

# Matrix Metalloproteinase-7 Coordinates Airway Epithelial Injury Response and Differentiation of Ciliated Cells

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Matrix metalloproteinase-7 (MMP7) expression is quickly up-regulated after injury, and functions to regulate wound repair and various mucosal immune processes. We evaluated the global transcriptional response of airway epithelial cells from wild-type and *Mmp7*-null mice cultured at an air-liquid interface. The analysis of differentially expressed genes between genotypes after injury revealed an enrichment of functional categories associated with inflammation, cilia, and differentiation. Because these analyses suggested that MMP7 regulated ciliated cell formation, we evaluated the recovery of the airway epithelium in wild-type and *Mmp7*-null mice *in vivo* after naphthalene injury, which revealed augmented ciliated cell formation in the absence of MMP7. Moreover, *in vitro* studies evaluating cell differentiation in air-liquid interface cultures also showed faster ciliated cell production under *Mmp7*-null conditions compared with wild-type conditions. These studies identified a new role for MMP7 in attenuating ciliated cell differentiation during wound repair.

**Keywords:** MMP7; matrilysin; microarray; wound repair

Like all epithelial surfaces, the lung epithelium has evolved to respond to the constant pressures exerted by the external environment, and is distinguished by an innate wound repair program initiated upon injury. Without such an efficient response, the body is left exposed to potential pathogenic invasion. More importantly, ineffective wound closure can lead to long-term adverse consequences such as chronic inflammatory states, fibrosis, and malignant transformations (1). Although the response to injury is essentially immediate, large wounds require time to repair. Therefore, injury is often accompanied by an inflammatory response that recruits leukocytes, which serve to scan for pathogens (2). Eventually, as wound healing nears completion, the associated inflammation resolves, returning the epithelium to a differentiated, homeostatic state.

Matrix metalloproteinases (MMPs) comprise a family of 25 or more zinc-dependent proteases that cleave the extracellular matrix and cell-surface proteins to regulate wound healing and a variety

## CLINICAL RELEVANCE

Matrix metalloproteinase-7 (MMP7) is an important mediator of lung inflammation and repair after injury. Here, we show that MMP7 also regulates ciliated cell differentiation in the airways.

of immune responses (3). In particular, MMP7 (matrilysin) plays a prominent role in the injury response of all mucosal epithelia. Expressed at low concentrations in the resting lung epithelium, MMP7 production is quickly induced in cells bordering the site of injury, stimulating cell migration and coordinating the inflammatory response (4–8).

MMP7 is spatiotemporally regulated after injury by mechanisms that have yet to be fully elucidated (9). On one level, its expression is restricted to areas of injury (5, 7, 10). MMP7 is also secreted as a zymogen that must be activated apparently by an allosteric-mediated autolytic process (3, 9, 11). The complexity of this regulation is further magnified by the temporal selectivity with which MMP7 cleaves its substrates. For example, syndecan-1 is shed early after injury to facilitate cell migration and neutrophil recruitment into the lungs (4, 6), whereas E-cadherin shedding is delayed and apparently functions to help resolve the inflammatory response by promoting the influx of immunosuppressive cells (8). In sum, MMP7 is a critical effector of lung epithelial repair and immunity.

Wound healing, injury, and inflammation are interconnected processes. In this study, we surveyed the epithelial cell transcriptome at baseline and after injury under wild-type (WT) and *Mmp7*-null (*Mmp7*<sup>−/−</sup>) conditions to understand how MMP7 regulates the global cellular response to injury. We found that mechanical injury to air-liquid interface cultures of the airway epithelium activated genes involved in proliferation and wound repair. By comparing the differential response of WT and *Mmp7*<sup>−/−</sup> epithelial cells to injury, we observed enrichment in developmental and morphogenesis pathways. Unexpectedly, our analyses discovered alterations in transcriptional programs involving ciliary function, which led us to uncover a novel role for MMP7 in the differentiation of ciliated cells after lung injury.

## MATERIALS AND METHODS

### Cell Culture

Male WT and *Mmp7*<sup>−/−</sup> C57BL/6 mice were used to create air-liquid interface (ALI) cultures (4, 12). Conditions of injury were created with three parallel scratches, using a sterile p-1000 pipette tip (Mettler Toledo, Columbus, OH). Injured and uninjured conditions were cultured for 24 hours in serum-free medium before RNA isolation (Trizol; Invitrogen, Carlsbad, CA). RNA quality was confirmed with a Bioanalyzer 2100 (Agilent, Santa Clara, CA). At 24 hours, wounds were incompletely closed in both genotypes.

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## Microarray Experiments

**Labeling and processing.** Biotin-labeled cRNA was hybridized to Affymetrix Mouse Genome 430A 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA) at the Center for Array Technology of the University of Washington. Detailed information about meeting the requirements for minimum information about a microarray experiment has been deposited at Gene Expression Omnibus (GSE38513).

**Correspondence analysis.** Multidimensional scaling of whole-genome transcriptional profiles of the 16 samples was performed using correspondence analysis (13).

**Differential gene expression.** Differential gene expression was assessed using a Bayesian implementation of the parametric *t* test, coupled with false discovery rate (FDR) analysis using a Q-value cutoff of less than 0.01 (14). We applied pairwise comparisons to identify significant differences between genotype and exposure: (1) we assessed injury-induced differences by comparing each genotype before and after injury separately (e.g., *Mmp7*<sup>-/-</sup><sub>injury</sub> versus *Mmp7*<sup>-/-</sup><sub>base</sub>, or WT<sub>injury</sub> versus WT<sub>base</sub>); (2) we compared differential gene expression between WT<sub>base</sub> versus *Mmp7*<sup>-/-</sup><sub>base</sub> samples; and (3) we identified injury-specific changes in gene expression, as will be described. First, we limited the analyses to differentially expressed genes between genotypes after injury (e.g., WT<sub>injury</sub> versus *Mmp7*<sup>-/-</sup><sub>injury</sub>), and then we excluded the genes differentially expressed only at baseline (e.g., WT<sub>base</sub> versus *Mmp7*<sup>-/-</sup><sub>base</sub>). However, genes differentially expressed between genotypes at baseline and demonstrating significant, genotype-specific changes after injury were included in our analysis. This approach excluded genes that showed differential expression *only* at baseline between WT and *Mmp7*<sup>-/-</sup> conditions, and captured all genotype-specific injury-responsive candidate genes.

**Functional enrichment.** Differentially expressed genes underwent functional analysis using the Database for Annotation, Visualization, and Integrated Discovery, based on an FDR cutoff of less than 0.01 (15).

## Naphthalene Injury

Male mice were treated with naphthalene (200 mg/kg) or corn oil vehicle, as previously described (4). Lungs were harvested on Days 7 and 14 after injury, and fixed at a pressure of 30 cm H<sub>2</sub>O with Karnovsky's fixative for scanning electron microscopy images, and with 10% formalin

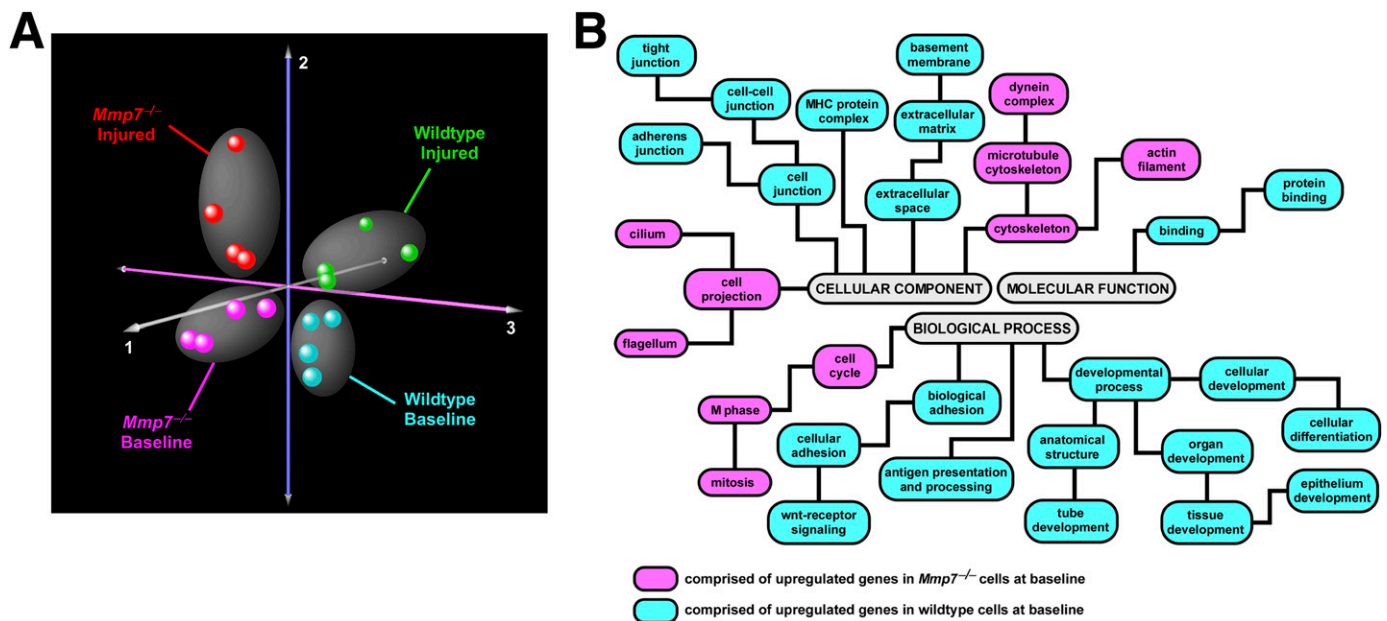
for histology. Airways were microdissected, and scanning electron microscopy was performed as previously described (16). Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington.

## RESULTS

### Injury Response Pattern of ALI Cultures

We have demonstrated that ALI cultures of *Mmp7*<sup>-/-</sup> airway cells mirror the reepithelialization defects seen *in vivo* in *Mmp7*<sup>-/-</sup> mice (4, 5, 7, 10). To evaluate the role of MMP7 in modulating the injury response of the airway epithelium in an unbiased manner, we transcriptionally profiled WT and *Mmp7*<sup>-/-</sup> ALI cultures at baseline and after scratch injury. An initial analysis based on gene expression variability across microarray experiments identified four distinct groups segregated according to genotype (WT, *Mmp7*<sup>-/-</sup>) and exposure (baseline or injury) (Figure 1A). These observations implied that MMP7 exerts widespread transcriptional influences in the lung epithelium at baseline and in response to injury.

We postulated that scratch injury would activate many common pathways in WT and *Mmp7*<sup>-/-</sup> airway epithelial cells. By comparing the transcriptional response of injured ALI cultures relative to their uninjured baseline state, we identified 542 differentially expressed genes in WT cultures, and 650 differentially expressed genes in *Mmp7*<sup>-/-</sup> ALI cultures (Q-value < 0.01). Approximately half of these injury-responsive genes were common to both genotypes. Functional enrichment analysis of these genes revealed that many similar processes were overrepresented in WT and *Mmp7*<sup>-/-</sup> ALI cultures after injury (*see* Table E1 in the online supplement). Indeed, the most significantly up-regulated genes after injury in both genotypes were those associated with cell proliferation (e.g., cyclin b1, cyclin b2, and cell division cycle-associated 3) and migration (e.g., fibronectin and  $\alpha$ -actinin) (Figure E1). Our analysis also revealed significant transcriptional differences between WT



**Figure 1.** (A) Correspondence analysis of wild-type (WT) and *Mmp7*-null (*Mmp7*<sup>-/-</sup>) airway epithelial transcriptome at baseline and in response to scratch injury at 24 hours. This analysis showed that the variability in gene expression was relatively small among biological replicates under each experimental condition, and that global changes occurred in gene expression, attributable to genotype (WT or *Mmp7*<sup>-/-</sup>) at baseline and after injury. (B) Differentially expressed genes between *Mmp7*<sup>-/-</sup> and WT cells at baseline (before injury) were functionally grouped according to their gene ontology (GO) annotation. The tree-like structure of GO is depicted for the statistically overrepresented functional categories (false discovery rate, < 0.01). Modules are colored according to whether they are comprised of up-regulated genes under *Mmp7*<sup>-/-</sup> (magenta) or WT (cyan) conditions. MHC, major histocompatibility complex; MMP7, matrix metalloproteinase-7.

and *Mmp7*<sup>-/-</sup> ALI cultures at baseline. We proceeded to investigate these differences further, and importantly, to adjust for them when assessing whether injury activated distinct pathways *between* genotypes.

Gene Expression Profiles of Airway Epithelial Cells Are Distinctly Altered by the Absence of *Mmp7*

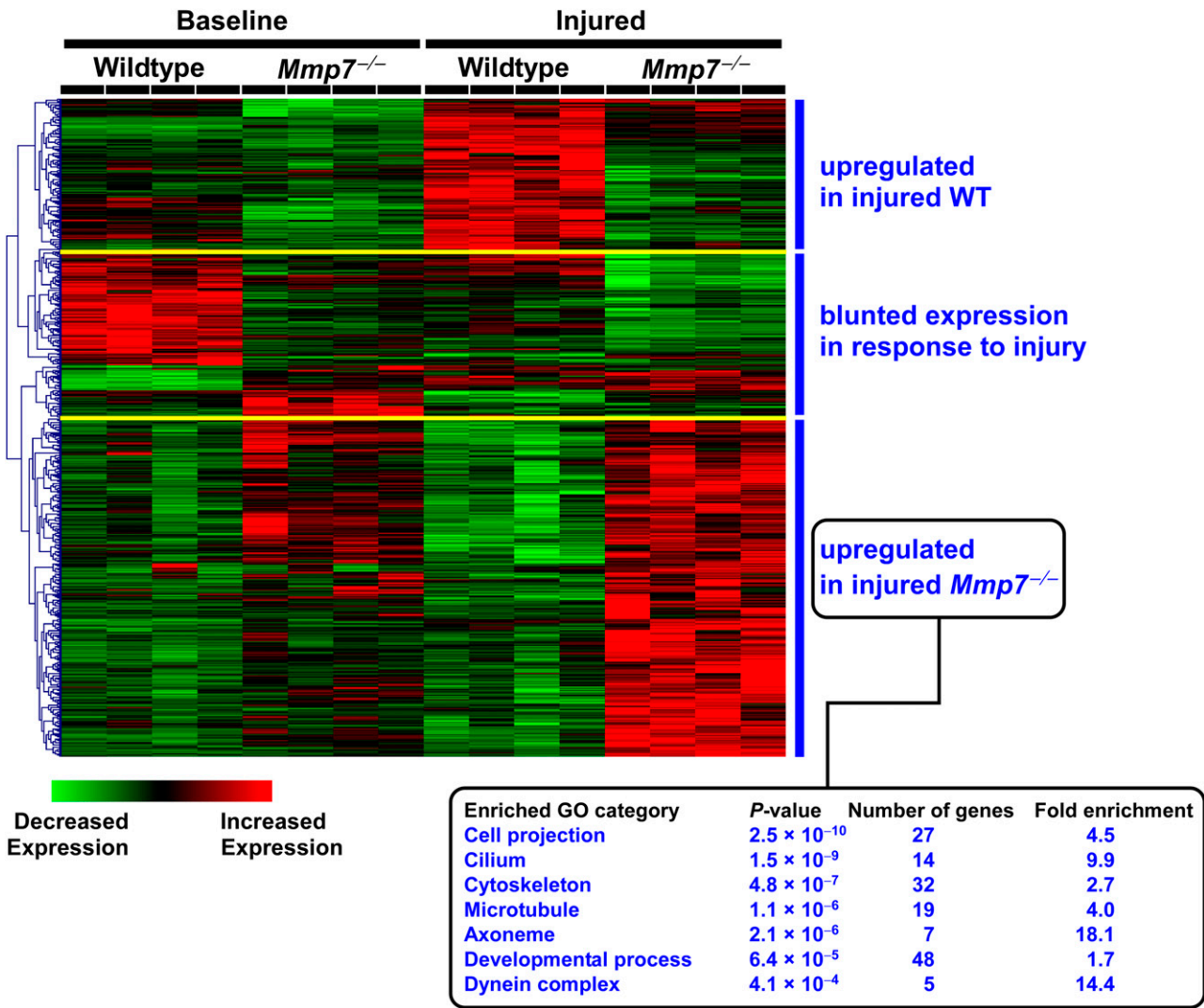
We identified many differentially expressed genes between WT and *Mmp7*<sup>-/-</sup> cells before scratch wound injury (Figure E2). These genes mapped to functionally diverse categories, and were highly enriched in developmental pathways such as branching morphogenesis and epithelial development, in structural components such as the dynein complex, and in processes involved with cellular junctions, adhesions, and projections (Figure 1B). These findings imply that despite their normal histologic appearance, *Mmp7*<sup>-/-</sup> epithelial cells have altered transcriptional states that may contribute to a dysregulated injury response.

Because significant differences in gene expression existed between genotypes before injury, we adjusted for these baseline differences to identify genotype-specific transcriptional programs in

response to injury (*see* MATERIALS AND METHODS). Approximately 500 injury-induced genes were differentially expressed between WT and *Mmp7*<sup>-/-</sup> airway epithelial cells. Figure 2 depicts the expression profiles of these genes, and shows several distinct clustering patterns corresponding to exposure (injury or baseline) and genotype (WT or *Mmp7*<sup>-/-</sup>) conditions. We verified these expression patterns with quantitative PCR for selected genes (Figure E3). The enrichment analysis of genes within the different expression patterns revealed distinct gene ontologies mapping to unique functional groups. Of particular interest was the subset of genes up-regulated after injury only in *Mmp7*<sup>-/-</sup> epithelial cells. This cluster was highly enriched in genes associated with cell projection, ciliary function and structure, and developmental processes.

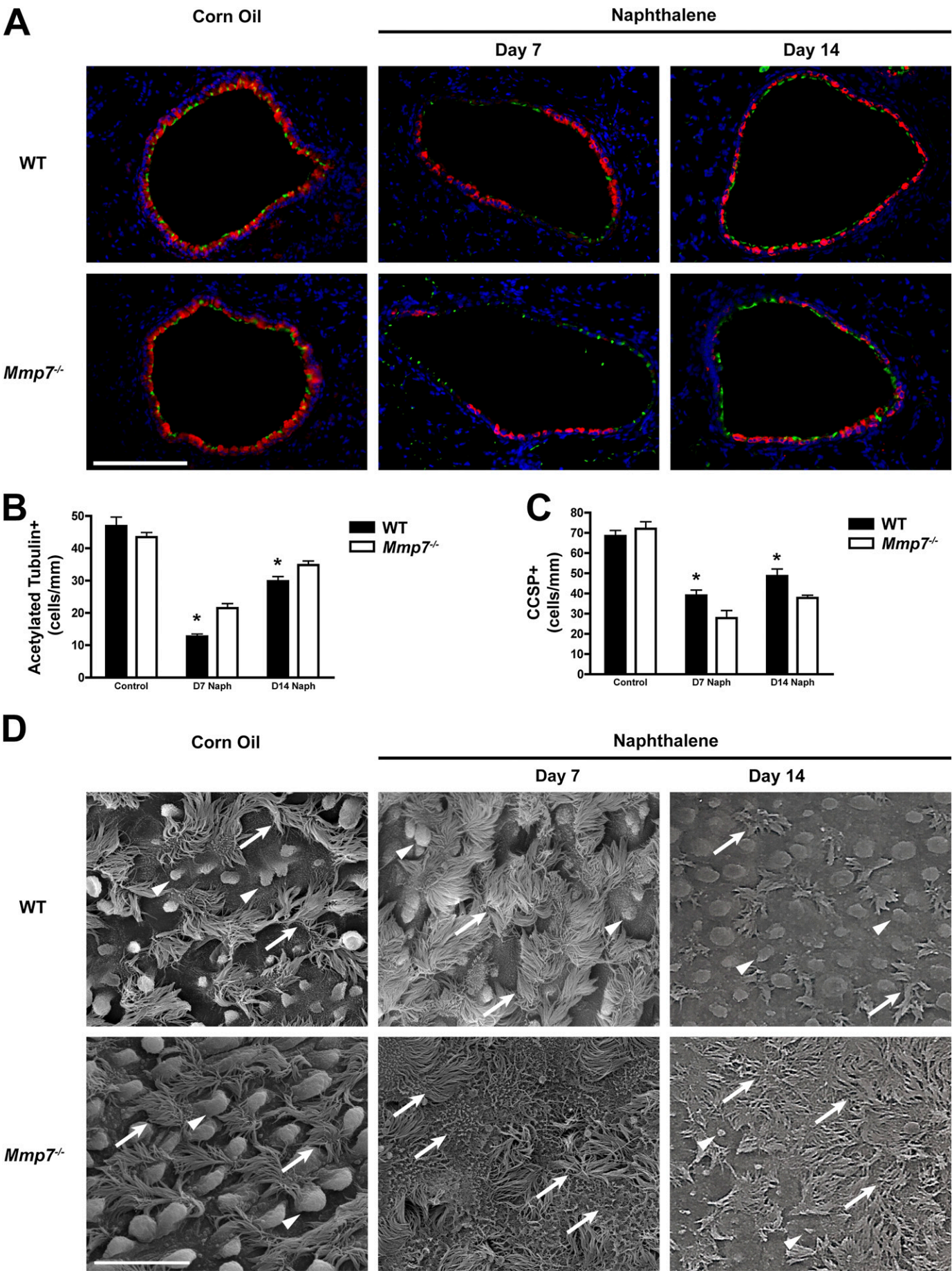
Airway Epithelial Differentiation Is Regulated by *Mmp7*

Because the transcriptional profiling analyses suggested that MMP7 plays a role in airway differentiation and ciliary function, we assessed whether MMP7 modulated ciliated cell formation by evaluating the recovery of airway epithelia *in vivo* after naphthalene



**Figure 2.** Baseline-adjusted, genotype-specific differential gene expression in response to injury. Several expression patterns are demarcated by the yellow lines: (1) genes that were primarily up-regulated in WT cells after injury, (2) genes that were differentially expressed at baseline between *Mmp7*<sup>-/-</sup> and WT cells, but lost this difference after injury, and (3) genes that were primarily up-regulated in *Mmp7*<sup>-/-</sup> cells in response to injury. Group 3 genes mapped to distinct functional groups, including processes involved in ciliary structure and function, as shown at lower right.





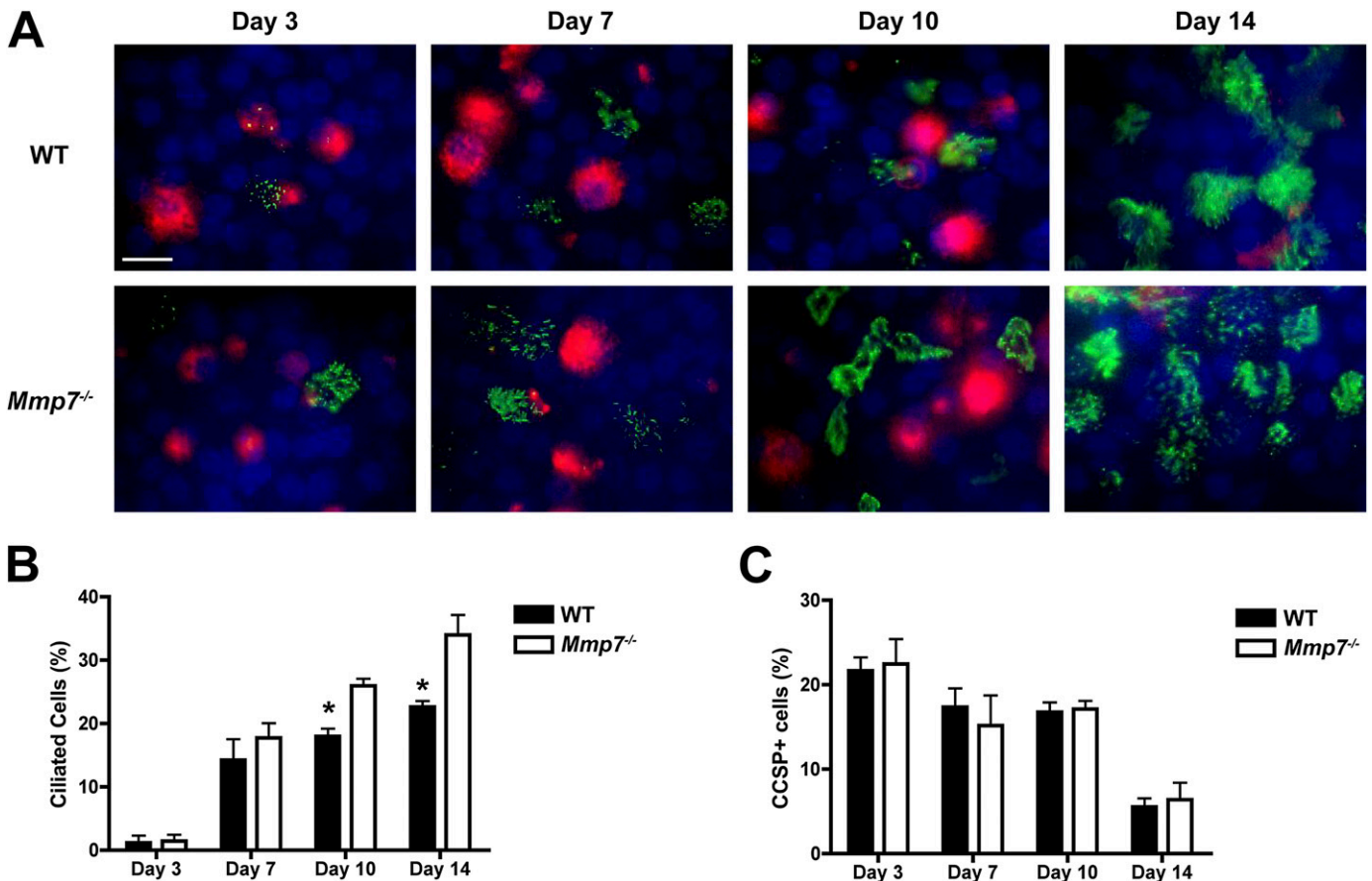
**Figure 3.** Altered recovery of ciliated cells in airway epithelia of *Mmp7*-null mice after naphthalene-induced lung injury. (A) Lung sections were immunostained for acetylated tubulin (green; Clone 6-11B-1; Sigma-Aldrich, St. Louis, MO) to mark ciliated cells, club cell-specific protein (CCSP) (red; Biovendor, Candler, NC) to identify club cells, and 4',6-diamidino-2-phenylindole (DAPI) (blue) as a nuclear stain. Epifluorescence images were captured using an Olympus BX-51 microscope (Olympus America, Center Valley, PA) with a  $\times 20/0.70$  air objective. Scale bar = 200  $\mu\text{m}$ . Acetylated tubulin<sup>+</sup> cells (B) and CCSP<sup>+</sup> cells (C) were counted per millimeter of airway length. Only airways between 200 and 350  $\mu\text{m}$  in diameter were evaluated. A minimum of five airways was randomly evaluated in various lung lobes for each sample. Four mice per genotype were evaluated at each time point. \* $P < 0.05$ . (D) Scanning electron micrographs of naphthalene-injured lungs. Dome-shaped club cells (arrowhead) are seen interspersed with ciliated cells (arrows) under uninjured (corn oil) conditions. Scale bar = 20  $\mu\text{m}$ .

injury. As depicted in Figure 3, uninjured conditions involve a normal distribution of club cells (Clara) and ciliated cells in both genotypes, consistent with previous descriptions (17, 18). However, distinct differences were observed between WT and *Mmp7*<sup>-/-</sup> conditions after injury. On Day 7 after injury, nearly twice as many ciliated cells were identified in *Mmp7*<sup>-/-</sup> airways compared with WT airways (Figure 3B;  $21.5 \pm 1.4$  versus  $12.7 \pm 0.7$  cells per millimeter, respectively). By Day 14 after injury, the difference was less dramatic, although more ciliated cells under *Mmp7*<sup>-/-</sup> conditions were still evident, compared with WT conditions ( $34.8 \pm 1.2$  versus  $29.8 \pm 1.5$  cells per millimeter, respectively). The total number of cells was similar between conditions, but was significantly lower on Day 14 after injury compared with uninjured control samples, suggesting that airway epithelia were not fully reconstituted 14 days after naphthalene injury (unpublished data). In contrast to ciliated cells, club cells were less abundant under *Mmp7*<sup>-/-</sup> conditions, compared with WT conditions on Day 7 ( $27.7 \pm 3.8$  versus

$39.0 \pm 2.7$  cells per millimeter, respectively) and Day 14 ( $37.8 \pm 1.3$  versus  $48.6 \pm 3.5$  cells per millimeter, respectively) after naphthalene injury (Figure 3C).

Although the numbers of ciliated cells were equalizing by Day 14 after naphthalene injury, the distribution of cells was different between conditions. By Day 14 after naphthalene injury, the WT airway regained the appearance of unperturbed epithelium with a homogeneous distribution of club and ciliated cells (Figure 3D). In contrast, *Mmp7*<sup>-/-</sup> mice maintained sheets of ciliated cells with patches of club cells. A similar finding was also evident in immunostained images for ciliated and club cells (Figure 3A).

To further evaluate the role of MMP7 in airway epithelial differentiation, we quantified the accumulation of ciliated cells in ALI cultures (Figure 4A). We found a more rapid accumulation of ciliated cells in *Mmp7*<sup>-/-</sup> conditions compared with WT conditions (Figure 4B). Indeed, by Day 14 of ALI culture conditions,



**Figure 4.** Ciliated cells appear more rapidly in airway epithelial cells differentiated at an air-liquid interface (ALI). (A) ALI cultures were immunostained for acetylated tubulin (green; Sigma-Aldrich) to mark ciliated cells, CCSP (red; Biovendor) to identify club cells, and DAPI (blue) as a nuclear stain. Epifluorescence images were captured using an Olympus BX-51 fluorescence/DIC microscope with a Plan Apo  $\times 60/1.4$  oil objective. Scale bar = 10  $\mu\text{m}$ . Ciliated cells (B) and club cells (C) were quantified under both conditions. Four independent cultures were evaluated per genotype at each time point. \* $P < 0.05$ .

34.0% of the cells in the *Mmp7*<sup>-/-</sup> ALI culture were ciliated, whereas only 22.6% of the cells were ciliated under WT conditions ( $P < 0.01$ ). Club-cell numbers were similar between conditions, and had characteristically diminished in abundance during 14 days in ALI culture (Figure 4C) (12).

## DISCUSSION

In the lungs, MMP7 has evolved as a conduit to channel the injury response and facilitate wound healing and tissue inflammation (4–8). Here, we profiled the global transcriptional response of injured airway epithelial cultures from WT and *Mmp7*<sup>-/-</sup> mice. We also observed significant differences in gene expression between WT and *Mmp7*<sup>-/-</sup> airway cells at baseline and after scratch injury. Unexpectedly, a functional analysis of differentially expressed genes after injury implicated MMP7 in airway epithelial development, and particularly, in ciliary function. We assessed the MMP7 modulation of ciliated cell differentiation, and found that ciliated cells were more abundant under *Mmp7*<sup>-/-</sup> conditions compared with WT conditions after naphthalene injury. Using the ALI culture model, which predominantly produces ciliated cells (12), we also found a more rapid development of ciliated cells in *Mmp7*<sup>-/-</sup> cultures compared with WT cultures. These data indicate that MMP7 suppressed the differentiation of ciliated cells in airway epithelia, both *in vitro* and *in vivo*.

Ciliated cells are terminally differentiated, and do not contribute to the repopulation of the airway epithelium (19, 20). Club cells at the bronchoalveolar ductal junction and associated with neuroendocrine bodies proliferate to regenerate cells in the terminal airways, and transdifferentiate to ciliated cells after naphthalene injury (19–22). In contrast, ALI cultures rely primarily on basal cells (23). Our *in vitro* and *in vivo* data suggest that MMP7 suppressed both these transit-amplifying cells (i.e., club cells and basal cells) from differentiating toward the ciliated cell lineage. Restraining the differentiation of ciliated cells after injury may involve beneficial effects for repair by leaving a larger pool of progenitor cells to divide and reconstitute the epithelial barrier. Moreover, cellular contents (e.g., actin and microtubules) are reserved for cell migration and division instead of ciliogenesis (24).

Notably, ciliated cells under the *Mmp7*<sup>-/-</sup> conditions were characterized by a patchy distribution within the airway epithelium 14 days after naphthalene injury, whereas in WT lungs, both ciliated and club cells were homogeneously distributed. A similar finding of coalescing ciliated cells was reported in female mice receiving multiple doses of naphthalene (16). However, these experiments used male mice and a single dose of naphthalene. We speculate that the patches of ciliated cells may reflect the profound defect in migration of epithelial cells deficient in MMP7 (5, 7, 10). Because the uninjured airway epithelium was indistinguishable between genotypes, we expect club cells to repopulate the *Mmp7*<sup>-/-</sup> airway epithelium eventually, and intercalate with ciliated cells. Therefore, although a lack of MMP7 alters the kinetics of differentiation, this effect may not be sufficient to change the baseline cellular distribution of the airway epithelium.

After naphthalene injury, club cells were less abundant under *Mmp7*<sup>-/-</sup> conditions compared with WT conditions. In comparison, ALI cultures demonstrated similar club-cell numbers between genotypes. These results indicate that MMP7 does not affect the differentiation of club cells from basal cells in ALI cultures. Club-cell proliferation could still be regulated by MMP7 in the *in vivo* airway. However, our previous study showed that MMP7 does not affect proliferation in repairing airways (7). Moreover, the total cell count in airway epithelia was similar between genotypes after naphthalene injury (unpublished data). Therefore, the decreased amount of club cells in the *Mmp7*<sup>-/-</sup>

airway compared with WT epithelium is likely attributable to a more robust transdifferentiation of club cells into ciliated cells and the relative depletion of this population of cells.

A major limitation of transcriptomic studies involves their inability to identify posttranscriptional modifications that regulate protein activity, such as proteolytic cleavage by MMPs. Although our data show that MMP7 regulates airway epithelial differentiation, we have not identified the substrates mediating these effects. Syndecan-1, an established substrate of MMP7, is a potential candidate (4, 6). Epithelial cells lacking syndecan-1 lose epithelial features and maintain a less differentiated state (25). Likewise, E-cadherin is another MMP7 substrate that exerts effects on epithelial differentiation (7, 26). More studies will need to be performed to determine whether MMP7 mediates its effects on the differentiation of ciliated cells through known or novel substrates.

In conclusion, we have identified MMP7 as a coordinator of diverse transcriptional programs, and as a key regulator of cellular differentiation after injury in the airway epithelium. The expression of MMP7 is quickly induced upon injury, and its expression is restricted to migrating epithelial cells (5, 7, 10). We therefore postulate that focal injuries in the lung concentrate MMP7 spatiotemporally to wounded areas and locally suppress differentiation, allowing for residual club cells to proliferate and migrate over the injury. As the epithelial surface is reconstituted, the expression of MMP7 decreases, allowing for the differentiation of ciliated cells in the epithelial barrier to occur.

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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