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Krt14 and Krt15 differentially regulate regenerative properties and differentiation potential of airway basal cells

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Abstract
Keratin expression dynamically changes in airway basal cells (BCs) following acute and chronic injury, yet the functional consequences of these changes on BC behavior remain unknown. In Bronchiolitis Obliterans (BO) following lung transplantation, BC clonogenicity declines which is associated with a switch from keratin15 (Krt15) to keratin14 (Krt14). We investigated the roles of these keratins using Crispr-KO in vitro and in vivo and found that Krt14-KO and Krt15-KO produce contrasting phenotypes in terms of differentiation and clonogenicity. Primary mouse Krt14-KO BCs failed to differentiate into club and ciliated cells, but had enhanced clonogenicity. By contrast, Krt15-KO did not alter BC differentiation, but impaired clonogenicity in vitro and reduced the number of label-retaining BCs in vivo following injury. Krt14, but not Krt15, bound the tumor suppressor stratifin (Sfn). Disruption of Krt14, but not of Krt15, reduced Sfn protein abundance and increased expression of the oncogene dNp63a during BC differentiation, while dNp63a levels were reduced in Krt15-KO BCs. Overall, the phenotype of Krt15-KO BCs contrasts that of Krt14-KO and resembles the phenotype in BO with decreased clonogenicity, increased Krt14 and decreased dNp63a expression. This work demonstrates that Krt14 and Krt15 functionally regulate BC behavior which is relevant in chronic disease states like BO.

Introduction

Respiratory diseases are among the most prevalent causes of death in the United States (1). Lung transplantation is the only effective treatment option for many end stage lung diseases; however, over 80% of lung transplant recipients develop chronic lung allograft dysfunction (CLAD) within 12 years of the operation (2). The most common form of CLAD is Bronchiolitis Obliterans (BO), which manifests in fibrotic occlusion of small airways (3). Airway epithelial injury is a risk factor for BO (3) which suggests that a failure in airway regeneration by stem cells likely contributes to the BO disease patho-progression. It has been demonstrated that depletion of airway epithelial basal stem cells precedes formation of BO-like lesions in murine tracheas after Cl₂ gas exposure (4). Airway basal stem cells can be depleted as a result of their
exhaustion over persistent cycles of epithelial repair due to chronic graft injury caused by infections, inflammation and immune responses of the host (5).

Airway basal cells (BCs) are recognized as resident stem cells of the surface airway epithelium (SAE) that can migrate, divide and differentiate after an injury (6). They are defined by expression of keratin5 (Krt5) – a type II keratin that must heterodimerize with a type I keratin to form intermediate filaments (IFs). Krt5 is the predominant type II keratin expressed in basal cells and can pair with either Krt14 and Krt15 (7). Krt15 expression in basal cells of uninjured airways has been suggested by scRNA-seq, while Krt14 expression is infrequent on the surface of homeostatic airways, but is abundant in submucosal glands (SMG) – a major secretory compartment and a niche for reserve airway stem cells (8, 9).

Our group and others observed that Krt14+ cell abundance increases on the airway surface in multiple lung pathologies, such as in Idiopathic Pulmonary Fibrosis (IPF), BO following lung transplantation, Restrictive Allograft Syndrome (RAS) following lung transplantation and in injury models like SO₂ and Cl₂ gas exposure, naphthalene and polidocanol injuries (8, 10-14). It is, however, unclear whether Krt14 appearance in the SAE reflects migration and expansion of glandular progenitors that express Krt14 at baseline or a change within surface airway basal cells to upregulate Krt14 expression. During a chronic injury, such as in BO, Krt14 appears in the surface airway BCs which correlates with a decline in their proliferative capacity in vitro (14). However, it is unknown whether this change in keratin expression alone can affect basal stem cell properties and fate. This manuscript offers a new paradigm to contextualize Krt14 expression dynamics and also evaluates Krt15 as the basal keratin alternative to Krt14. We show that Krt14 and Krt15 expression dynamics have a functional impact on basal cell behavior (proliferation, maintenance and differentiation).

Overall, Krt14 and Krt15 are similar in their amino acid composition (~81% amino acid homology), however, there are important biochemical differences between them. For example, Krt14 has a Cysteine residue in position 373 (Alanine in Krt15) which has been shown to form
interfilamentous di-sulfate bonds (15-17). These cross-linkages have been shown to alter nuclear shape and act as recruitment sites for signal cascades in differentiating keratinocytes (15). This mechanism might be conserved in airway basal cells as well.

We hypothesized that changes in Krt14 and Krt15 expression not only indicate regenerative state of airway BCs, but also actively influence cell fate decisions. In this work, we demonstrate that disruption of Krt14 and Krt15 in mouse airway BCs lead to opposite phenotypes. Specifically, the loss of Krt14 enhances the cells' proliferative capacity, but impairs ciliated and club cell differentiation, while the loss of Krt15 impairs proliferative capacity and does not impede differentiation. This change is accompanied by nuclear enlargement and elongation in differentiating Krt14-KO, but not in Krt15-KO BCs. These differentiating Krt14-KO BCs have decreased levels of tumor suppressor stratifin (Sfn, also known as 14-3-3sigma) and upregulated levels of oncogene dNp63a. By contrast, Krt15-KO primary airway BCs have a decreased dNp63a expression early in their differentiation. Krt14 whole body knockout mice have poor neonatal survival, reduced body weight, visually smaller lungs and tracheal submucosal glands as well as decreased club cell abundance. Krt15-KO mice, conversely, appear phenotypically normal with the exception of a mild hair loss defect. We additionally observed a decrease in label-retaining tracheal basal cells 21 days after injury in Krt15-KO mice. Lastly, we show that the phenotype observed in Krt15-KO BCs functionally resembles the phenotype of BO following lung transplantation (14), where basal cells have increased Krt14, decreased Krt15 and dNp63a expression and impaired proliferative capacity. Cumulatively, these data suggest that Krt14 and Krt15 influence BC behavior that controls proliferation, quiescence, and/or differentiation in response to injury.

**Results**

Krt14 and Krt15 expression dynamically changes following airway injury *in vivo* and *in vitro*
Upregulation of Krt14 expression in the SAE has been reported in multiple lung pathologies and injury models (8, 10-14). However, considering that keratins are obligate heterodimers and Krt5 (type II) is expressed at homeostasis, it remains unclear which other type I keratin serves as a heterodimerization partner to Krt5 at homeostasis in the absence of Krt14. To begin answering this question we analyzed the published scRNAseq literature and identified Krt15 as a likely candidate (18-22). Additionally, Krt15 has been previously shown to be expressed in murine tracheal basal cells (23). To verify these previous findings and extend them to humans and ferrets we successfully localized Krt15 and Krt14 in the large airways of mice, ferrets, and humans. At homeostasis, Krt15 expression was mostly limited to basal cells on the airway surface and in the gland ducts; Krt14 was expressed in gland ducts and in the myoepithelial cells (MECs) of gland tubules and acini (Figure S1A-D). αSMA was absent on the airway surface and in the gland ducts, but present in the myoepithelial cells surrounding tubules and acini at homeostasis which was consistent between mice, ferrets, and humans (Figure S1A-D).

Next, we confirmed that Krt14 and Krt15 dynamically change their expression in several injury model systems. We observed that at homeostasis, Krt15 is a predominant type I keratin in the ferret airway surface basal cells, while Krt14 expression on the airway surface is infrequent (Figure 1A, Figure S1). Krt14 dramatically expands its expression domain as early as 3 days after a polidocanol injury in ferrets, with continued Krt15 expression (Figure 1B). In chronic and persistent lung injuries, such as in RAS and BO, Krt15 expression largely disappears, while Krt14 persists in basal cells of large airways (Figure 1C-F). We observed a similar trend in human OB following lung transplantation, where Krt14 expressing BCs become more abundant and Krt15 expressing BCs decline in chronically injured airways of patients with BO compared to control patients with no CLAD (Figure S2A,B). The regions contrasting Krt14 and Krt15 expression showed an apparent decrease in Krt15 staining intensity and stratification of Krt14+ regions in human BO (Figure S2C,D,F,G). Morphometric quantification confirmed that OB
airway surface contained fewer Krt15+ and more Krt14-expressing cells than no CLAD airways (Figure S2E). BO diagnosis in patients was made based on CT scans, pulmonary function tests and H&E histology of the lung specimens (Figure S3).

For BC functional studies, it was important to establish a baseline for Krt14 and Krt15 expression in vitro. Cell culture conditions are often compared to a constant wound healing state, therefore, we were not surprised to observe that Krt14 is expressed in the majority of primary airway BCs as early as 7 days in culture (passage 1) (Figure 1G,I), and becomes ubiquitously expressed by passage 5 (Figure 1H,I). By contrast, Krt15 is expressed in almost all of the primary BCs on passage 1 (Figure 1E,I) and is gradually lost in the majority of basal cells by passage 5 (Figure 1H,I). These trends were similar between mouse and ferret SAE which were cultured in SAGM and Pneumacult Ex+ media respectively (Figure 1I,J). Overall, these changes resemble the trends observed during injury regeneration in vivo.

Krt14 and Krt15 expression during wound healing in an ex vivo tracheal injury model

The question remained whether the increased abundance of Krt14+ BCs in the SAE during wound healing was due to the migration of Krt14+ progenitors from glands or enhanced Krt14 expression of resident BCs in the SAE. To address this question, we used an ex vivo brush injury model of the ferret trachea to examine dynamic changes in keratin expression in a highly controlled setting (Figure 2A). We hypothesized that migration of Krt14+ gland progenitors onto the surface and upregulation of Krt14 in resident SAE BCs were both responsible for the increased abundance of Krt14+ BCs. We observed that on day 2 after partial brushing of the luminal surface of the explant, Krt14 staining was more intense at the edges of the scratch (area to the left of dotted orange line in Figure 2H-J) in cells which incorporated Ethynyl-deoxy-Uridine (EdU) and also expressed lower levels of Krt15 (Figure 2B,E,H). By day 5 post-injury, the expression domain of Krt14 widened and correlated with a broader band of EdU incorporation (Figure 2C,F,I). By day 15, we noticed that the center of the scratch, which
was already covered with Krt14+ cells, retained less EdU (pulsed for 24h on day 3) than the periphery of the scratch marked by higher levels of Krt15 expression (Figure 2D,G,J). This suggests that most of the Krt14+ cells in the center of the scratch either proliferated after the EdU pulse or had diluted the EdU to the point that it became undetectable. By contrast, Krt15+ cells that stayed approximately at the initial scratch boundary retained high levels of EdU. This observation suggests that Krt14/Krt15 expression might distinguish the transit amplifying progenitor fate of BCs from the label-retaining stem cell fate.

To test whether the increased Krt14 abundance on the airway surface is partly due to migration of Krt14+ gland progenitors, we removed the entire SAE by brushing. We observed Krt14+ epithelial cells emerge onto the airway surface from submucosal glands during the 15-day recovery, which was enough to re-epithelialize the majority of the explant surface (Figure S4A-D). The re-epithelialized airway surface on day post injury 15 (DPI15) had overall larger cells, which were mostly Krt14+/Krt15-, unlike in the uninjured explants on day 2 and day 15, which had compact Krt15+ cells (Figure S4E-G). We additionally observed Krt14+/αSMA+ MECs on the surface of injured explants on day 15. Thus, we conclude that glandular contribution to the airway surface repair can at least partially account for appearance of Krt14 on the injured surface epithelium. These observations are consistent with prior work demonstrating surface repair by glandular progenitors in mice and ferrets in vivo (8, 9, 24, 25).

**Generation and gross phenotype of Krt14 and Krt15 knockout mouse models**

To establish a functional link between basal cell keratins and progenitor cell fate during regeneration, we generated Krt14 and Krt15 knockout mice by removing exon 3 from Krt14 and Krt15 genes. Deletion of exon 3 causes a frameshift with a stop codon in exon 4 resulting in loss of exons 3-8 in both keratins. Some of the Krt15-KO mice displayed patchy hair loss in animals that were older that 8 weeks (Figure S5E,F), but were otherwise phenotypically normal with similar body weight compared to their heterozygous littermates (Figure S5H). By contrast,
Krt14-KO mice had significantly lower body weight, visually smaller lungs compared to their heterozygous littermates, frequent unilateral and bilateral ear defects, and increased mortality before and after weaning (Figure S5I-K). Relatively few Krt14-KO mice survived weaning and all of these animals were used for immunolocalization studies. Immunolocalization of Krt15 and Krt14 expression in the trachea confirmed its absence in Krt15-KO and Krt14-KO mice, respectively (Figures 3F and 4B, S5A-B), suggesting that the mutant transcripts are most likely degraded by nonsense-mediated decay. We additionally validated Krt15 knockout using primary airway basal cells at passage 0. Krt15 heterozygous mice had Krt15 and Krt14 co-expression on day 3 in culture, at passage 0. As expected, we observed no filamentous staining for Krt15 and increased intensity of Krt14 staining in Krt15-KO cells, which was additionally confirmed by western blot (Figure S5C-D,G). These studies could not be performed on Krt14-KO basal cells due to poor survival of the mice.

**Evaluation of the airway phenotype of Krt14-KO and Krt15-KO mice**

Further examination of tracheas from Krt14-KO mice revealed the presence of ciliated cells, but fewer club cells, on the airway surface (Figure 3A-F). Submucosal glands of Krt14-KO mice – where Krt14 is normally expressed at homeostasis – expressed less Scgb1a1 than the glands of Krt14 heterozygous (Krt14-Het) mice (Figure 3C-D,I) and were smaller in size (when normalized by the mouse body weight) (Figure 3G-H,K), but a similar % of αSMA+ MECs in the glands compared to Krt14-Hets (Figure 3L). Overall, the tracheal surface epithelium of Krt14-KO mice had a trend towards higher levels of Krt15 compared to their Krt14-Het littermates (Figure 3G,H,J). By contrast, Krt15-KO and Krt15-Het mice had no reduction in gland size (Figure S5A,B) and similar abundance of ciliated and club cells (Figure S6A-E) similarly to Krt15-KO ALI cultures in vitro (Figure S6F,G).

We next sought to evaluate the airway epithelial regenerative responses in Krt15-KO mice after a naphthalene injury. Since Krt15 expression and proliferative capacity of BCs both
decline in human and ferret BO, we hypothesized that Krt15-KO mice would have fewer label
retaining basal cells following injury. To test this, we subjected Krt15-KO and Krt15-Het mice to
a moderate Naphthalene injury (250 mg/kg) and pulsed with EdU on DPI3. We observed that
Krt15-KO and Krt15-Het mice had a decreased abundance of Krt5+/EdU+ cells on DPI21, but
not on DPI7 (Figure 4A-C,D), suggesting a potential defect in basal stem cell maintenance
and/or retention after injury. The abundance of EdU+ non-basal cells (Krt5-) was not different
between genotypes (Figure 4E).

Krt15 and Krt14 Crispr KO

In order to study the functional significance of Krt15 loss, we performed Crispr KO in
passage 2 primary airway basal cells, most of which were still expressing Krt15. For Krt14-KO
experiments we used passage 5 cells, which had strong ubiquitous expression of Krt14 and
relatively weak Krt15 expression by immunofluorescent staining (Figure 1F). We used the cells
from transgenic mice that express Cas9 from H11 locus (H11-Cas9) and ROSA26-LoxP-
Tomato-LoxP-EGFP reporter (ROSA-TG) to rapidly knockout Krt15 or Krt14 on a cell population
level after a gRNA transfection. We co-transfected passage 2 or passage 5 cells using a mix of
gRNAs against LoxP and Krt15 or Krt14 respectively. We then performed FACS to collect
EGFP+ cells, under an assumption that the reporter conversion from Tomato to EGFP would
correlate with disruption of the Krt15 or Krt14 loci (Figure 5A). We verified Krt14-KO efficiency
by western blot (Figure S6A), qPCR (Figure S7B), immunofluorescent staining (Figure S6C),
and sequencing of Krt14 gene locus (Figure S7D) which had 92% of alleles knocked out. In
some instances, we noticed re-expression of Krt15 in advanced passage Krt14-KO cells (Figure
S8A-B). Similarly, we verified Krt15-KO efficiency by immunofluorescent staining (Figure S9A)
and sequencing of Krt15 gene locus (Figure S9B) which had 93% of alleles with frameshift-
containing indels.
Immediately after FACS, 500 cells per well were seeded in a colony formation efficiency (CFE) assay on irradiated 3T3-J2 fibroblasts. We observed that Krt15-KO cells formed fewer colonies that were also smaller in size compared to the scrambled gRNA-treated control cells (Figure 5B,C,F,G), suggesting that loss of Krt15 is detrimental for BC proliferative capacity. A similar decline in proliferative capacity was observed in the primary cells from ferrets that develop BO (14). In this work, we demonstrated that BCs of ferrets with BO and RAS had elevated levels Krt14 and decreased Krt15 in their SAE (Figure 1A-D). In contrast, Krt14-KO cells formed more colonies that were larger in size compared to the scrambled gRNA-treated controls (Figure 5D,E,H,I). Overall, loss of Krt15 or Krt14 led to the opposite outcomes in terms of proliferative capacity. Krt15-KO led to a decline in clonogenicity and resulted in basal keratin profile similar to that of chronic injury and BO (Krt14+/Krt15-). While Krt14-KO improved clonogenicity and resulted in basal keratin profile similar to that of healthy homeostatic airways (Krt15+/Krt14-).

**Nuclear elongation in differentiating Krt14-KO BCs**

We also evaluated the ability of BC progenitor cells to differentiate at an Air-Liquid Interface (ALI). To approach this question and allow for more flexibility in immunostaining with cell-specific markers, we co-transfected BCs with gRNAs against Tomato and Krt14 and subsequently sorted for non-fluorescent cells. We observed that the areas of culture lacking Krt14 had effectively no Scgb1a1+ club cells, compared to Krt14+ regions of the culture (Figure 6A,B,F). Thus, Krt14 appears to have cell-autonomous BC functions in club cell differentiation. We additionally noticed a difference in nuclear size and shape in Krt14-KO cells compared to Krt14+ ones during differentiation (Figure 6A-D). Overall, the abundance of club and ciliated cells was significantly decreased in Krt14-KO cultures (Figure 6F-G, Figure S10A,B). Nuclear shape and differentiation of Krt15-KO basal cells into ciliated and club cells was unaltered (Figure S6F,G).
Krt14 and Krt15 selectively impact migration of SMG cells as compared to SAE cells.

In an effort to further define the roles of Krt14 and Krt15, we tested how the loss of each keratin affects cells migration in vitro. To simulate migration of SMG progenitors through the glandular ducts onto the airway surface, we performed a transwell migration assay, in which cells migrate through 8 µm pores towards TGFβ1, a chemoattractant (Figure S11A). We observed that migration of Krt15-KO and Krt15-Het SAE was similar, while the Krt15-KO SMG cells migrated more efficiently compared to Krt15-Het controls (Figure S11B-C). We also tested Krt14-KO and WT cells in a competitive migration assay, where we seeded Krt14-KO and WT cells at ~1:1 ratio and stained the underside of the transwell after 12h to record the new Krt14-KO:WT ratio in the migrated cell population (Figure S11D). We observed that the KO:WT ratio shifted in favor of Krt14-KO in migrated SMG cells compared to the input ratio, but remained similar in the migrated SAE population (Figure S11E-F). Overall, Krt14-KO and Krt15-KO in SMG, but not in the SAE cells facilitated migration in vitro. The observed effects might be explained by decreased cellular and nuclear rigidity in the absence of Krt14+ and Krt15+ intermediate filaments in the KO SMG cells.

Krt14-KO airway basal cells lose Sfn and upregulate p63, while Krt15-KO cells retain Sfn and downregulate p63 early in differentiation

In order to understand the mechanism by which Krt14 and Krt15 differentially influence the fate of BCs, we looked at the key biochemical differences between these two structurally similar keratins. One notable residue was cysteine 373, which is conserved in Krt14, but is replaced by alanine in Krt15. The importance of this residue for proliferation and differentiation of keratinocytes was elegantly described by Guo et al (15). Using a proteomics screen the authors discovered 14-3-3-sigma (Sfn) as the top Krt14 interacting protein. They demonstrated that proper intracellular distribution of Sfn is dependent on C373 of Krt14 in vivo. Sfn and p63
have been shown to play opposing roles in epidermal tumorigenesis and Sfn heterozygous mice have increased p63 levels in their hyperproliferative epidermis (26, 27). This could be due to dNp63a being targeted for proteasomal degradation by Sfn (28). Therefore, we tested the hypothesis that Sfn abundance and/or distribution would be affected by the loss of Krt14 and whether it would result in p63 upregulation. We observed a marked decrease in Sfn staining in Krt14-KO, but not in Krt15-KO basal cells (Figure 7A-C, D). We demonstrated that Sfn interacts with Krt14, but not with Krt15 using proximity ligation assay (Figure 7E). Additionally, we observed elevated nuclear p63 as well as increased abundance of p63 transcripts in Krt14-KO cultures early in differentiation (Figure 7F-I). The effect on p63 was the opposite in Krt15-KO, where we observed a reduction of p63 on protein and mRNA levels (Figure 7J,K, ). Functionally, we performed a Crispr-KO of dNp63 in primary mouse SAE and observed a reduction in clonogenicity compared to the scrambled control (Figure 7L), which was similar to Krt15-KO. This suggests that unlike Krt15-KO, Krt14-KO SAE might favor prolonged proliferation over differentiation through a mechanism involving downregulation of the tumor-suppressor Sfn and upregulation of an oncogene p63.

Discussion

We show that Krt14 and Krt15 expression in surface airway BCs functionally impacts proliferative potential and differentiation. Since Krt14 is upregulated in the SAE after injury, it likely demarcates transit-amplifying cells (8, 10-14). Transit-amplifying cells have a limited capacity to replicate and are primed to differentiate after a limited number of divisions (29). We observed that Krt14-KO primary airway BCs have increased clonogenic capacity (Figure 3) and impaired club and ciliated cell differentiation (Figure 4, 6). Therefore, Krt14 appears necessary to prime airway BCs for differentiation by limiting their replicative potential as progenitors. Krt14’s counterpart – Krt15 – is expressed in homeostatic Krt5+ BCs, which have been shown to serve as stem cells (6). Krt15-KO early passage primary BC cultures acquire the keratin
profile similar to that of exhausted progenitors in CLAD (Krt14+/Krt15-). Freshly-harvested primary progenitors from ferrets that developed CLAD showed a decreased clonogenic potential in CFE assays (14), which is similar to what we observe in Krt15-KO cells. Additionally, airway BCs in CLAD show decreased p63 abundance (14), which is similar to what we see in differentiating Krt15-KO cells and opposite of that in Krt14-KO cells. Overall, Krt14-KO cells resemble the keratin profile of homeostatic airway BCs (Krt15+/Krt14-), which had the highest clonogenic potential in CFE assays by Swatek et al. (14), similar to Krt14-KO CFE assays in this study.

Prior in vitro Krt14 loss of function studies primarily used squamous epithelial cell lines, like HaCaT and AW13516 (30, 31). These studies have demonstrated that knockdown of Krt14 results in defects in both proliferation and differentiation. Our data on Krt14-KO in primary airway BCs does not show decreased proliferation and instead shows enhanced proliferative capacity in the absence of Krt14. It is important to consider that genetic compensation by other keratins (such as Krt15) is triggered by nonsense-mediated decay and would only occur in a knockout, but not in a knockdown, which could explain the discrepancy. The observed difference in phenotypes can also suggest that Krt14 has context-dependent functions in different types of epithelia.

In humans and mice, frameshift-inducing Krt14 mutations cause epidermolysis bullosa simplex (EBS) – a disease characterized by skin blistering which can also be caused by mutations in Plectin (32-35). Elongation and enlargement of the nuclei that we observed in Krt14-KO basal cells during their differentiation (Figure 6) closely resembles the phenotype of Plectin KO cells (36). Plectin binds keratin intermediate filaments and attaches them to nucleoskeleton through LINC complex as well as linking the filaments with one another and with integrins that mediate cell’s attachment to extracellular matrix (37, 38). The origin of altered nuclear shape in Krt14-KO BCs might be due to compromised attachment of IFs to nucleoskeleton by Plectin. Altered nuclear size and shape can affect trafficking of Yap which...
might explain the increased proliferative capacity and impaired club and ciliated cell differentiation of Krt14-KO BCs observed in our system. Specifically, Elosegui-Artola et al. have demonstrated that elongated nuclei favored nuclear import of Yap – a pro-proliferation transcription factor (39). Future work is needed to further elucidate this relationship in our system.

Previous reports have indicated perinatal lethality in Krt14-KO mice lacking exon 1, but some of the animals were able to survive post weaning age when their epidermal phenotype became less severe (33). Even though the authors demonstrated that Krt15 is able to heterodimerize with Krt5 and form IFs, Krt15 levels did not increase in their knockout model. In contrast to that study, the Krt14-KO mice in our study had overall less severe phenotype with an apparent increase in Krt15 staining intensity in Krt14-KO mouse trachea (Figure 6). This may be due to genetic compensation for the loss of Krt14 by upregulation of Krt15. Unlike the previously-described Krt14-KO mouse model where the authors removed exon 1 of Krt14, our model is subject to nonsense-mediated decay because we removed exon 3 of Krt14 and therefore the transcriptional initiation site was intact (33). Notably, rare human cases of Krt14 loss-of-function mutations are compensated by upregulation of Krt15 and display a much less severe phenotype than the Krt14-KO mice characterized by Lloyd et al (40, 41). Recent reports have described a mechanism where nonsense-mediated decay can lead to transcriptional compensation by closely related gene homologs exhibiting sequence similarity with the mutated gene’s mRNA (42). This mechanism may explain the findings of enhanced Krt15 expression in Krt14-KO BCs.

We generated Krt15 whole body KO mice by deleting exon 3 (the same way as for Krt14-KO mice). These mice were viable and fertile and had a similar body weight as their heterozygous littermates. We noticed that approximately half of the adult Krt15-KO mice (after 8 weeks of age) had partial hair loss on their flanks, head, back or chest (Figure S5). This can be attributed to a defect in maintenance of follicular stem cells which are normally marked by Krt15
(43). Krt15 lineage tracing and genetic ablation studies using Krt15-Cre and iDTR (inducible diphtheria toxin receptor) mice have been carried out in the intestine and esophagus. These works reveal that Krt15 marks a radiation-resistant population of long-lived progenitors with enhanced clonogenicity (44, 45). This is in line with our observation that Krt15-KO airway basal stem cells have a decreased clonogenicity in vitro and decreased long-term label retention in airway BCs after injury in vivo (Figure 4, Figure 5).

The recent article by Guo et al, where the researchers looked at keratinocyte proliferation and differentiation in response to C373A mutation in Krt14, has partly informed the hypothesis in our model system. Pierre Coulombe’s group observed enhanced proliferation, impaired differentiation, altered nuclear shape and increased instances of nuclear fragmentation in the epidermis of Krt14C373A mutant mice (15). This phenotype was similar to our own observations in our Krt14-KO airway basal cells which had enhanced clonogenicity, impaired club and ciliated cell differentiation and abnormal nuclear shape and size during differentiation. We propose that Krt15, which has Alanine in place of Cysteine 373, compensates for the loss of Krt14 in our system and subsequently resembles the C373A mutant Krt14 that Coulombe group used in their study (15).

Using a proteomics screen, Coulombe and colleagues identified 14-3-3 family members as the most abundant interactors with Krt14, in particular, 14-3-3 sigma (Sfn) was the most abundant K14 binding protein and it had much weaker interaction with C373A mutant Krt14 compared to WT (15). Consistent with these findings, we observed a robust protein-protein interaction between Krt14 and Sfn, but not between Krt15 and Sfn (Figure 7), which is logical considering the equivalent residue to C373 of Krt14 is Alanine in Krt15. Guo et al have demonstrated abnormal distribution of Sfn in Krt14 C373A mutant epidermis, which they propose to be the reason for enhanced nuclear Yap localization and subsequent phenotypes of enhanced proliferation and impaired differentiation (15). We also observed a near complete absence of Sfn in our Krt14-KO, but not in Krt15-KO cells (Figure 7). Sfn functions as a tumor
suppressor, its downregulation leads to immortalization of primary human keratinocytes by locking them in a stem-cell-like state (46). Sfn has also been shown to target dNp63a for proteasomal degradation in response to DNA damage (28). Sfn heterozygous mice (+/Er) display hyperproliferative epidermis, increased nuclear p63 and Yap (27). Increased p63 can shift the balance in favor of prolonged proliferation instead of differentiation by repressing Notch signaling (26, 47-49), which is a pathway that specifies club cells in the airways (50). Consistent with this literature, we observed enhanced proliferative capacity, impaired differentiation, decreased Sfn, and increased p63 after a knockout of Krt14 in airway BCs. By contrast Krt15-KO resulted in impaired proliferative capacity, increased Krt14, and decreased p63, which overall resembles the BC’s phenotype in BO (14).

Our lead working model postulates that changes in Krt14/Krt15 content alters BC behavior by modifying Sfn abundance, which in turn impacts signal transduction cascades that balance proliferation and differentiation (i.e., dNp63 nuclear export and proteasomal degradation). Notably, we observed increased dNp63 protein and mRNA levels in Krt14-KO, which cannot be solely explained by impaired degradation of dNp63 protein. However, dNp63 nuclear export is not the only way Sfn can mediate its role as a tumor suppressor. For example, Sfn has also been shown to enhance p53 activity (51), which can further suppress dNp63 transcriptionally (52). Therefore, Krt14-induced changes in dNp63 transcript levels could be indirectly affected by Sfn action on p53.

Conclusions

Overall, this work offers a new insight into the functional significance of Krt14/Krt15 switching in airway BCs in BO and after injury and provides evidence that keratin composition can affect the fate of airway progenitors. Since this work does not exhaustively prove certain mechanistic details of our proposed working model, further exploration is needed on the molecular networks regulated by Krt14 from Krt15 and how these networks divergently impact...
BC behavior. Such studies have the potential to uncover novel pathways for BC reprogramming in the context of chronic lung disease states. Limits on passaging Krt14-KO primary BCs currently impose experimental limitations (e.g., Krt14 rescue experiments), thus future mechanistic studies would greatly benefit from a conditional knockout approach by enabling temporal regulation of each keratin during BC proliferation and differentiation. Such conditional knockout models would also facilitate interrogation of Krt14 and Krt15 functions during airway injury and repair in the absence of developmental compensation.

The findings we provide in this manuscript show how the changes in Krt14 and Krt15 expression can influence the fate and behavior of airway basal cells *in vitro* and *in vivo* with some insights into the potential mechanisms driving these phenotypes. This study is limited in its ability to confirm a cause-and-effect relationship between the injury-associated basal keratin changes and airway fibrosis in BO, but it lays the groundwork for testing these relationships in the future. The present manuscript relies heavily on *in vitro* and murine models of Krt14-KO and Krt15-KO which are insufficient to test the direct relevance to human BO because mice do not develop BO lesions in the injury models available to us. In the future, conditional Krt15-KO and Krt14-KO ferret lung transplantation models can be used to interrogate potential causative effects of Krt14 and Krt15 expression in BO. Nonetheless, our current findings provide insights into airway epithelial injury repair processes which are universally relevant to many airway pathologies.

**Materials and Methods**

**Animal studies**

All animal studies were approved by the University of Iowa Institutional Animal Care and Use Committee (IACUC). The following strains of mice on C57BL/6 background were used:

- B6J.129(Cg)-Igs2tm1.1(CAG-cas9*)Mmw/J (H11-Cas9), B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-
tdTomato-EGFP)J (ROSA-TG), B6.129(Cg)-Krt15^Tm1ex3 (Krt15-KO), B6.129(Cg)-Krt14^Tm1ex3 (Krt14-KO). See the Supplemental Methods for details on how the mice were injured with naphthalene, and pulsed with 5-ethyl-2'-deoxyuridine.

**Primary cell isolation and culture**

Cells from the SAE were isolated from 3-6 mouse tracheas using enzymatic digestion as described in Supplemental methods. Cells were cultured in in Small Airway Growth Medium (SAGM), in F-media (for CFE assays) or in PneumaCult ALI media (for ALI assays) (more details in the Supplemental Methods).

**Crispr/Cas9 knock out in vitro**

Crispr/Cas9 knockouts were performed in primary SAE cells isolated from H11-Cas9: Rosa-TG mice. Cells were transfected with a 1:1 mixture of gRNAs against the reporter (LoxP or Tomato) and against the gene of interest (Krt14 or Krt15) using Lipofectamine RNAiMax. Cells were fluorescently sorted and the knock out efficiency was determined as described in Supplemental Materials and Methods.

**Generation of Krt14 and Krt15 knock out mice**

Krt14 and Krt15 knock out mice were generated at the University of Iowa Genome Editing Core Facility by pronuclear injection of C57Bl6j zygotes with Crispr/Cas9 RNPs containing gRNAs targeting intronic sequences around exon 3 of either Krt14 or Krt15 genes. The resulting chimeric F0 mice were back crossed to wild-type C57Bl6j and the F1 male offspring was used in breeding with wild-type C57Bl6j after the confirmation of the knockout by sanger sequencing.

Pronuclear-stage embryos were collected in KSOM media (Millipore; MR101D) and injected with RNPs. 15 to 25 embryos were immediately implanted into the oviducts of pseudo-pregnant ICR females (see Supplemental Methods for details).
**ALI differentiation, and colony forming efficiency assays**

Primary cells from H11-Cas9: Rosa-TG mice were transfected with the specified gRNA and fluorescently sorted prior to ALI differentiation and colony forming efficiency (CFE) assays.

For ALI assays, FACS sorted cells were expanded in SAGM to obtain sufficient numbers and then seeded at a density of $3 \times 10^5$ cells per well on a 0.33 cm$^2$ polyester (0.45µm pores) transwell membrane (Corning) in SAGM media (Lonza) for at least 24h until confluency was reached. Cultures were then transitioned to an ALI (day 0) and SAGM media was replaced with basolateral PneumaCult ALI (Stem Cell Technologies). Cells are fixed, stained, and imaged on the indicated days of the assay (see Supplemental Methods for details).

For CFE assays, immediately after FACS, 500 cells were seeded onto a monolayer of irradiated 3T3-J2 fibroblasts in 12-well plates. Wells were fixed on day-7 following seeding, stained, and imaged to quantify (see Supplemental Methods for details).

**Ex vivo scratch assays and whole mount ferret trachea staining**

Wild-type adult ferret tracheas were dissected along the membranous side and scratched with a stiff nylon brush (diameter=2mm). Explants were then cultured in F-media. On specified days, the tracheas were pulsed with 10 µM EdU (Thermo Fisher Scientific). On specified days, the explants were fixed, stained, cleared, and imaged *en face* as whole mounts (more details can be found in Supplemental Methods).

**Immunofluorescence**

Mouse tracheas were resected and fixed in 4% paraformaldehyde (PFA) for 2h, washed in PBS, and embedded in paraffin or in OCT frozen blocks. Longitudinal sections were cut, fixed in PFA.
for 20 min, and stained as described in the Supplemental Methods. EdU was detected using Click-iT EdU Cell Proliferation Kit for Imaging (Thermo Fisher Scientific) according to the accompanying protocol with an increased incubation time when performed on whole mount ferret tracheas. A complete list of primary antibodies used can be found in Table S2 of the Supplemental Methods.

**Western Blot analysis**

Protein samples were fractionated using NE-PER kit (Thermo Fisher Scientific) following the provided protocol. Protein concentration was measured using Pierce BCA assay kit (Thermo Fisher Scientific). Samples were run on a SDS PAGE gel under reducing conditions and transferred onto Amersham Protran 0.45 nitrocellulose membrane (GE healthcare), which was subsequently probed using the specified antibodies and imaged using Ai600 imager (GE healthcare). Band intensity was analyzed using ImageJ and normalized to loading control and to the WT control.

**Quantitative PCR analysis**

RNA was isolated from cells using RNeasy kit (Qiagen) and converted into cDNA using High-Capacity cDNA RT kit (Applied Biosystems) according to the manufacturer’s instructions. qPCR reactions were set up using Power SYBR (Applied Biosystems) and run on CFX connect Real-Time PCR detection system (Bio-Rad). The expression data was normalized using delta-delta-CT method. See the supplemental methods for details and the primer sequences.

**Transwell migration assays**

Migration assays were performed using primary mouse SAE or SMG cells which were seeded to confluence (3x10^5 cell/per well) into 804G-coated transwells (d=6.5 mm, pore size 8 µm. Corning, cat# 353097) in SAGM with TGFβ1 (10 ng/ml, PeproTech) in the bottom chamber,
which served as a chemoattractant. Cells on the underside of the transwell were fixed and stained after 12h of migration. More details are in the supplemental materials and methods.

Proximity ligation assay

The assay was carried out using Duo Link PLA kit (Millipore Sigma, Cat# DUO92202). The assay was carried out on permanox chamber slides (Lab-Tek, Cat# 177445) according to the provided protocol. Primary antibody incubation was carried out o/n at 4°C. Experimental samples and negative control were run on the same slides. See the supplemental materials and methods for more details.

Statistical analysis

Results are reported as mean +/- SEM, individual dots on the graphs represent biological replicates unless stated otherwise in the legend. Statistical analysis was conducted using Prism version 8 (GraphPad Software) when N equaled the number of independent animals unless stated otherwise in the legend. The statistical tests used are stated in each figure legend. Significant differences between 2 groups were assessed using a 2-tailed T-test. Data were considered significant at \( P<0.05 \).

Study approval

All procedures were approved by the University of Iowa IACUC and conducted according to the NIH’s *Guide for the Care and Use of Laboratory Animals* (National Academies Press, 2011). Collection of human samples was approved by the Institutional Review Board at the University of Iowa.

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Data availability statement

The datasets generated and/or analyzed in the present study are available from the corresponding author on reasonable request.
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Figure 1. **Dynamic changes in Krt14 and Krt15 expression after an injury and in vitro**

(A-C) Immunofluorescent staining of a cartilaginous ferret airway from a healthy control ferret (A), a ferret 3 days after polidocanol injury (B) and a ferret that developed a form of chronic lung allograft dysfunction (CLAD) after lung transplantation (C). (D-F) Quantified protein expression of p63 (D), Krt14 (E), and Krt15 (F) in large airways. (G-H) Murine SAE cells show a different basal keratin profile on passage 1 (G) vs passage 5 (H). (I-J) Quantification of Krt14 and Krt15 abundance in primary SAE from mice (I) and ferrets (J) grown with dual SMAD inhibition in SAGM and Pneumacult Ex+ respectively. Graphs show mean +/- SEM, N=6 ferrets (D-F) or N≥3 cell pools (I-J). Significance was determined by one-way ANOVA and Tukey’s multiple comparison test (D-F) or by two-way ANOVA and Sidak’s multiple comparison test (I-J). * – P<0.05, ** – P<0.01, *** – P<0.001, **** – P<0.0001. Scale bars in A-C are 50 µm, in G-H are 20 µm.
Figure 2. Krt14 is upregulated at the edges of a regenerating wound ex vivo

(A) Experimental design: ferret tracheal explants were excised, cut longitudinally along the membranous portion of the trachea and cultured submerged in F-media for the indicated time. Day 2 samples were cultured with EdU starting on day 0. Remaining samples were pulsed with EdU for 24h on day 3. (B-D) Staining intensity plots of the right halves of the explants collected on days 2, 5 and 15 after injury. X-axis represents the distance from the center of the scratch. (E-G) Overview micrographs of the scratch on days 2, 5 and 15 after injury. (H-J) Close up micrographs of the boxed regions from the explants on day 2 (H), day 5 (I), and day 15 (J).

Images are representative of N≥3 representative experiments with explants from different animals. Orange dashed lines represent the original wound edge. Scale bars in E-G are 500 µm, Scale bars in H-J are 50 µm.
Figure 3. Krt14-KO mice have reduced glandular size, Scgb1a1 gland secretions and club cells abundance.

(A-B) Immunofluorescent staining of 8-week-old Krt14-Het (A, C) or Krt14-KO (B, D) mouse tracheas for Ac-Tubulin and Scgb1a1. (E-F) Quantification of club cell (E) and ciliated cell (F) abundance in the SAE. (G-H) Immunofluorescent staining of 8-week-old Krt14-Het (G) or Krt14-KO (H) mouse tracheas for Krt14, Krt15 and αSMA. (I) Quantification of Scgb1a1+ SMG cells.

Quantification of the SMG size between Krt14-Het and Krt14-KO normalized to body weight. (L) Quantification of % of αSMA+ cells in the SMGs of Krt14-KO and Krt14-Het mice. Graphs show mean +/- SEM, N≥3 independent mice. Statistical significance was determined by 2-tailed T-test. Scale bars in A-B are 50 µm, in A'-B' scale bars are 20 µm, in E-F scale bars are 200 µm.
Figure 4. Krt15-KO leads to a decline in label retaining basal cells 21 days after injury. (A-B) 5-week-old Krt15-Het or Krt15-KO mice were injured with 250 mg/kg Naphthalene and pulsed with EdU daily for the first 3 days immediately after injury. Immunofluorescent staining of the tracheas from Krt15-Het (A) or Krt15-KO (B) mice 21 days post injury. Yellow arrows indicate Krt5+ EdU+ cells. (C) The overall abundance of EdU+/Krt5+ cells in the SAE was quantified on DPI7. (D-E) Abundance of EdU+ Krt5+ cells (D) and EdU+ Krt5- cells (E) was quantified for DPI21. Graphs show Mean +/- SEM, N ≥ 4 independent mice. Significance was determined by 2-tailed T-test. Scale bars are 100 µm.
Figure 5. Knockouts of Krt15 and Krt14 have the opposite effects on clonogenic potential of primary SAE in vitro.

(A) The outline of CRISPR KO experimental design. Passage 2 mouse SAE cells were used for Krt15-KO, passage 5 for Krt14-KO. Cells from ROSA-TG:H11-Cas9 mice were co-transfected with a 50:50 mix of LoxP and Krt15 or Krt14 gRNAs, 7 days later EGFP+ cells were sorted and seeded for CFE assay (500 cells/well of a 12-w plate). (B-E) Wells were fixed, stained and imaged 7 days after the start of the CFE assay. (F, H) Colony formation efficiency was estimated by counting colonies with over 50 cells in them. (G, I) Total colony area was calculated in ImageJ. Graphs show mean +/- SEM, N ≥ 3 independent transfections, dots represent different technical replicates (different wells). Significance was determined by mixed effect model Nested 2-tailed T-test. Scale bars are 1000 µm.
Figure 6. Differentiating Krt14-KO cells have enlarged and elongated nuclei and impaired club and ciliated cell specification.

Passage 5 ROSA-TG:H11-Cas9 cells were transfected with a mix of Krt14 and Tomato gRNA’s, after 7 days sorted for Tomato-/EGFP- cells and grown on ALI for 21 days. (A-B) Immunofluorescent staining for K14, nuclei (HO342) and Scgb1a1 was performed en face on day 21 cultures. (C-D) Nuclear aspect ratio and size were quantified in Metamorph from cells grown on the ALI for 21 days. (E-F) Fold change in of Scgb1a1 and Ac-Tub area staining was quantified using ImageJ. (G) Representative confocal micrographs of Krt14-KO and WT cultures on ALI day 21. Graphs shows mean +/- SEM, N≥5 transwells. Graphs in E and F show N≥3 experiments pulled, graph in C is a representative of N=2 experiments and the graph in D shows N=1 experiment (cells collected from 3-4 mice were used per experiment). Significance was determined by 2-tailed T-test in C and D and by mixed effects model Nested 2-tailed T-test in E and F. Scale bars are 50 µm.
Figure 7. Krt14-KO airway basal cells lose Sfn and upregulate p63, while Krt15-KO cells downregulate p63 early in differentiation

(A-C) Immunofluorescent staining of Krt14 and Sfn in Krt14-WT (A), Krt14-gRNA treated cells (B) and Krt15-gRNA treated primary airway basal cells (C). (D) Quantification of immunofluorescent staining for Sfn from panels A-C. (E) Proximity ligation assay (PLA) of the interaction between Krt14 or Krt15 and Sfn or negative control (NC) in p2 healthy Human SAE and quantification of the PLA signal. (F-G) Western blot analysis of the cytosolic (F) and nuclear (G) fractions collected from Krt14-WT and Krt14-KO airway basal cells on ALI day 5. (H) Normalized quantification of western blot band intensity. (I) qPCR analysis of Krt14-KO and Krt14-WT differentiating cultures on ALI day 5. (J) Western blot analysis of the nuclear fraction collected from Krt15-KO airway basal cells on ALI day 5. (K) qPCR analysis of Krt15-KO and Krt15-WT differentiating cultures on ALI day 5. (L) Quantification of colony forming efficiency of dNp63-KO vs Scrambled-KO primary mouse SAE cells. Crispr-KO and CFE assays were performed similarly to Krt15-KO in figure 5. Graphs in E,H,I,L show mean +/- SEM from N=3 independent pools of primary cells. Graph in K shows mean +/- SEM of technical replicates from a cell pool isolated from 3 independent Krt15-WT or KO mice. Lanes in F, G, and J were run on the same gel but were noncontiguous. Statistical significance in was determined by two-way ANOVA, Sidak’s multiple comparison test (H,I,K) or, by one-way ANOVA, Tukey’s multiple comparison test (D,E) or by 2-tailed T-test in panel L (P<0.0001). Scale bars are 20 µm.
Graphical abstract caption: Krt14 replaces Krt15 during chronic airway injury in BO which correlates with decreased proliferative potential. Krt14-KO in vitro enhances proliferative potential and inhibits club cell differentiation while Krt15-KO reduces proliferative potential and allows for normal differentiation. These two contrasting phenotypes are accompanied by reduced Sfn and increased dNp63a levels in Krt14-KO and with the opposite changes in Krt15-KO. We propose a mechanism whereby di-sulfite bonds form between Krt14+, but not between Krt15+ filaments during differentiation to enable binding and stabilization of Sfn which can then mediate degradation of dNp63a resulting in the observed phenotypes.