Targeted Injury of Type II Alveolar Epithelial Cells Induces Pulmonary Fibrosis

Thomas H. Sisson¹, Michael Mendez², Karen Choi¹, Natalya Subbotina¹, Anthony Courey¹, Andrew Cunningham¹, Aditi Dave¹, John F. Engelhardt³, Xiaoming Liu³, Eric S. White¹, Victor J. Thannickal¹, Bethany B. Moore¹, Paul J. Christensen², and Richard H. Simon¹

¹Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Hospital, Ann Arbor;

²Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Veterans Affairs Medical Center, Ann Arbor, Michigan; and ³Department of Anatomy and Cell Biology, University of Iowa, Iowa City, Iowa

Rationale: Ineffective repair of a damaged alveolar epithelium has been postulated to cause pulmonary fibrosis. In support of this theory, epithelial cell abnormalities, including hyperplasia, apoptosis, and persistent denudation of the alveolar basement membrane, are found in the lungs of humans with idiopathic pulmonary fibrosis and in animal models of fibrotic lung disease. Furthermore, mutations in genes that affect regenerative capacity or that cause injury/apoptosis of type II alveolar epithelial cells have been identified in familial forms of pulmonary fibrosis. Although these findings are compelling, there are no studies that demonstrate a direct role for the alveolar epithelium or, more specifically, type II cells in the scarring process.

Objectives: To determine if a targeted injury to type II cells would result in pulmonary fibrosis.

Methods: A transgenic mouse was generated to express the human diphtheria toxin receptor on type II alveolar epithelial cells. Diphtheria toxin was administered to these animals to specifically target the type II epithelium for injury. Lung fibrosis was assessed by histology and hydroxyproline measurement.

Measurements and Main Results: Transgenic mice treated with diphtheria toxin developed an approximately twofold increase in their lung hydroxyproline content on Days 21 and 28 after diphtheria toxin treatment. The fibrosis developed in conjunction with type II cell injury. Histological evaluation revealed diffuse collagen deposition with patchy areas of more confluent scarring and associated alveolar contraction.

Conclusions: The development of lung fibrosis in the setting of type II cell injury in our model provides evidence for a causal link between the epithelial defects seen in idiopathic pulmonary fibrosis and the corresponding areas of scarring.

Keywords: diphtheria toxin; lung; collagen; scarring

Idiopathic pulmonary fibrosis (IPF) is a progressive scarring disorder of the lung for which there are no established therapies. A better understanding of the pathogenesis of IPF is critical for the identification of new therapeutic targets. The epithelial-mesenchymal hypothesis of IPF suggests that this disease is the result of successive injuries to the alveolar capillary membrane with ineffective reconstitution of a normal epithelium (1, 2).

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Correspondence and requests for reprints should be addressed to Thomas H. Sisson, M.D., Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical Center, 1150 West Medical Center Drive, 6301 MSRB III, Ann Arbor, MI 48109-5642. E-mail: tsisson@umich.edu

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Alveolar epithelial cell abnormalities are a common feature of pulmonary fibrosis. A mutation in a type II alveolar epithelial gene product that causes cell toxicity is associated with familial lung scarring.

What This Study Adds to the Field

We have established a causal relationship between type II alveolar epithelial cell injury and interstitial collagen accumulation.

Support for this hypothesis comes in part from the histologic evaluation of biopsies taken from patients with IPF. In fibrotic foci, the proposed areas of active scarring, there are prominent defects in the alveolar epithelium including hyperplasia and denudation (3–5). The importance of epithelial cell injury and apoptosis in the pathogenesis of pulmonary fibrosis is also supported by several different animal models that consistently demonstrate these defects (6–9).

At the core of the epithelial-mesenchymal hypothesis of pulmonary fibrosis is the contention that type II alveolar epithelial cells fail to repair the damaged epithelium as a result of ineffectual proliferation, migration, and/or differentiation, and this leads to interstitial scarring (1). The persistent denudation of the epithelium in IPF lesions suggests that residual type II cells are unsuccessful in their reparative efforts. This ineffectual reconstitution of the epithelium is postulated to drive fibrosis by inducing the proliferation and differentiation of fibroblasts and the deposition of collagen. *In vitro* evidence demonstrates that fibroblast differentiation and collagen production is enhanced in epithelial cell/fibroblast cocultures by injury to the epithelial cell component (10).

The importance of the alveolar epithelium in the pathogenesis of lung fibrosis is further implicated by the observation that epithelial cell growth factors protect against scarring in animal models (11–13). There is evidence linking a familial form of IPF with a mutation in the surfactant protein C gene. The defective product that results from this mutation was found to be toxic to the type II alveolar epithelial cells that express the mutated gene, and the correlation of this defect with fibrosis specifically implicates this specific constituent of the epithelium in the pathogenesis of lung fibrosis (14).

Although many studies have suggested a link between an injury to alveolar epithelial cells and the development of pulmonary fibrosis, there are no studies that directly test whether they are mechanistically related. We hypothesized that a targeted injury of

type II cells would be sufficient to cause pulmonary fibrosis. To test this hypothesis, we used the transgenic model of diphtheria toxin-mediated tissue-specific injury. With this approach, exogenously administered diphtheria toxin (DT) binds to and damages cells expressing the human precursor of heparin binding-EGF-like growth factor (proHB-EGF), also known as the diphtheria toxin receptor (DTR) (15, 16). The toxin cannot adhere to rodent proHB-EGF because of differences in the amino acid sequence at the DT binding site (16). Mice are therefore resistant to the cytotoxic effects of DT. To target type II alveolar epithelial cells with this model, we generated transgenic mice that express DTR off of the type II cell-specific surfactant protein C (SPC) promoter. Using this strategy, we found that administration of DT to these transgenic animals caused significant pulmonary fibrosis as assessed by hydroxyproline and histology. This new model offers an additional approach to studying the pathogenesis of disorders that result in alveolar scarring. Portions of these results have been presented previously in abstract form.

METHODS

Generation of Transgenic Mice

To target type II alveolar epithelial cells for injury by DT, an expression cassette containing the murine SPC promoter and the DTR gene (SPC-DTR) was generated by cloning the DTR cDNA (a gift from Dr. Kenji Kohno) into the pEGFP-N1 vector (Clontech) using the EcoRI restriction site in the multicloning sequence. The murine SPC promoter (a gift from Dr. Stephan Glasser) was inserted into the vector using NheI and XhoI restriction sites. The expression cassette was then cleaved from the plasmid backbone and microinjected into C57BL/6 mouse eggs, which were implanted into pseudo-pregnant mothers. Resultant mice possessing the transgenic construct (founders) were bred with 6- to 8-week-old C57BL/6 partners. This cross resulted in litters consisting of heterozygous transgenic and wild-type (WT) pups, and the offspring from these pairings were used in subsequent experiments. All mice were given water and food *ad libitum*. All protocols were approved by the University Committee on Use and Care of Animals.

Assessment of Mouse Genotypes

To determine the genotypes of offspring from SPC-DTR and C57BL/6 crossings, we purified DNA from mouse tail biopsies. To assess for the presence of the SPC-DTR construct, we performed a PCR reaction with the following primers: upper primer, 5'-TCGTGGGG-CTTCTCATGT TTAGGT-3'; lower primer, 5'-CGGCGGGTCTTGAGTTGC-3' (product size, 579 bp). We assessed the integrity of the purified DNA by simultaneously running a PCR reaction with primers specific for the β -globin gene (upper primer, 5'-CCAATCTGCTCACACAGGATA GAGAGGGCAGG-3'; lower primer, 5'-CCTTGAGGC-TGTCCAA GTGATTCAGGC CATCG-3'; product size, 494 bp). The PCR conditions were the same for each primer pair: 94°C for 8 minutes, then 94°C for 1 minute followed by annealing at 67°C for 2 minutes followed by elongation at 72°C for 2 minutes. The latter three steps were repeated for 30 cycles. The reaction was completed at 72°C for 10 minutes.

In Vitro Confirmation of the Integrity of the SPC-DTR Expression Cassette

MLE-12 cells, a cell line derived from murine alveolar epithelial cells that express SPC, were plated at a density of 5×10^3 cells per well in a 96-well plate and grown to 80% confluency in Hites media + 10% fetal bovine serum. Following the manufacturer's guidelines, we used the Eugene reagent (Roche Molecular Biochemicals, Indianapolis, IN) to transfect the cells with the SPC-DTR expression cassette-containing pEGFP-N1 vector. Control cells received equivalent volumes of Eugene reagent alone or phosphate buffered saline (PBS). The cells under the different conditions were incubated for 24 hours, after which their media was changed to Hites + 2% fetal bovine serum (100 μ l) with or without DT (0.1 μ g/ml). After another 24 hours, the mitochondrial activity of the

cultures was assessed with an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Promega, Madison, WI) following the manufacturer's instructions.

RT-PCR for DTR

Total RNA was purified from 100-mg pieces of lung, heart, kidney, spleen, and liver using the Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The purified RNA (500 ng/50 μ l reaction) was then subjected to RT-PCR with primers specific for the DTR message (*see above* for sequence). The RT-PCR reaction conditions were as follows: 48°C for 45 minutes for one cycle followed by 94°C for 1 minute, 67°C for 2 minutes, and 72°C for 2 minutes, repeated for 30 cycles. The resultant product was analyzed by electrophoresis on a 1.5% agarose gel.

Type II Alveolar Epithelial Cell Isolation

Type II epithelial cells were isolated from WT and SPC-DTR transgenic mice using a previously described protocol (17, 18). Briefly, after proteolytic digestion of lung tissue, the single-cell suspensions of lung tissue were depleted of macrophages using biotinylated anti-CD32 and anti-CD45 antibodies followed by streptavidin-coated magnetic particles and negative selection in a magnetic separator. The negative cells were plated overnight in 100-mm culture plates. The type II alveolar epithelial cells comprised the nonadherent population and were recovered for culture and mRNA extraction.

Diphtheria Toxin Administration

Six- to 8-week-old SPC-DTR transgenic and WT mice were intraperitoneally injected with DT once daily for up to 14 days (Sigma Chemical, St. Louis, MO) at doses of 10.0 and 12.5 μ g/kg in 100 μ l of PBS. Control transgenic and WT mice were injected for the same duration with 100 μ l of PBS alone. Mice were killed after the last DT injection or maintained for an additional 7 or 14 days. At the time of death, the right lung was removed for analysis of hydroxyproline content or RNA isolation, and the left lung was inflation-fixed at 25 cm H₂O and processed for histology.

Hydroxyproline Assay

Hydroxyproline content of the lung was measured as previously described with modifications (19). Briefly, at the time of death, lungs were excised from the experimental animals and homogenized in 0.5 ml of PBS. We added 0.5 ml of 12 N HCl to the homogenate and hydrolyzed the samples at 120°C for 24 hours. Thereafter, 5 μ l of each sample was combined with 5 μ l citrate/acetate buffer (238 mM citric acid, 1.2% glacial acetic acid, 532 mM sodium acetate, 85 mM sodium hydroxide) in a 96-well plate. We added 100 μ l of chloramine T solution (0.282 g chloramine T to 16 ml of citrate/acetate buffer, 2.0 ml of n-propanol, and 2.0 ml ddH₂O) and incubated the samples for 30 minutes at room temperature. Next, 100 μ l of Ehrlich's reagent (2.5 g *p*-dimethylaminobenzaldehyde added to 9.3 ml of n-propanol and 3.9 ml of 70% perchloric acid) was added, and the samples were incubated at 65°C for 30 minutes. The absorbance of each sample was then measured at 550 nm. Standard curves for the experiment were generated using known concentrations of reagent hydroxyproline (Sigma Chemical Co.).

Lung Histology

DTR transgenic and WT mice were treated with 100 μ l of intraperitoneal DT (10 μ g/kg) or an equal volume of PBS daily for 14 days. On Days 21 and 28, the left lung was inflation-fixed at 25 cm H₂O pressure with 10% neutral-buffered formalin, removed *en bloc*, fixed in 10% neutral-buffered formalin overnight, and paraffin embedded. Eight-micron sections were stained using Masson's trichrome and picrosirius red methods.

Real-Time PCR of Total Lung RNA for DTR, SPC, and Glyceraldehyde-3-phosphate Dehydrogenase

Total RNA was purified from 30 mg of lung tissue on Days 3, 7, and 14 after the initiation of DT treatment using the Absolutely RNA RT-PCR kit per the manufacturer's directions (Stratagene, La Jolla, CA). The total RNA was reverse transcribed to cDNA, and specific PCR products were generated using Brilliant SYBR Green RT-PCR master mix kit, 1 step (Stratagene), as per the manufacturer's instructions. cDNA conversion, amplification, and data analysis were performed using the

Mx3000P real-time PCR system computerized cycler (Stratagene) as previously described (20). We used the following primers that were selected using software available at <http://labtools.stratagene.com> and were synthesized and HPLC purified (Invitrogen Life Technologies): DTR (upper primer: 5'-GGAATCGGCTGG-GGACTGCTACCTCTGA-3'; lower primer: 5'-AGTTCAGCGTGTC-CGGCGAGGGCGAGG-3'; product size 239 bp), SPC (upper primer: CATCGTTGTGTATGACTACCA; lower primer: CCTGAAGTTCTGGAGTTTCT; product size 130 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (upper primer: TATGTCGTGGAGTCTACTGGT; lower primer: GAGTTGTCATATTTCTCGTGG; product size 149 bp). Primers were used at 75 nM each in 25- μ l reactions. Cycle parameters were as follows: 40 minutes at 55°C (reverse transcription step); 10 minutes at 95°C (denaturation step); and 40 cycles composed of 30 seconds at 95°C, 1 minute at 58°C, and 30 seconds at 72°C. Control wells containing no template were used to exclude the presence of contaminating DNA and to identify potential primer-dimer products from the dissociation curve analysis. The total RNA from each sample lung was analyzed for SPC and GAPDH mRNA in separate triplicate wells. The fluorescence values of the threshold cycles were collected at the end of the annealing step from each reaction. The threshold cycle values from GAPDH amplification were used to normalize specific mRNA quantification. Data are expressed as the relative change in specific mRNA expression in the DT-treated WT and transgenic animals compared with a pooled sample from PBS-treated transgenic mice, used as a calibrator. To correct for possible volume differences, transparency of the caps, or other well-to-well differences, the passive reference dye 5(6)-carboxy-X-Rhodamine-C5-maleimide was used in all reactions.

RT-PCR of Total RNA from Isolated Type II Alveolar Epithelial Cells

To assess for specific expression of the transgene, type II alveolar epithelial cells (AECs) were isolated from WT and transgenic mice as described above. Purified RNA (50 ng/50 μ l reaction) from these cells was reverse transcribed and amplified with primers specific for DTR, SPC, and GAPDH as described above. The RT-PCR reaction conditions were as follows: 48°C for 45 minutes for one cycle followed by 94°C for 1 minute, 67°C for 2 minutes, and 72°C for 2 minutes repeated for 30 cycles. The resultant products were analyzed by electrophoresis on a 2.2% FlashGel DNA cassette (Lonza, Rockland, ME) using the FlashGel system (Lonza).

In Vitro DT Treatment of Isolated Type II AECs

Isolated type II AECs from WT and DTR transgenic mice were plated on fibronectin-coated 96-well plates at a density of 200,000 cells/well. The cultures were then maintained in DMEM with 0.1% fetal calf serum to maintain SPC expression. After 48 hours in culture, a time point when the cells were still expressing SPC, a subset of wells were exposed to DT at a dose of 1.0 μ g/ml. Control wells received an equal volume of PBS. Twenty-four hours after DT exposure, the mitochondrial activity of the cultures was assessed with an MTT assay as described above.

Type II AEC Quantification

Immunohistochemistry was used to identify type II AECs within lung tissue sections. WT and SPC-DTR mice were treated with DT (10 μ g/kg) or PBS (100 μ l) for 1, 3, or 7 days ($n = 3-4$ at each time point). One hour before the animals were killed, bromodeoxyuridine (BrdU) was injected intraperitoneally. The left lung was then inflation-fixed at 25 cm H₂O pressure with 10% neutral-buffered formalin, removed *en bloc*, fixed in 10% neutral-buffered formalin overnight, and paraffin embedded. Eight-micron sections were deparaffinized in xylene and rehydrated in graded ethanol washes. The sections then underwent antigen retrieval by boiling for 20 minutes in citrate buffer (pH 6.0). For the purpose of BrdU staining, the specimens were treated with 2N HCl followed by 0.1 M borax buffer. The sections were then blocked with 5% donkey serum and stained with primary antibodies to BrdU (1:200) and SPC (1:2,000). An alkaline phosphatase-labeled anti-mouse secondary antibody was applied at a concentration of 1:250 for BrdU staining. At the same time, a biotin-labeled donkey anti-rabbit secondary antibody was applied for SPC staining. The Vector Laboratories alkaline phosphatase kit was used to produce a red color in BrdU-positive cells, and a Vector Laboratories DAB peroxidase substrate kit was used to generate a brown color in SPC-positive cells.

Statistical Analysis

The data are presented as means $\pm$ SEM. Statistics were performed using GraphPad Prism software. Mean values from the different groups were compared using one way ANOVA with *post hoc* pairwise comparisons using the Bonferroni correction. Survival difference between groups was analyzed using Kaplan-Meier curves and the log rank test.

RESULTS

Transfection of MLE-12 Cells with SPC-DTR

To assess the functionality of the SPC-DTR expression cassette before generating transgenic mice, we transfected MLE-12 cells with the pEGFP-N1 vector containing this gene construct. Two different ratios of transfection reagent and plasmid were used to optimize the efficiency of transgene expression. MLE-12 cells were chosen based on their murine origin (suggesting that they should be inherently resistant to DT) and their capacity to express SPC. Separate groups of control cells were left untreated or received the transfection reagent alone. All groups were then exposed to saline or DT (0.1 μ g/ml) for 24 hours. Thereafter, the cells were analyzed for viability using an MTT assay. We found that only cells transfected with the SPC-DTR expression cassette had a statistically significant DT-induced decrease in MTT activity (Figure 1). Control groups exposed to transfection reagent alone or left untreated displayed no change in viability with exposure to DT as compared with PBS.

In Vivo Expression of DTR mRNA

After confirming the fidelity of the SPC-DTR construct *in vitro*, we generated transgenic mice with the expression cassette on a C57BL/6 background. Four transgenic founders that were PCR positive for SPC-DTR originated from the initial microinjection procedure. Three of these founders successfully passed the transgene along to their offspring in a Mendelian pattern of inheritance resulting in three separate lines. The transgene was maintained in a heterozygous state by breeding SPC-DTR-positive mice with C57BL/6 partners. To confirm that DTR gene expression was lung specific, total RNA was isolated from the lung, heart, liver, kidney, and spleen of transgene-positive mice from two of the three lines. Littermates that were PCR negative for the transgene served as controls. RT-PCR was then performed with primers specific for the DTR message. RT-PCR of template RNA isolated from the lungs of three separate transgenic mice representing two of the three founder lines produced bands of expected size (Figure 2). On the other hand, template RNA isolated from other organs of these same mice yielded no bands in the reaction. RT-PCR of template RNA extracted from all organs of nontransgenic littermates (including the lungs) was negative for the DTR message. Based on these results, descendants from these two founder lines were used for subsequent studies.

To assess further for *in vivo* expression of DTR, we isolated type II alveolar epithelial cells identified by SPC staining from transgenic and WT mice. We then analyzed the DTR expression in these cells by extracting total RNA and performing real-time PCR. With primers specific for the DTR transcript, we identified expression only in the type II cells isolated from transgenic animals (Figure 3). The transgenic and WT cells demonstrated equivalent expression of the GAPDH house keeping gene. Both groups expressed equivalent amounts of SPC message.

Diphtheria Dose Explorations

After confirming lung-specific expression of DTR in our transgenic animals, we performed pilot experiments in which a single DT injection or two DT injections separated by 1 week were performed. Using doses of 50 μ g/kg per injection, we found no

Figure 1. Functional integrity of the murine surfactant protein C promoter and the diphtheria toxin receptor gene (SPC-DTR) expression cassette. MLE-12 cells were grown to 80% confluency in a 96-well plate in 100 μ l of media and treated with transfectant reagent alone, with transfectant reagent + the SPC-DTR expression cassette, or with phosphate-buffered saline (PBS) alone for 24 hours. The cells were washed, and subsets from each treatment group were exposed to diphtheria toxin (DT) or an equal volume of PBS for 24 hours. The metabolic activity of the cells was assessed with

a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are reported as the absorbance at 550 nm (mean $\pm$ SEM; n = 6 for each treatment condition). Each group is compared using a Student's *t* test.

obvious phenotypic change in the transgenic mice when analyzed 3 and 14 days after treatment (data not shown). Thereafter, to maximize our chance of observing an effect of DT in our transgenic mice, we administered daily injections of the toxin for 2 weeks and found consistent abnormalities in the transgenic animals using a range of doses from 10 to 25 μ g/kg per injection. Daily doses of 10.0 and 12.5 μ g/kg were used for the experiments reported below.

Weight Loss with Diphtheria Toxin Treatment

To assess for a treatment effect of DT dosing, we measured the body weights of transgenic and control animals over 4 weeks. DT and PBS were injected daily for 2 weeks, and the mice were monitored for an additional 2 weeks. Because no difference was seen between WT and transgenic mice receiving PBS, these groups were pooled for presentation. The administration of DT to the transgenic mice resulted in a marked weight loss that was initially detectable after 5 days of treatment (Figure 4). The rate of weight loss stabilized after the DT dosing was discontinued, but by 4 weeks, this group had lost on average approximately 1.5 g. DT-exposed WT and the PBS-treated groups gained weight, and on average these mice weighed 4.0 g more than the DT-treated transgenic mice at the end of the study period.

Figure 2. Tissue diphtheria toxin receptor (DTR) mRNA expression. Total RNA was isolated from lung, kidney, spleen, liver, and heart tissue of adult transgene-positive surfactant protein C ([SPC]-DTR+) and transgene-negative (SPC-DTR-) mice. The RNA was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) for expression of the DTR message with primers specific for this gene construct. The resultant products of the RT-PCR were separated on a 1.5% agarose gel and photographed. Each result is from a single mouse from two out of three reproductive founder lines.

Survival after Diphtheria Toxin Treatment

To assess whether the targeted type II AEC injury would cause mortality, transgenic and WT mice were treated with 10 or 12.5 μ g/kg of DT daily for 14 days. A control group of WT mice received intraperitoneal PBS daily for 14 days. The mice were observed daily for an additional week off of therapy; moribund mice were killed promptly. Daily injections of PBS did not cause death. WT mice treated with DT also exhibited no mortality. In contrast, DT exposure at 12.5 μ g/kg caused mortality in the transgenic group beginning on Day 18 and approaching 25% by 3 weeks, although this did not reach statistical significance (*P* = 0.12). The 10 μ g/kg dose of DT resulted in no mortality in the WT or transgenic groups.

Development of Pulmonary Fibrosis after Diphtheria Toxin Treatment

To assess the effect of DT on lung fibrosis, two groups of transgenic and WT mice received 10 μ g/kg of DT daily for 14 days. A separate group of control mice comprised of DTR-expressing and WT animals received daily PBS injections for 14 days. On Days 7, 14, 21, and 28, mice in each treatment arm were analyzed for lung collagen content using a hydroxyproline assay. After 7 days of treatment, there was no appreciable accumulation of collagen in the transgenic mice compared with

Figure 3. Type II alveolar epithelial cell diphtheria toxin receptor (DTR)mRNA expression. Type II alveolar epithelial cells were isolated from transgenic-positive surfactant protein C ([SPC]-DTR+) and wild-type (WT) (SPC-DTR-) control mice. RNA was extracted from the isolated cells and analyzed by real-time polymerase chain reaction with primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), SPC, and DTR. The products of the reverse transcription-polymerase chain reaction were separated on a 2.2% FlashGel cassette and photographed.

Figure 4. Weight loss associated with diphtheria toxin treatment. Transgenic surfactant protein C diphtheria toxin receptor (SPC-DTR) and wild-type (WT) mice were treated with daily intraperitoneal doses of diphtheria toxin (DT) at 10 $\mu\text{g/kg}$ in 100 μl of phosphate buffered saline (PBS) for 14 days. A control group of mixed transgenic and WT mice (SPC-DTR and WT) received daily intraperitoneal injections of 100 μl PBS for 14 days. The mice were weighed daily during the injections and then for a subsequent 14 days. Data are reported as the change in weight from Day 0 (mean $\pm$ SEM; $n = 6$ mice per group). The weight curves were compared by linear regression analysis. The slopes between SPC-DTR/DT and WT/DT significantly differ ($P < 0.001$), as do the curves between SPC-DTR/DT and SPC-DTR and WT/PBS ($P < 0.001$).

control mice (Figure 5). On Day 14, the SPC-DTR mice demonstrated a statistically significant increase in lung collagen content compared with the PBS-treated control group. On Days 21 and 28, treatment with DT induced a further increase in lung hydroxyproline in the SPC-DTR mice (Figure 5). On Day 21, the amount of lung collagen was approximately twofold greater in the DT-treated transgenic group when compared with the

control mice. In contrast, WT mice that received DT had similar levels of lung collagen as the PBS-treated control group.

The quantitative increase in lung collagen content was associated with qualitative changes in alveolar histology. The lungs of the DT-exposed (10 $\mu\text{g/kg}$) transgenic mice contained fibrotic lesions on Day 28, which were comprised of thickened alveolar walls, the accumulation of spindle-shaped cells, and the deposition of blue-staining collagen (Figure 6A). In general, the collagen accumulation occurred diffusely as demonstrated by picrosirius red staining (Figure 6B). Scattered areas of more confluent regions of fibrosis were occasionally observed. In the DT-treated WT mice, there were no areas of fibrosis, and the lungs were indistinguishable from PBS-injected control mice. A similar pattern of pathologic change was observed in mice treated with 12.5 $\mu\text{g/kg}$ of DT, but the confluent lesions were more severe (Figure 6C).

The Effect of Diphtheria Toxin on Type II AEC Function

To assess whether the DT-induced fibrosis occurred in conjunction with type II alveolar epithelial cell dysfunction, we measured the change in expression of type II cell-specific gene products after DT administration. Specifically, we administered the toxin daily to transgenic and nontransgenic mice for 3, 7 and 14 days. These time points were chosen to span the duration of the DT exposure. A separate group of DTR-positive mice was treated with PBS for the same duration and served as a control. After the last dose of DT, total RNA was extracted from the lungs and analyzed for DTR mRNA levels (Day 3) and for SPC mRNA levels (Days 3, 7, and 14) using real-time PCR. DT treatment of the transgenic animals caused a significant reduction in DTR expression relative to the PBS-treated transgenic group (Figure 7A) at the early time point. The DT-treated WT group had no transgene expression because these mice did not possess the transgenic construct. There was also a small decrease (10%) in mean SPC expression at Day 3 in the DT-treated transgenic group, but this was not statistically different from the control PBS-treated mice or from the DT-treated nontransgenic group. On Days 7 and 14,

Figure 5. Lung hydroxyproline content after diphtheria toxin treatment. In two separate experiments, transgenic surfactant protein C diphtheria toxin receptor⁺ (SPC-DTR⁺) and wild-type (WT) mice (SPC-DTR⁻) were treated with daily intraperitoneal doses of diphtheria toxin⁺ (DT⁺) at 10 $\mu\text{g/kg}$ in 100 μl of phosphate buffered saline for 14 days. A control group of mixed transgenic and WT (SPC-DTR^{+/+}) mice received daily intraperitoneal injections of 100 μl phosphate buffered saline (DT⁻) for 14 days. In the first experiment, the right lung from each mouse was excised and analyzed for hydroxyproline content on Day 21 ($n = 6-8$ per group). In the second experiment, the right lung from each mouse was excised and analyzed for hydroxyproline content on Day 28 ($n = 6$ per group). Data are reported as the mean hydroxyproline content ($\mu\text{g/ml}$) $\pm$ SEM. The three groups are compared using a one-way analysis of variance with a Bonferroni's *post hoc* multiple comparison test.

Figure 6. Lung histology after diphtheria toxin treatment. Transgenic surfactant protein C diphtheria toxin receptor (SPC-DTR) and wild-type (WT) mice were treated with daily intraperitoneal doses of (A and B) diphtheria toxin (DT) at 10 $\mu\text{g/kg}$ or (C) 12.5 $\mu\text{g/kg}$ in 100 μl of phosphate buffered saline for 14 days. A control group of transgenic mice (SPC-DTR) received daily intraperitoneal injections of 100 μl phosphate buffered saline for 14 days. On Day 28, the left lung was fixed at 25 cm H_2O and cut into 8- μm sections. Sections were stained using the Masson's Trichrome method (A and C) or with Picrosirius Red (B). Representative regions of the lung at 10 $\times$ and 20 $\times$ magnification are shown.

however, the expression of SPC was significantly reduced by DT administration in the transgenic mice as compared with DT-treated WT mice (Figure 7B).

To further assess the effect of DT on transgenic AECs, we isolated type II cells from uninjured SPC-DTR and WT mice. These cells were then maintained in culture for 2 days (a time point when they were still expressing SPC), and subsets were exposed to DT at a dose of 1.0 $\mu\text{g/ml}$. After 24 hours of exposure, we measured mitochondrial activity of the cultures via an MTT assay. DT treatment of the DTR-expressing cells produced a significant 22% reduction in MTT activity as compared with a 7% reduction in the control group (Figure 8).

The Effect of Diphtheria Toxin on Type II Cell Numbers

DT-mediated tissue-specific injury has been associated with cell depletion and cell dysfunction (21). To determine whether exposure of the SPC-DTR transgenic mice to the toxin resulted in type II cell loss, we isolated type II cells from uninjured transgenic and WT mice. Cells were maintained in culture for 2 days, and

subsets were exposed to DT at a dose of 1.0 $\mu\text{g/ml}$. After 24 hours of exposure, we counted the number of live and dead cells after trypan blue staining. Despite the decrease in MTT activity at this point, we found no statistical difference in the percent of dead cells in the DT-treated transgenic culture as compared with control transgenic cells (DT: $17 \pm 6\%$ vs. PBS: $12 \pm 1\%$). There was no evidence of DT-induced cell death in the WT cultures (DT: $6 \pm 4\%$ vs. PBS: $13 \pm 6\%$).

To assess the effect of DT treatment on type II cell numbers *in vivo*, we performed SPC and BrdU immunohistochemistry on lung sections of transgenic mice after 1, 3, and 7 days of DT treatment. Control groups included WT mice treated with DT and WT mice treated with PBS for the same duration. We then counted in a blinded fashion the number of SPC-positive and SPC-BrdU-double positive cells per 40 $\times$ high powered field (HPF). At each of the time points, we found no difference in the total number of SPC-positive cells per HPF in any group. On the other hand, the SPC-DTR mice had more SPC-BrdU double positive cells per HPF on Day 7 than the two control groups (Figure 9).

Figure 7. Diphtheria toxin receptor (DTR) and surfactant protein C (SPC) expression after diphtheria toxin treatment. Transgenic (SPC-DTR⁺) and WT (SPC-DTR⁻) mice were treated with daily intraperitoneal doses of diphtheria toxin (DT⁺) at 10 μ g/kg in 100 μ l of PBS for 3, 7, and 14 days. Control groups of transgenic mice (SPC-DTR⁺) received daily intraperitoneal injections of 100 μ l phosphate buffered saline (DT⁻) for 3, 7, and 14 days. After the last DT injection,

total RNA was isolated from the lungs of each mouse and analyzed by real-time polymerase chain reaction with primers specific (A) for the DTR or (B) for SPC. The expressions of DTR and SPC for each sample were normalized to the corresponding expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are reported as the relative expression of DTR and SPC compared with the control group of mice (mean $\pm$ SEM; n = 5–9 mice per group). The groups are compared using a one-way analysis of variance with a Bonferroni's *post hoc* multiple comparison test.

DISCUSSION

Administration of DT to mice expressing a lung specific DTR transgene caused scarring of the lung as assessed by lung collagen content and histological appearance. This induction of fibrosis was associated with a decrease in animal survival in a DT dose-dependent fashion and a decrease in the expression of type II cell gene products (i.e., SPC and DTR). However, the fibrosis and the changes in alveolar epithelial functions occurred in the absence of a detected decrease in type II cell number. Taken together, our results demonstrate that injury of the type II epithelium can directly induce pulmonary fibrosis.

The DT-mediated cell ablation strategy has been successfully used to model a variety of disorders, including glomerulosclerosis with the depletion of podocytes, osteoporosis with the depletion of osteocytes, acute hepatitis with the depletion of hepatocytes, and cardiomyopathy with the depletion of cardiomyocytes (16, 22–24). This model has also been used to deplete inflammatory cell populations including dendritic cells and monocyte/macrophages. In our investigation, 2 weeks of daily DT exposure reproducibly induced pulmonary fibrosis. The 10- μ g/kg dose of DT is in the range of what others have used in studies to ablate podocytes (20) and to specifically target neurons (23), hepatocytes (15), osteocytes (23), and monocytes/macrophages (21). A significantly higher dose (5,000 μ g/kg) was used to ablate cardiomyocytes (24). Our dosing approach differs from many of these prior studies (e.g., hepatocytes, cardiomyocytes, podocytes, and osteocytes), in that more than one administration of DT was used to achieve the fibrotic phenotype. A similar prolonged administration was used to assess the role of monocytes/macrophages in atherosclerotic plaque formation (21). There are several possible explanations for why repeated doses of DT were required for a phenotype in our model. One potential explanation is that alveolar progenitor cells can rapidly divide in response to cell injury, thus minimizing the consequences from a single dose of DT. Our data showing increased type II cell proliferation at Day 7 of DT exposure are consistent with this possibility. The most likely progenitors are residual uninjured type II cells that are not exposed to or are resistant to DT, although it is possible that the injured cells can also proliferate. A similar capacity for cell replacement has been seen in DTR-expressing monocyte/macrophages and splenic dendritic cells that recover to pretreatment numbers within 6 days after DT-induced depletion (25). Because of this ability to regenerate depleted cells, a more prolonged

dosing may be required to achieve persistent alteration of function. In contrast, the kidney has a limited capacity to regenerate podocytes, and therefore one dose of DT in rats that express podocyte-specific DTR causes glomerulosclerosis (22). Another potential mechanism for why multiple DT doses were required to generate pulmonary fibrosis is that the level of DTR expression may be relatively low in our transgenic mice. In the model of acute hepatitis, the amount of DTR expressed in the different founder lines was inversely correlated with the dose of toxin required to cause hepatic injury and death (16). We must also consider the possibility that type II cells become resistant to DT after multiple administrations. This scenario was also postulated to explain why prolonged DT dosing produced only a 50% decrease in peripheral monocytes that were engineered to express DTR (21). Cleavage of the receptor from the surface of expressing cells by proteases may provide a mechanism of resistance (26). Finally, a shorter course of DT treatment may be sufficient to cause significant type II cell depletion and resultant fibrosis in our model. Thus far, we have compared only single doses of DT with the 14-day administration protocol. Our observations that DTR

Figure 8. MTT activity after DT treatment of primary type II alveolar epithelial cells. Type II alveolar epithelial cells were isolated from transgenic surfactant protein C diphtheria toxin receptor⁺ (SPC-DTR⁺) and wild-type (WT) control mice. The cells were maintained in culture in a 96-well plate for 3 days, and subsets were treated with diphtheria toxin (DT) (1.0 μ g/ml) or an equal volume of phosphate buffered saline for 24 hours. The mitochondrial activity of the cells was then assessed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are reported as the decrease in MTT activity of the DT-treated cells compared with PBS-treated cells (n = 8) $\pm$ SEM. The WT and SPC-DTR groups are compared using Student's *t* test.

Figure 9. Immunohistochemical staining for SPC and BrdU. Transgenic surfactant protein C diphtheria toxin receptor (SPC-DTR) and wild type (WT) mice were treated with daily intraperitoneal doses of diphtheria toxin (DT) at 10 μ g/kg in 100 μ l of phosphate buffered saline for 1, 3, and 7 days. A group of WT mice received no treatment and served as the Day 0 control group. One hour before death, each animal received an intraperitoneal injection of bromodeoxyuridine (BrdU). After the last DT injection, the lungs from each animal were fixed by distending at 25 cm H₂O with 10% neutral-buffered formalin, removed *en bloc*, further fixed in 10% neutral-buffered formalin overnight, and embedded in paraffin. Eight-micron sections were immunostained for SPC and BrdU. The number of SPC-positive (brown stained) and SPC-BrdU double-positive (brown-red stained) cells per 40 $\times$ high-powered field were counted. *Upper panel:* Representative images of DT-treated WT and SPC-DTR mice. Arrows point to BrdU-positive cells, and arrowheads point to double-stained cells. *Lower panel:* Graphic representation of (A) the number of SPC-positive cells and (B) SPC-BrdU double-stained cells per high-powered field. Results are reported as the mean $\pm$ SEM ($n = 3-4$ per

group). The WT and SPC-DTR groups are compared using a two-way ANOVA. The effect on genotype on SPC-BrdU is statistically significant ($P < 0.05$). There is no effect of genotype or day on the number of SPC-positive cells ($P =$ not significant).

expression is significantly decreased after 3 days of toxin exposure and SPC expression is decreased after 7 days of treatment suggest that a shorter course of treatment may be adequate to induce scarring.

DT mediates cell toxicity by catalyzing the ADP-ribosylation of elongation factor 2, thereby interfering with protein synthesis (16). This disruption of protein synthesis is classically reported to induce apoptotic cell death. However, the DT model has also been shown to induce autophagy in cardiomyocytes (24). Furthermore, when this approach was used to target osteocytes, electron microscopic examination revealed that the DT-induced injury could lead to necrosis as well as apoptosis (23). Cell-specific targeting with DT can also induce cellular dysfunction without causing death, as was demonstrated for monocyte/macrophage injury in an atherosclerosis model (21). Targeting the monocyte/macrophage population with DT in this report not only reduced the number of cells but also impaired LDL uptake by 54% in the remaining viable cells. In our studies, we demonstrate an alteration of type II cell function after DT treatment of the transgenic mice. DT may also induce type II cell death based on several observations. First, we found that type II cell proliferation increases in the SPC-DTR animals after DT exposure while the total number of type II cells remains constant. The stable number of total cells in the setting of proliferation suggests that the dividing cells are replacing a transiently depleted subset of the type II epithelium. Second, our *in vitro* results demonstrated a trend toward increased cell death in isolated transgenic type II cells after 24 hours of DT treatment. Perhaps a longer *in vitro* exposure to the toxin would enhance this effect.

One other prior report has examined the effect of DT-mediated lung cell ablation (27). In this model, the DT receptor was expressed by the lysozyme M promoter with the intent of depleting macrophages. The authors found DT administration at doses of 10 and 40 μ g/kg to cause acute lung injury and death in

the transgenic mice within 4 to 6 days. Evaluation of histological sections revealed a loss of type II cells and macrophages. The authors then performed a WT bone marrow transplant into the transgenic group to replace the DT-sensitive alveolar macrophages with DT-resistant cells. Treatment of these mice with DT caused acute lung injury with loss of type II cells even though the alveolar macrophages were now resistant to the toxin. This led the authors to conclude that the DT-induced acute lung injury was the result of an insult to the type II epithelium. Several explanations may account for why the ablation of type II pneumocytes in this model caused a different phenotype as compared with our model. First, expressing DTR off of the lysozyme M promoter may not be specific for type II cells as is the SPC promoter (28). As a result, other cells comprising the alveolar wall in addition to macrophages and type II cells may be damaged. Second, the more severe phenotype observed in the lysozyme M model may be the result of this promoter inducing higher levels of DTR expression in the type II cells or an increased number of DTR-expressing type II cells. One might predict that the more efficient targeting of a substantial proportion of the type II cells or a more diffuse injury to the alveolar wall would cause acute lung injury and death. Regardless of the mechanism of acute lung injury in their model, no data regarding the development of fibrosis in response to the insult were presented.

The mechanism by which impaired type II function leads to fibrosis requires further study. One possibility is that injury impairs the homeostatic ability of the lung to replace type I cells lost during normal attrition. Damage of the type II epithelium may also lead to the loss of important signals that suppress lung fibroblasts proliferation and collagen production. For example, alveolar epithelial cells are an important source of prostaglandin E₂, which has been shown to inhibit multiple aspects of the fibroproliferative response, including fibroblast chemotaxis, fibroblast proliferation, and collagen synthesis (29-33). A loss of

type II alveolar epithelial cells could diminish intraalveolar levels of this antifibrotic mediator. Finally, the residual injured type II epithelium may be a source of profibrotic factors such as TGF- β that directly activate local fibroblasts.

Establishing a causal link between type II cell loss and lung scarring provides new insights into the pathogenesis of familial and sporadic cases of IPF. First, our findings support the conclusions of Thomas and colleagues that the SPC mutation they identified in familial IPF, which reduces type II cell function and viability, predisposes to fibrosis (14). Our findings also provide support for a casual relationship between the type II cell apoptosis seen in IPF biopsies and the development of alveolar scarring (32). The link between telomerase mutations and pulmonary fibrosis identified in multiple kindreds implies that some key cell in the lungs of patients with IPF has decreased proliferative capacity (34, 35). From our data, we speculate that the type II alveolar epithelial cell may be this critical cell. Aberrant type II cell reparative capacity could also explain why IPF is diagnosed in older adults (36–38) in whom senescence of these alveolar progenitor cells may prevent adequate replacement when epithelium is damaged during normal daily life.

In summary, we have generated a new mouse model of pulmonary fibrosis caused by the targeted depletion of type II alveolar epithelial cells. Our findings provide direct evidence specifically linking the targeting of type II cells for injury to the development of lung fibrosis. Based on our findings and mounting clinical evidence, we speculate that alveolar epithelial cell damage may play a central role in the pathogenesis of IPF. Our model should be useful for elucidating the downstream mechanisms by which targeting of type II cells leads to fibroblast proliferation, collagen deposition, and alveolar disruption.

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.

References

- Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001;134:136–151.
- Selman M, Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killers. *Proc Am Thorac Soc* 2006;3:364–372.
- Katzenstein AA. Pathogenesis of “fibrosis” in interstitial pneumonia: an electron microscopic study. *Hum Pathol* 1985;16:1015–1024.
- Kasper M, Haroske G. Alterations in the alveolar epithelium after injury leading to pulmonary fibrosis. *Histol Histopathol* 1996;11:463–483.
- Kawanami O, Ferrans VJ, Crystal RG. Structure of alveolar epithelial cells in patients with fibrotic lung disorders. *Lab Invest* 1982;46:39–53.
- Adamson IY, Bowden DH. Bleomycin-induced injury and metaplasia of alveolar type 2 cells: relationship of cellular responses to drug presence in the lung. *Am J Pathol* 1979;96:531–544.
- Hagimoto N, Kuwano K, Miyazaki H, Kunitake R, Fujita M, Kawasaki M, Kaneko Y, Hara N. Induction of apoptosis and pulmonary fibrosis in mice in response to ligation of Fas antigen. *Am J Respir Cell Mol Biol* 1997;17:272–278.
- Wang R, Ibarra-Sunga O, Verlinski L, Pick R, Uhal BD. Abrogation of bleomycin-induced epithelial apoptosis and lung fibrosis by captopril or by a caspase inhibitor. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L143–L151.
- Budinger GR, Mutlu GM, Eisenbart J, Fuller AC, Bellmeyer AA, Baker CM, Wilson M, Ridge K, Barrett TA, Lee VY, et al. Proapoptotic Bid is required for pulmonary fibrosis. *Proc Natl Acad Sci USA* 2006;103:4604–4609.
- Morishima Y, Nomura A, Uchida Y, Noguchi Y, Sakamoto T, Ishii Y, Goto Y, Masuyama K, Zhang MJ, Hirano K, et al. Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. *Am J Respir Cell Mol Biol* 2001;24:1–11.
- Atabai K, Ishigaki M, Geiser T, Ueki I, Matthay MA, Ware LB. Keratinocyte growth factor can enhance alveolar epithelial repair by nonmitogenic mechanisms. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L163–L169.
- Dohi M, Hasegawa T, Yamamoto K, Marshall BC. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. *Am J Respir Crit Care Med* 2000;162:2302–2307.
- Kim HJ, Sammak PJ, Ingbar DH. Hepatocyte growth factor stimulates migration of type II alveolar epithelial cells on the provisional matrix proteins fibronectin and fibrinogen. *Chest* 1999;116:94S–95S.
- Thomas AQ, Lane K, Phillips J III, Prince M, Markin C, Speer M, Schwartz DA, Gaddipati R, Marney A, Johnson J, et al. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med* 2002;165:1322–1328.
- Naglich JG, Metherall JE, Russell DW, Eidel L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* 1992;69:1051–1061.
- Saito M, Iwakawa T, Taya C, Yonekawa H, Noda M, Inui Y, Mekada E, Kimata Y, Tsuru A, Kohno K. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol* 2001;19:746–750.
- Corti M, Brody AR, Harrison JH. Isolation and primary culture of murine alveolar type II cells. *Am J Respir Cell Mol Biol* 1996;14:309–315.
- Lazar MH, Christensen PJ, Du M, Yu B, Subbotina NM, Hanson KE, Hansen JM, White ES, Simon RH, Sisson TH. Plasminogen activator inhibitor-1 impairs alveolar epithelial repair by binding to vitronectin. *Am J Respir Cell Mol Biol* 2004;31:672–678.
- Sisson TH, Hanson KE, Subbotina N, Patwardhan A, Hattori N, Simon RH. Inducible lung-specific urokinase expression reduces fibrosis and mortality after lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L1023–L1032.
- Punturieri A, Alviani RS, Polak T, Copper P, Sonstein J, Curtis JL. Specific engagement of TLR4 or TLR3 does not lead to IFN- β -mediated innate signal amplification and STAT1 phosphorylation in resident murine alveolar macrophages. *J Immunol* 2004;173:1033–1042.
- Stoneman V, Braganza D, Figg N, Mercer J, Lang R, Goddard M, Bennett M. Monocyte/macrophage suppression in CD11b diphtheria toxin receptor transgenic mice differentially affects atherosclerosis and established plaques. *Circ Res* 2007;100:884–893.
- Wharram BL, Goyal M, Wiggins JE, Sanden SK, Hussain S, Filipiak WE, Saunders TL, Dysko RC, Kohno K, Holzman LB, et al. Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene. *J Am Soc Nephrol* 2005;16:2941–2952.
- Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab* 2007;5:464–475.
- Akazawa H, Komazaki S, Shimomura H, Terasaki F, Zou Y, Takano H, Nagai T, Komuro I. Diphtheria toxin-induced autophagic cardiomyocyte death plays a pathogenic role in mouse model of heart failure. *J Biol Chem* 2004;279:41095–41103.
- Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, et al. In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* 2002;17:211–220.
- Furukawa N, Saito M, Hakoshima T, Kohno K. A diphtheria toxin receptor deficient in epidermal growth factor-like biological activity. *J Biochem* 2006;140:831–841.
- Miyake Y, Kaise H, Isono K, Koseki H, Kohno K, Tanaka M. Protective role of macrophages in noninflammatory lung injury caused by selective ablation of alveolar epithelial type II cells. *J Immunol* 2007;178:5001–5009.
- Cross M, Mangelsdorf I, Wedel A, Renkawitz R. Mouse lysozyme M gene: isolation, characterization, and expression studies. *Proc Natl Acad Sci USA* 1988;85:6232–6236.
- Lipchik RJ, Chauncey JB, Paine R, Simon RH, Peters-Golden M. Arachidonate metabolism increases as rat alveolar type II cells differentiate in vitro. *Am J Physiol* 1990;259:L73–L80.
- Bitterman PB, Wewers MD, Rennard SI, Adelberg S, Crystal RG. Modulation of alveolar macrophage-driven fibroblast proliferation by alternative macrophage mediators. *J Clin Invest* 1986;77:700–708.
- Elias JA, Rossman MD, Zurier RB, Daniele RP. Human alveolar macrophage inhibition of lung fibroblast growth: a prostaglandin-dependent process. *Am Rev Respir Dis* 1985;131:94–99.
- Goldstein RH, Polgar P. The effect and interaction of bradykinin and prostaglandins on protein and collagen production by lung fibroblasts. *J Biol Chem* 1982;257:8630–8633.

33. Korn JH, Halushka PV, LeRoy EC. Mononuclear cell modulation of connective tissue function: suppression of fibroblast growth by stimulation of endogenous prostaglandin production. *J Clin Invest* 1980;65:543–554.
34. Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, Rosenblatt RL, Shay JW, Garcia CK. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci USA* 2007;104:7552–7557.
35. Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA III, *et al.* Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med* 2007;356:1317–1326.
36. Flaherty KR, Mumford JA, Murray S, Kazerooni EA, Gross BH, Colby TV, Travis WD, Flint A, Toews GB, Lynch JP III, *et al.* Prognostic implications of physiologic and radiographic changes in idiopathic interstitial pneumonia. *Am J Respir Crit Care Med* 2003;168:543–548.
37. Flaherty KR, Thwaite EL, Kazerooni EA, Gross BH, Toews GB, Colby TV, Travis WD, Mumford JA, Murray S, Flint A, *et al.* Radiological versus histological diagnosis in UIP and NSIP: survival implications. *Thorax* 2003;58:143–148.
38. Veeraraghavan S, Latsi PI, Wells AU, Pantelidis P, Nicholson AG, Colby TV, Haslam PL, Renzoni EA, du Bois RM. BAL findings in idiopathic nonspecific interstitial pneumonia and usual interstitial pneumonia. *Eur Respir J* 2003;22:239–244.