithelium repair. After naphthalene exposure, Clara cells are killed and sloughed into the airspace. Remaining cells consist primarily of FOXJ1-stained cells that flatten and spread over the injured bronchiolar surface, and naphthalene-resistant bronchiolar cells that are considered progenitor cells. During the repair process, the progenitors proliferate, migrate, and undergo differentiation into Clara cells and/or ciliated cells. Surviving ciliated cells migrate and resume normal cuboidal-columnar shape, making the space for cells produced by proliferation of progenitors. Differentiation of new cells and the redifferentiation of squamous cells derived from surviving ciliated cells produce the normal columnar epithelium that consists of ciliated and nonciliated respiratory epithelial cells. STAT3 signaling within the respiratory epithelium is required for restoration of cell shape and numbers after bronchiolar injury.

in the periphery of CCSP-positive cell clusters. This pattern of proliferation occurred in both *Stat3^{Δ/Δ}*, *Gp130^{Δ/Δ}* and control mice, indicating that initial proliferation of surviving progenitors and their redifferentiation to CCSP-expressing phenotype was relatively independent of STAT3 signaling.

In the study of Rawlins et al,⁷ naphthalene was administered to mice in which ciliated cells were lineage-labeled via the FOXJ1 promoter. During repair, lineage-labeled cells were clustered together and separated by regions of unlabeled proliferating cells. In *Stat3^{Δ/Δ}* mice, the clustering of CCSP-stained and FOXJ1-stained squamous cells persisted, and squamous FOXJ1-stained cells failed to recover their normal columnar shape. In the absence of injury, cell shape and size were unaltered in *Gp130^{Δ/Δ}* and *Stat3^{Δ/Δ}* mice. It is, however, not clear from our studies, whether the failure to resume columnar shape after injury is related to an effect of Stat3 on processes regulating cell shape rather than a defect in migration that influences cell density and, in turn, cell shape. The *in vitro* migration assays demonstrated an important role for STAT3 in lung epithelial cell migration and wound repair (Figure 10). We speculate that during repair, cell migration and restoration of cuboidal morphology may create the space required for newly divided cells to repopulate the airways.

Squamous metaplasia, cell proliferation, and redifferentiation are orchestrated to maintain barrier function and repair the epithelium following injury. A number of cytokines, growth factors, and other polypeptide mediators, including IL-6 family members, are produced following injury to influence the repair of the bronchiolar epithelium. Whereas GP130-STAT3 signaling regulates diverse aspects of acquired innate defense, for example, T-cell proliferation and B-cell differentiation,^{11,46} GP130-STAT3 also regulates many airway epithelial cell processes, including surfactant homeostasis, cell survival, and apoptosis.^{16,17} In the present study, we show that the GP130-STAT3 signaling functions in a cell-autonomous manner and plays a critical role in the repair of airway epithelium, influencing epithelial cell migration, density, and shape.

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