Reparative Capacity of Airway Epithelium Impacts Deposition and Remodeling of Extracellular Matrix

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Defective epithelial repair in the setting of chronic lung disease has been suggested to contribute to uncontrolled extracellular matrix (ECM) deposition and development of fibrosis. We sought to directly test this hypothesis through gene expression profiling of total lung RNA isolated from mouse models of selective epithelial cell injury that are associated with either productive or abortive repair. Analysis of gene expression in repairing lungs of naphthalene-exposed mice revealed prominent clusters of up-regulated genes with putative roles in regulation of the extracellular matrix and cellular proliferation. Further analysis of tenascin C (*Tnc*), a representative matrix protein, in total lung RNA revealed a transient 4.5-fold increase in mRNA abundance 1 day after injury and a return to steady-state levels by Recovery Day 3. *Tnc* was deposited by the peribronchiolar mesenchyme immediately after injury and was remodeled to basement membrane subtending the bronchiolar epithelium during epithelial repair. Epithelial restitution was accompanied by a decrease in *Tnc* mRNA and protein expression to steady-state levels. In contrast, abortive repair using a transgenic model allowing ablation of all reparative cells led to a progressive increase in *Tnc* mRNA within lung tissue and accumulation of its gene product within the sub-epithelial mesenchyme of both conducting airways and alveoli. These data demonstrate that the ECM is dynamically remodeled in response to selective epithelial cell injury and that this process is activated without resolution in the setting of defective airway epithelial repair.

Keywords: airway epithelium; repair; Clara cell; extracellular matrix; fibrosis

The epithelial–mesenchymal trophic unit (EMTU) is a complex arrangement of epithelial cells, fibroblasts, smooth muscle, and extracellular matrix (1). Dynamic interactions between these components are critical for the process of lung development and continue into adulthood for regulation of normal tissue homeostasis and repair (2, 3). Chronic lung injury leads to permanent alterations to both cellular and extracellular components of the EMTU that lead to permanent declines in lung function. This is clearly seen in chronic lung diseases such as asthma and chronic obstructive pulmonary disease. Pathological changes to lung tissue that are associated with these diseases include fibroproliferation, accumulation and remodeling of extracellular matrix components, and altered epithelial cell function, which together lead to airway obstruction (4–6). However, the complexity of these interactions in the intact lung, and the inability to

CLINICAL RELEVANCE

This study demonstrates *in vivo* that airway epithelial reparative capacity regulates extracellular matrix (ECM) deposition and turnover. As such, this study suggests that excessive ECM deposition in chronic fibroproliferative lung disease may be a result of defective epithelial repair.

effectively model these interactions *in vitro*, have led to difficulties in identifying critical factors that initiate the process of pathological tissue remodeling. Roles for epithelial cell dysfunction in the initiation and/or progression of this process have been proposed, although not tested *in vivo* (1, 7).

The airway epithelium provides both a physical and biological barrier that functions to protect the host against invading microorganisms and inhaled pollutants (8–12). The epithelium of bronchiolar airways turns over with very slow kinetics and is maintained in the steady-state by the infrequent proliferation of an abundant facultative progenitor cell that is commonly referred to as the Clara cell (13–16; A. Giangreco and coworkers, unpublished data). Injury resulting in the depletion of Clara cells, such as that resulting from exposure to the aromatic hydrocarbon naphthalene, is repaired through the activation of local tissue stem cells residing at airway branch point associated neuroepithelial bodies (NEBs) and the bronchioalveolar duct junction (BADJ) (14, 17–20). Further studies have revealed that stem cells localized at the BADJ, termed bronchioalveolar stem cells (BASCs), can be identified *in vivo* by co-expression of Clara cell secretory protein (CCSP) and surfactant protein C (SPC), isolated according to cell surface phenotype, and may have the *in vitro* capacity to generate cells of both bronchiolar and alveolar lineages (20). Further research is necessary to understand the microenvironmental cues that regulate stem cell-mediated epithelial repair.

Roles for matrix remodeling in regulation of airway repair are suggested from studies investigating mouse models carrying null alleles for selected matrix metalloproteinases (MMPs). MMPs are a family of membrane-associated or secreted proteases that, upon activation, have the ability to degrade components of the ECM, epithelial cell junctions, and liberate tethered growth and chemotactic factors in response to wounding (21). In recent work, MMPs have been shown to play key roles in regulating epithelial repair in the lung (22, 23). Chen and coworkers demonstrated that tissue inhibitor of metalloproteinase (TIMP)1 functions to inhibit airway epithelial repair, raising the possibility that increased levels of TIMP1 that are observed in diseases such as obliterative bronchiolitis may both attenuate epithelial reparative capacity and promote fibrosis (23). These studies reinforce the concept that epithelial defects within the EMTU may initiate uncontrolled fibroproliferation and ECM deposition (1). *In vitro* studies have shown that inhibition of epithelial repair can augment pro-fibrotic signaling pathways, thereby supporting this model (24). However, this paradigm has yet to be tested directly.

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In this study we test the hypothesis that epithelial reparative capacity is a key regulator of extracellular matrix remodeling. We used previously validated *in vivo* models of Clara cell ablation to demonstrate that ECM deposition, as measured by Tenascin C (*Tnc*) mRNA and protein expression, is dynamically and reversibly regulated during repair of epithelial lesions in airways. In contrast, we show that loss of epithelial reparative capacity results in continued deposition of ECM without resolution. This study provides the first *in vivo* evidence that ECM deposition is a dynamic component of the EMTU during productive airway epithelial repair and suggests that defects to epithelial reparative capacity directly lead to matrix deposition through alterations within the EMTU.

MATERIALS AND METHODS

Animal Housing

Mice used in this study were housed in an AAALAC-certified institutional vivarium under specific pathogen-free conditions, 12-hour light/dark cycle, provided food and water *ad libitum*, and sentinels screened quarterly for pathogens. All mouse experimental procedures were approved by Duke and University of Pittsburgh IACUC.

Models of Airway Injury

Ganciclovir administration. Adult FVB/N C57BL/6 mice were exposed to ganciclovir (GCV) as previously described (25, 26). Cytovene IV (GCV sodium; Hoffman-LaRoche, Inc., Nutley, NJ) was dissolved in pyrogen-free saline and administered chronically through a 14 d mini-osmotic pump (ALZET Osmotic Pumps, #2109D; ALZA Corp., Palo Alto, CA), resulting in a dosage of 4.5 mg/kg ganciclovir daily. Body weights were measured to evaluate in real time the extent of injury.

Naphthalene administration. Male adult FVB/N (The Jackson Laboratory, Bar Harbor, ME) mice were injected intraperitoneally with naphthalene (Sigma, St. Louis, MO) dissolved in sterile Mazola corn oil as previously described (18). In brief, mice were injected with naphthalene (dosage indicated in text) in the morning (8:00–10:00 A.M.) to normalize to diurnal fluctuations in glutathione. Body weights were measured to evaluate in real time the extent of injury.

Tissue Processing

At the time of killing, mice were anesthetized through an intraperitoneal injection of ketamine and xylazine. After entry into a surgical plane, mice were dissected and the thoracic cavities opened. Plasma was collected from the right atrium using a 1-ml tuberculin syringe followed by separation at $10,000 \times g$ for 10 minutes in a BD Microcontainer plasma separator tube (BD Microtainer; Becton Dickinson, Franklin Lakes, NJ). Mice were exsanguinated through scission of the inferior vena cava and trachea cannulated with an intravenous catheter (BD Insyte; Becton Dickinson). Bronchoalveolar lavage (BAL) fluid was collected in 1 ml of PBS. The left lung and accessory lobe were resected and homogenized in 2 ml 4M GIC and RIPA buffer with 1 mM PMSF for total RNA and protein isolation, respectively. The remaining right lung lobes were inflation-fixed at constant pressure for 20 minutes in 10% (vol/vol) buffered formaldehyde (Ricca Chemical Co., Arlington, TX). The right cranial lobe was cryopreserved in optimal cutting temperature (OCT) medium, sectioned in 5- μ m increments with a cryostat (Microm HM550; Microm, Waldorf, Germany), and adhered to positively charged microscope slides (Unifrost Plus Microscopic Slides, EMSC200W+; Ever Scientific, Morgantown, PA).

RNA Isolation and Microarray Analysis

RNA was isolated from left lung according to protocol by Chomczynski and Sacchi (27). RNA quality was determined using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent, Foster City, CA). All Microarray analysis was performed at the University of Pittsburgh Cancer Center Clinical Microarray core using Codelink 20K Mouse Bioarrays (Applied Microarrays, Tempe, AZ) according to manufacturers' suggested protocol. Bioinformatic analysis was conducted as previously described

(28). Differentially expressed genes were determined using the Scoregenes software package (Jerusalem, Israel). Relative change in gene expression was determined in a pairwise comparison of treatment to control. A significant change in gene expression passed the following criteria: *t* test *P* value < 0.01, threshold number of misclassifications (TNOM) = 0, and regulation $\geq$ 2-fold relative to control. Genes fulfilling these criteria were hierarchically clustered using Scoregenes and visualized as a heat-map in Treeview (Eisen Laboratory, LBL, Berkeley, CA). Microarray analysis is MIAME compliant and will be deposited into GEO at the time of publication.

TaqMan PCR

Determination of relative mRNA abundance in total lung RNA was determined using quantitative real-time PCR according to established protocol. In brief, RNA was reverse-transcribed using a High-Capacity cDNA Synthesis Kit (Applied Biosystems, Foster City, CA) and a Veriti Thermocycler (Applied Biosystems). cDNA, RealMaster Mix (Qiagen, Valencia, CA), and gene-specific Taqman FAM-labeled probes and primers (Applied Biosystems) were combined into a 96-well optical plate using the EpMotion 5070 automated pipetting system (Eppendorf, Hamburg, Germany) to increase precision of real-time data. Real-time PCR was conducted on a Realplex⁴ EppgradientS (Eppendorf) according to protocol. The $\Delta\Delta C_T$ for real-time PCR was calculated to determine relative mRNA abundance (29). An unpaired one-tailed Student's *t* test was used to determine statistical significance.

Enzyme-Linked Immunosorbent Assay

We assayed CCSP protein in BAL and plasma samples with a CCSP sandwich ELISA. In brief, we IgG purified (Millipore, Billerica, MA) goat anti rat-CCSP antiserum (capture antibody), diluted in ELISA coating buffer, and incubated in a 96-well polystyrene high binding plate overnight (Costar 3590; Thermofisher, Pittsburgh, PA). After sample and rCCSP standard incubation, we probed with rabbit anti rat-CCSP antiserum (primary detection antibody) and goat anti rabbit-HRP (Bio-Rad, Hercules, CA). Absorbance was measured at 450 nm using a microplate reader (EL808 Ultra Microplate Reader; Bio-tek Instruments, Inc., Winooski, VT).

Immunofluorescence and Microscopy

Four-color immunofluorescence imaging was conducted using the following primary/secondary antibodies and nuclear stains. **Primary antibodies:** goat anti rabbit-CCSP (In House), rabbit anti-human *Tnc* (Chemicon, Millipore), Mouse_{IgG2a} anti- α -smooth muscle actin (Sigma). **Secondary antibodies:** donkey anti-goat 660 (Invitrogen, Carlsbad, CA), donkey anti-rabbit 488 (Invitrogen), goat anti-mouse_{IgG2a}-594 (Invitrogen). **Nuclear stain:** DAPI (Sigma). Tissue sections were post-fixed in 100% ethanol for 10 minutes and rehydrated with sequential 3-minute washes in 95, 85, and 70% ethanol. The sections were incubated with Trypsin (Cellgro, Manassas, VA) for 5 minutes at 37°C and blocked in 5%BSA/PBS for 30 minutes. Primary antibody was diluted in 5%BSA/PBS and applied to each section overnight at 4°C in a humidified chamber followed by secondary antibody incubation in 5%BSA/PBS for 1 hour. Cross-reactivity of secondary antibodies was eliminated by blocking with normal goat serum. The slides were cover-slipped with DAPI (Sigma) and Fluoromont G mounting media (Southern Biotech, Birmingham, AL). Microscopic analysis was conducted using a Zeiss Observer.Z1 inverted fluorescent microscope (Carl Zeiss, Inc., Gottingen, Germany) and images processed in AxioVision Release 4.6.3 (Carl Zeiss) software.

RESULTS

Airway Epithelial Injury and Repair Regulates Pro-Mitotic Signaling Pathways and ECM Dynamics

Naphthalene-induced acute Clara cell injury is repaired through activation of endogenous bronchiolar stem cells (14, 19). During repair both the epithelium, the initial target site of injury, and the underlying peribronchiolar mesenchyme proliferate throughout the reparative phase (19). To better define the cellular and molecular dynamics of the reparative process after naphthalene exposure, genome-wide mRNA expression profiling was per-

formed. Wild-type FVB/N adult male mice were exposed to 275 mg/kg naphthalene and RNA isolated from total lung homogenate from unexposed control and 1, 2, 3, or 6 days after injury and used to screen oligonucleotide microarrays ($n = 4$ /exposure group). Bioinformatic analysis identified a total of 4,751 transcripts with a significant (> 2 -fold) change in gene expression. Genes were hierarchically clustered according to expression and displayed in Figure 1A as a heat-map. Using these selection criteria the gene expression signature of the repairing airway was determined to comprise 3,552 up-regulated and 1,199 down-regulated gene transcripts (Figure 1A, † and ‡, respectively).

We previously established that the majority of down-regulated genes directly reflected loss of Clara cells and provided new tools to investigate the dynamics of Clara cell loss and regeneration (28). In this study, we focused solely on up-regulated gene transcripts, specifically those involved in regulation of cell proliferation and matrix remodeling. Relative mRNA abundance of representative genes from nodes 89, 176, and 534 (Figure 1B) were analyzed by real-time PCR to validate the bioinformatic process as well as verify gene expression changes to candidate signaling pathways (Figures 2A–2F, 2G, and 2H, respectively). Node 89, 176, and 534 gene names and fold change relative to control are listed in Tables E1, E2, and E3 in the online supplement, respectively. Genes selected for validation included *Akp2*, a potential marker for tissue stem cells (Figure 2A), pro-mitotic signaling molecules (Figures 2B, 2C, and 2G), ECM modifiers (Figures 2D and 2E), and ECM (Figures 2F and 2H). Each of these genes demonstrated

significant changes in gene expression over the time course of naphthalene-induced airway injury and repair that were predicted by microarray analysis.

We next sought to determine the expression pattern of selected genes identified by microarray screening through the processes of airway injury, repair, and resolution. Changes in the mRNA abundance of amphiregulin (*Areg*), tumor necrosis factor receptor superfamily member *Tnfsfr12a*, and *Tnc* were evaluated by real-time PCR analysis of total lung RNA isolated from unexposed controls, 1, 2.5, 4, 8, and 12 hours or 1, 2, 3, 5, 10, and 30 days after naphthalene (250 mg/kg) injury (Figure 3). Naphthalene-induced airway injury resulted in a rapid and dramatic increase in the abundance of *Areg* mRNA that was elevated by 3.1-fold 1.0 hour after exposure and reached a maximum increase of 12.8-fold 8.0 hours after exposure (Figure 3A). Messenger RNA abundance for *Areg* remained significantly elevated during the reparative phase after naphthalene exposure that included recovery day 5, and returned to near steady-state levels by Days 10 and 30 (Figure 3A). Expression of *Tnfsfr12a* mRNA showed a similar profile of expression to that of *Areg*, differences being a delay in gene induction and more rapid return to baseline levels (Figure 3B). Peak induction of *Tnfsfr12a* occurred at the same 8-hour repair time point as observed for *Areg*, achieving more than 6-fold the level observed in steady-state lung. Expression of *Tnc* mRNA was significantly increased by 2.5-fold at 8.0 hours after injury, achieved peak levels of 5.8-fold steady-state levels at Recovery Day 1, and return to near steady-state levels by 5 days (Figure

Figure 1. The gene expression signature of repairing airway epithelium. RNA from control (0) and 1, 2, 3, or 6 days after naphthalene-induced injury was isolated from whole lung homogenate and used for microarray analysis ($n = 4$ /exposure group). Statistically significant gene expression changes relative to control were clustered and presented as a heat map. Red and green indicate down- and up-regulated gene expression relative to control, respectively. Heat map representation of (A) all significant gene clusters and (B) magnification of the most informative gene clusters. († and ‡ denote clusters that are up-regulated and down-regulated relative to control, respectively).

Figure 2. Real-time PCR validation of microarray results. Messenger RNA abundance in total lung RNA samples from control (0) and 1, 2, 3, or 6 days after naphthalene injury was determined by real-time PCR analysis using Taqman probes and primers. Clusters were validated by quantifying mRNA abundance of representative genes from (A–F) Node 89, (G) Node 176, and (H) Node 534. Data are presented as mean $\pm$ SEM. * unpaired student's t-test $P < 0.05$.

3C). These data indicate that robust and transient up-regulation of transcripts encoding pro-mitotic signaling molecules and ECM occurs after naphthalene-induced Clara cell ablation and the ensuing phase of tissue repair.

ECM Is Reversibly and Dynamically Regulated during Airway Epithelial Repair

We next sought to define the kinetics of *Tnc* protein accumulation during airway injury and repair. *Tnc* was localized by immunofluorescence and the relationship to epithelial renewal and alterations in peribronchiolar mesenchyme evaluated through colocalization with CCSP (Figures 4A, 4D, and 4E) and α -smooth muscle actin (α -SMA; Figures 4C–4E), respectively. Localization of *Tnc* immunoreactivity within the steady-state epithelium revealed a subset of weakly positive mesenchymal cells that were positive for the myofibroblast/smooth muscle cell marker α -SMA (Figures 4B–4D). No *Tnc*-immunoreactive protein was detected within other cell types of the airway or alveolar compartments. Magnification of Figure 4D revealed the intense colocalization of *Tnc* and α -SMA (Figure 4E). Acute Clara cell injury, as assessed by CCSP immunolocalization, was prominent by Day 1 (Figures 4F, 4I, and 4J). The distribution and abundance of *Tnc* immunoreactivity was dramatically altered during the early phase of repair after naphthalene exposure. Analysis of *Tnc* immunoreactivity at 1 day after exposure, the time point showing peak *Tnc* mRNA

accumulation, revealed intense up-regulation in the subepithelial mesenchyme (Figures 4G, 4I, and 4J). Colocalization with α -SMA revealed that deposition of *Tnc* occurred within the underlying mesenchyme, showing a broader range of expression than that defined by SMA immunoreactivity (Figures 4I and 4J).

To further define the dynamics of matrix remodeling that accompanied airway injury and repair, *Tnc*, CCSP, and SMA were localized by immunofluorescent staining of lung tissue at 3, 5, and 30 days after injury (Figures 5A–5E, 5F–5J, and 5K–5O, respectively). Domains of epithelial regeneration were defined by small clusters of CCSP(+) cells observed in terminal bronchioles at Recovery Day 3 (Figures 5A, 5D, and 5E). These repairing regions showed evidence of expansion at Recovery Day 5 (Figures 5F, 5I, and 5J), with further expansion noted by Recovery Day 30 (Figures 5K, 5N, and 5O). This pattern of repair closely mirrored the kinetics of cell proliferation and renewal observed in previous studies (14, 28). During this period of epithelial renewal, *Tnc*-immunoreactive protein showed an overall decrease in abundance by Day 3, showing evidence for reorganization to the basement membrane immediately subtending the repairing airway epithelium (Figure 5E). A continuing decline in *Tnc*-immunoreactive protein was observed by Day 5, with residual immunoreactivity observed in the subepithelial basement membrane (Figure 5J). The abundance and distribution of *Tnc* immunoreactivity returned to near steady-state levels by Recovery Day 30 (Figures 5K–5O). This pattern of

Figure 3. Pro-mitotic signaling pathways and extracellular matrix (ECM) are dynamically regulated during acute Clara cell injury, productive repair, and resolution. Total lung RNA was isolated from control (0) and 1, 2.5, 4, 8, or 12 hours (solid bars) and 1, 2, 3, 5, 10, or 30 days (open bars) after injury. Real-time PCR analysis was

conducted to measure mRNA abundance of gene transcripts representative of (A, B) pro-mitotic signaling pathways and (C) ECM. Data are presented as mean $\pm$ SEM. * unpaired student's t-test $P < 0.05$.

Figure 4. Extracellular matrix is deposited during productive airway epithelial repair. Clara cell secretory protein (CCSP), tenascin-C (*Tnc*), and α -smooth muscle actin (α -SMA) were immunolocalized on formalin-fixed and cryopreserved lung tissue isolated at (A–E) control (0) and (F–J) 1 day after naphthalene. Representative pseudo-colored photomicrographs are shown of DAPI (gray) merged with (A, F) CCSP (magenta), (B, G) *Tnc* (green), or (C, H) α -SMA (red). (D, I) Four-color merged images of control and 1 day after injury. (E, J) Magnification of areas denoted by a dashed rectangles in D and I (asterisk denotes colocalization, arrow indicates *Tnc* subtending α -SMA-positive cells). Representative images of the distal airway epithelium at $\times 400$ magnification (scale bar = 50 μ m).

Tnc-immunolocalization was also observed in the subepithelial region of proximal airways (Figure E1). Based on this dynamic pattern of altered *Tnc* immunoreactivity, we conclude that nascent extracellular matrix is deposited after injury, reorganized to basement membrane subtending regenerating and injured airway epithelium, and returns to steady-state levels upon completion of successful airway epithelial repair. These data indicate that ECM is reversibly and dynamically regulated during productive airway epithelial repair.

Deficiency in Epithelial Repair Leads to Persistent Matrix Deposition

Chronic lung injury has been associated with uncontrolled fibroproliferation and deposition of ECM components, includ-

ing *Tnc* (30). Even though defects in epithelial repair have been proposed as a contributing factor in fibroproliferation and matrix deposition, this has not been demonstrated directly. We sought to determine whether the transient increases in matrix deposition observed during productive repair of injured airways would be altered in a model of unproductive repair, in which repair was abrogated due to loss of tissue stem/progenitor cells. To achieve this, transgenic mice (CCtk) expressing *Herpes simplex virus* thymidine kinase (HSV-tk) in CCSP-expressing cells were exposed to GCV to injure the airway epithelium and abrogate repair (19). This model results in irreversible injury of all CCSP(+) epithelial cells and has been shown to result in alveolar dysfunction as well as fibroproliferation (26). RNA was isolated from unexposed control (CCtk[–]) and CCtk (+) mice 3, 6, or 9 days after chronic GCV exposure to compare the gene expression signatures of injury/productive repair with injury/unproductive repair. Bioinformatic analysis identified 1,729 gene transcripts that were regulated by greater than 2-fold between GCV-injured CCtk mice relative to untreated control mice. These genes were hierarchically clustered and are presented as a heat-map in Figure 6A. The abortive airway epithelial repair gene signature is composed of 1,656 genes whose mRNAs were increased by 2-fold or greater and 73 mRNA species showing greater than 50% decreases in their abundance (Figure 6A). Down-regulated gene transcripts were previously used to identify new and existing markers for mature Clara cells (28). In an effort to further compare the productive and unproductive gene signatures, we focused on genes showing a robust pattern of mRNA induction displayed in node 278, which comprised 278 genes (Figure 6B and Table E4). Analysis of mRNA abundance for selected genes by real-time RT-PCR recapitulated results obtained by microarray analysis (Figures 6C–6J). A difference between productive and abortive repair was observed for *Akp2* and *Errfi1* (compare Figure 2 with Figure 6), neither of which showed changes in mRNA abundance with abortive repair (Figures 6C and 6I, open bars). These data support the notion that stem cell amplification and inhibition of EGFR signaling has been abrogated with loss of epithelial reparative capacity (31, 32). The abundance of *Areg* mRNA was chronically elevated in the abortive repair model compared with the transient increase observed in mice displaying productive repair (Figure 6D). In contrast, the mRNA for *Tnfrsf12a* was transiently up-regulated in both injury models (Figure 6E). Genes whose products participate in remodeling of the extracellular matrix were chronically up-regulated in mice showing abortive repair, though less abundant (Figures 6F and 6G), as were those for extracellular matrix molecules *Tnc* and *Lgals1*. These data indicate that there are significant differences in the abortive repair gene signature compared with productive repair that have potential to impact tissue remodeling.

To further explore the dynamics of extracellular matrix remodeling observed during abortive airway epithelial repair, the abundance and distribution of *Tnc* expression was determined as a function of Clara cell abundance in control and GCV-exposed CCtk mice. CCSP abundance was measured in total lung RNA, bronchiolar lavage fluid, and serum to assess injury of Clara cells using molecular endpoints (Figures 7A–7C). Ganciclovir exposure resulted in a reduction in CCSP mRNA abundance to 7.82% and 0.84% of control levels at Days 6 and 10, respectively. This was paralleled by decreases in CCSP content within airway lining fluid, which was reduced to 0.52% of control and undetectable levels at Exposure Days 6 and 10, respectively. Evaluation of *Tnc* mRNA abundance by real-time PCR revealed a 3.44- and 9.70-fold up-regulation at Exposure Days 6 and 10, respectively. These results suggest that ECM, as assessed by *Tnc* mRNA abundance, is continually

Figure 5. Epithelial repair is associated with matrix remodeling leading to restoration of steady-state levels and distribution. CCSP, *Tnc*, and α -SMA were immunolocalized on formalin-fixed and cryopreserved lung tissue isolated at (A–E) 3, (F–J) 5, and (K–O) 30 days after naphthalene injury. Pseudo-colored images of DAPI (gray) merged with (A, F, K) CCSP (magenta), (B, G, L) *Tnc* (green), or (C, H, M) α -SMA (red) of representative distal airway epithelium. (D, I, N) Four-color merged images of 3, 5, and 30 days after injury. (E, J, O) Magnification of areas denoted with by dashed rectangles in D, I, and N (asterisk denotes *Tnc* positive basement membrane independent of sub-tending α -SMA cells, arrowhead indicates *Tnc* sub-tending injured epithelium associated with underlying α -SMA cells, and arrow indicates *Tnc* localized to basement membrane sub-tending regenerating units). Representative images of the distal airway epithelium at $\times 400$ magnification (scale bar = 50 μ m).

deposited during defective epithelial repair. To verify that this was true at the level of protein accumulation *Tnc*-immunoreactive protein was measured by immunofluorescent colocalization with CCSP and α -SMA. Analysis of control tissue revealed abundant CCSP(+) epithelial cells (Figures 7E, 7H, and 7Q) and the stereotypic distribution of *Tnc* (Figures 7F, 7H, and 7Q), and SMA (Figures 7G, 7H, and 7Q). CCSP-immunoreactive cells were greatly diminished 6 days after ganciclovir exposure (Figures 7I, 7L, and 7R) and was accompanied by increased *Tnc*-immunoreactive protein sub-tending the airway and alveolar epithelium (Figures 7J, 7L, and 7R). The abundance and distribution of α -SMA immunoreactivity remained unchanged 6 days after chronic injury (Figures 7K, 7L, and 7R). A further decrease in the abundance of CCSP-immunoreactive cells was observed after 10 days of ganciclovir exposure (Figures 7M, 7P, and 7S). This was associated with further accumulation of *Tnc*-immunoreactive protein at the basement membrane sub-tending airway epithelium as well as peribronchiolar and alveolar mesenchyme (Figures 7N, 7P, and 7S). No changes in α -SMA immunoreactivity were observed (Figures 7O, 7P, and 7S). These results indicate that defective airway epithelial reparative capacity results in chronic ECM deposition in airway and alveolar compartments. These data collectively provide the first experimental evidence that defects in epithelial reparative capacity are sufficient to drive excessive ECM deposition.

DISCUSSION

Defects in epithelial maintenance have been postulated to contribute to uncontrolled ECM deposition in fibroproliferative chronic lung diseases (24, 33). However, direct supportive evidence is lacking. We have addressed this problem through the use of mouse models in which selective airway injury is associated with either productive or unproductive repair. Using genome-wide expression profiling we identified clusters of regulated genes that included those encoding pro-mitotic signaling molecules and components of the extracellular matrix. Using *Tnc* as a prototypical matrix protein, we show that its abundance is transiently up-regulated during acute injury and productive repair, and that this process continues without resolution in the setting of airway injury with defective repair. These data are the first to provide direct experimental evidence that ECM is dynamically regulated in the repair response to selective epithelial cell injury and that epithelial repair defects are sufficient to drive excessive matrix deposition typically observed in chronic fibroproliferative lung diseases.

Increased deposition of *Tnc* has recently been shown to be a marker of chronic fibroproliferative lung disease, such as obliterative bronchiolitis (OB) (30). Furthermore, gene expression analysis of tissue samples from nonsmoking patients with COPD revealed major alterations to gene transcripts involved in ECM deposition (34). Our observation that *Tnc* can be

Figure 6. The gene expression signature of injured airway epithelium with defective repair. RNA from control (0, unexposed CcTk[−]) or CcTk(+) mice exposed to ganciclovir (GCV) chronically for 3, 6, or 9 days was isolated from total lung homogenate and mRNA abundance determined using microarray analysis ($n = 4$ /exposure group). Statistically significant gene expression changes relative to control were clustered and presented as a heat map. Red and green indicate down- and up-regulated gene expression relative to control, respectively. Heat map representation of (A) all significant gene clusters and (B) magnification of the most informative gene clusters (Node 278). († and ‡ denote clusters that are up-regulated and down-regulated relative to control, respectively). (C) mRNA abundance of genes representative of Node 278 were determined in total lung RNA samples from control (0) and 3, 6, or 9 days of chronic GCV exposure by real-time PCR analysis using Taqman probes and primers. Graphs with open bars indicate genes that were not regulated during chronic GCV exposure and therefore not present in Node278, but were included for comparison to productive repair. Y-axis scaling was adjusted to Figure 2 graphs to allow for comparison of fold-change in productive versus unproductive repair. Data are presented as mean $\pm$ SEM. * unpaired student's t-test $P < 0.05$.

deposited in response to epithelial cell injury but is only resolved after repair suggests that restoration of epithelial homeostasis is a critical factor in resolving otherwise persistent matrix accumulation. ECM deposition is controlled by the combined regulation of protein synthesis and degradation. Studies in human lung indicate that *Tnc* is expressed in cells of the subepithelial mesenchyme during development and in disease (35, 36). *In vitro* studies also indicate that fibroblasts are a source of *Tnc* expression (37). It is likely that the increased expression of *Tnc* in our studies occurs in the subepithelial mesenchyme as a result of fibroblast activation secondary to epithelial cell injury.

Our data demonstrate that transcriptional activation of *Tnc* occurs rapidly after injury and is immediately attenuated after initiation of productive repair. This is paralleled by a dramatic increase in *Tnc*-immunoreactive protein within the subepithelial mesenchyme. As productive repair ensues, *Tnc* pools were initially reduced, resulting in either residual or reorganization of *Tnc* protein at the basement membrane. Basement membrane-associated *Tnc* was later depleted at the time of epithelial

renewal. These data imply that matrix deposition after epithelial injury and productive repair occurs in three stages: (1) transient synthesis of nascent *Tnc* during the injury phase, (2) mesenchymal degradation, and (3) epithelial-associated degradation during repair. However, in the setting of unproductive repair, our data demonstrate that *Tnc* mRNA abundance and production are chronically elevated in both airway and alveolar compartments. We have previously shown that chronic defects in airway repair results in disruption of alveolar homeostasis (26) and speculate that these changes are the basis for elevated matrix deposition.

Repair of acute epithelial lesions in airways is a highly regulated process involving a complex interaction between multiple cell types as well as the underlying ECM (1, 38). Lesions induced by exposure to naphthalene are highly selective for conducting airways due to its bioactivation within Clara cells, the abundant pool of facultative progenitors. The ensuing repair process involves proliferation of naphthalene-resistant cells that have been considered to be the local population of tissue stem cells (14). Regulation of stem cell activity is likely mediated by

Figure 7. Multi-compartmental deposition of extracellular matrix in lungs of mice with nonrepairing conducting airway epithelium. Control (0, unexposed CCtk[−]) or CCtk(+) mice were administered GCV chronically for 6 or 10 days. (A) CCSP mRNA abundance in total lung RNA as measured by real-time PCR. (B, C) CCSP protein in bronchoalveolar lavage fluid and serum was measured using an enzyme-linked immunosorbent assay. (D) *Tnc* mRNA abundance in total lung RNA as measured by real-time PCR. * unpaired student's t-test $P < 0.05$. CCSP, *Tnc*, and α -SMA were immunolocalized on formalin-fixed and cryopreserved lung tissue isolated at (E–H) Control (0, unexposed CCtk[−]), (I–L) 6, and (M–P) 10 days after chronic GCV administration. Pseudo-colored images of DAPI (gray) merged with (E, I, M) CCSP (magenta), (F, J, N) *Tnc* (green), or (G, K, O) α -SMA (red) of representative distal airway epithelium. (H, L, P) Four-color merged images of control, 3, and 10 days of GCV treatment, respectively. (Q–S) Magnification of areas denoted with dashed rectangles in H, L, and P, respectively (asterisk indicates colocalization of *Tnc* and α -SMA, arrow indicates *Tnc* in basement membrane subtending injured airway epithelium, and arrowhead indicates *Tnc* deposited in the alveolar compartment). Representative images of the distal airway epithelium at $\times 400$ magnification (scale bar = 50 μ m).

concerted intrinsic and extrinsic cues derived from the surrounding cellular and acellular environment, referred to as a stem cell niche (39). Currently the role of the ECM in regulation of niche dynamics and stem cell function has yet to be elucidated in the lung. In this study we show that during productive epithelial repair, the ECM molecule *Tnc* is dynamically regulated, including reorganization to basement membrane subtending regenerating and injured epithelium. Several studies have focused on the role of *Tnc* in regulation of the stem cell niche, and suggest that *Tnc* may regulate both proliferation and developmental progression of neural stem cells in the developing central nervous system (40, 41). These data are consistent with those from other studies demonstrating a critical role for *Tnc* in regulating hematopoiesis (42). Recent studies have implied that *Tnc* is up-regulated after injury and is required for both proliferation and migration of scratch-wounded cultured astrocytes (43). Structural modeling and *in vitro* models have indicated that *Tnc*-EGF domains can bind to EGFR and promote migration independently of effects on cell proliferation (44, 45). Together these data suggest that the deposition of *Tnc* and reorganization to basement membrane subtending injured and regenerating epithelium may play a critical role in niche dynamics through regulation of proliferation and differentiation or migration of

epithelial progenitors and nascent epithelium, respectively. Future studies will test these observations in the setting of airway epithelial repair.

Since we extracted RNA from total lung homogenates, a caveat to our study is the underestimation of changes in gene expression that occurs during the process of airway injury and repair. Despite this caveat, other novel findings of the present study were the identification of several prometotic signaling pathways regulated during airway epithelial repair. These included amphiregulin, a ligand for the epidermal growth factor receptor (EGFR), and *Tnfrsf12a*, both of which have been shown to regulate hepatic progenitor cells (46–48). Future studies will investigate the contribution made by these signaling pathways in regulation of airway repair and remodeling.

The airway epithelium provides a primary barrier between the host and the environment and plays an essential role in immunomodulation in the lung. As such, immediate and effective repair after injury is required for maintaining lung homeostasis. Chronic fibroproliferative lung diseases are characterized by a remarkable deposition of ECM protein. In this study, we provide the first experimental evidence to suggest that airway epithelial reparative capacity regulates ECM dynamics. Furthermore, we demonstrate that dynamic regulation of ECM

is a component of productive airway epithelial repair and may play an essential role in regulation of this process.

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

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