ORIGINAL ARTICLE

CRISPR–Cas9-mediated gene knockout in primary human airway epithelial cells reveals a proinflammatory role for MUC18

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Targeted knockout of genes in primary human cells using CRISPR–Cas9-mediated genome-editing represents a powerful approach to study gene function and to discern molecular mechanisms underly underlying complex human diseases. We used lentiviral delivery of CRISPR–Cas9 machinery and conditional reprogramming culture methods to knockout the MUC18 gene in primary human nasal airway epithelial cells (AECs). Massively parallel sequencing technology was used to confirm that the genome of essentially all cells in the edited AEC populations contained coding region insertions and deletions (indels). Correspondingly, we found mRNA expression of MUC18 was greatly reduced and protein expression was absent. Characterization of MUC18 knockout cell populations stimulated with TLR2, 3 and 4 agonists revealed that IL-8 (a proinflammatory chemokine) responses of AECs were greatly reduced in the absence of functional MUC18 protein. Our results show the feasibility of CRISPR–Cas9-mediated gene knockouts in AEC culture (both submersed and polarized), and suggest a proinflammatory role for MUC18 in airway epithelial response to bacterial and viral stimuli.

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INTRODUCTION

Functional studies of a gene or multiple genes in primary human cells are critical to elucidate the pathological mechanisms underlying complex human diseases. In the past decade, many studies have utilized RNA interference (RNAi) technology to effectively knockdown genes of interest.1 However, this knockdown approach does not result in complete loss of gene/protein expression (knockout (KO)) and can often result in off-target effects.2 Thus, methods for complete KO of a gene in human cells, especially in primary cells, are urgently needed.

By using clustered regularly interspaced short palindrome repeats-associated Cas9 nuclease (CRISPR–Cas9) technology, several groups of investigators have successfully generated gene KOs and made sequence level nucleotide changes in both human transformed and induced pluripotent stem cells (iPSCs).3-5 Moreover, CRISPR–Cas9 machinery has recently been used successfully to edit the genome of primary mouse cells in vivo.6-8 However, this technology has only been applied to a few primary human cell types currently used in culture to study specific disease processes.9 Application of this technology to primary airway epithelial and other primary cell types is limited by low transfection efficiencies, a requirement for multiple passages/proliferation, and harsh selection conditions required to achieve an edited primary cell population.

Human primary airway epithelial cells (AECs) are the first line of host defense against hazardous inhaled environmental factors such as pathogens and pollutants. The ability to KO genes in AECs would allow functional analysis of single genes in the epithelial response to these environmental risk factors.

MUC18, also known as CD146 or melanoma cell adhesion molecule (MCAM), is a 113 kD transmembrane glycoprotein and is a member of the immunoglobulin superfamily.10,11 MUC18 is composed of an extracellular domain, a single transmembrane domain and a short (63 amino acids) cytoplasmic tail. MUC18 overexpression was initially identified in human malignant melanoma cells and thought to promote tumor metastasis.12-14 Our recent publications15,16 demonstrated that MUC18 is upregulated in asthmatic and COPD patient airway epithelial cells. MUC18 protein is expressed by basal and ciliated airway epithelial cells.15 Our findings further suggest that MUC18 is critical to bacteria-induced murine lung inflammation.15 However, whether MUC18 promotes airway epithelial inflammatory responses to pathogens or toll-like receptor (TLR) agonists, mimicking pathogen infections, remains unclear.

In the current study, we detail for the first time, generation of primary human nasal airway epithelial cells knocked out for a gene (here MUC18), using CRISPR–Cas9 technology. We use the MUC18 KO cells to demonstrate a proinflammatory function of MUC18 in response to stimulation with various TLR agonists. Our workflow provides a strategy to produce gene KO’s in primary airway epithelial cells, and our results reveal a function of MUC18 in the airway epithelium that may be important to multiple airway diseases.
RESULTS
MUC18-targeted KO strategy
Preliminary studies indicated that primary airway epithelial cells are difficult to transfect at high efficiency and low toxicity. Consequently, a lentiviral transduction strategy was used to introduce the CRISPR–Cas9 machinery. A recently developed lentiviral vector, which expresses the sgRNA, Cas-9 nuclease and puromycin resistance gene was used (Figure 1). The gRNA was designed to target Cas9 machinery immediately downstream of the MUC18 start codon. Targeting at this site will create double-stranded breaks repaired by nonhomologous end joining that will result in frame shift insertions and deletions (indels), and thus ‘knockout’ functional MUC18 protein (Figure 1). Random integration of the lentiviral expression cassette ensures stable expression of the MUC18-targeting CRISPR–Cas9 and puromycin selection machinery. The application of puromycin allows the selection of cells with successful integration and has previously been shown to eventually lead to a mixed population (with respect to a specific indel) of biallelically edited cells.

Initial generation of MUC18 KO nasal airway epithelial cells
Passage 3 primary human nasal AECs (AEC-1) were infected in standard growth conditions, with addition of a ROCK inhibitor. Transduction efficiencies were determined using a green fluorescent protein (GFP) expressing control virus and were near 100%. Owing to the limited proliferative capacity of primary AECs and the need to select the infected cell population, the cells were transitioned into modified Schlegel culture conditions. This culture method involves the growth of epithelial cells on an irradiated fibroblast feeder layer, with specialized media additives, and a ROCK inhibitor. Several studies have revealed this method allows near unlimited proliferation and passage of several epithelial cell types without transformation and loss of primary cell characteristics. We generated puromycin-resistant fibroblasts to allow puromycin selection of AECs without disruption of the feeder layer. Cells were seeded at low density (1–3.2 × 10^5 per 100-mm dish) throughout culture to aid in selection. In total, puromycin selection was applied across five AEC passages (39 days in this experiment), which based on experiments using this system in cell lines should have been adequate time for selection and CRISPR-mediated cutting of the MUC18 target site (Supplementary Figure 1). A PCR amplicon was designed across the MUC18 cut site to examine generation of indels. High resolution melt (HRM) analysis of PCR products from P8 GFP control and MUC18 CRISPR–Cas9-treated cells revealed significant differences in the melting behavior of CRISPR–Cas9-treated cell amplicons, characteristic of sequence differences in the CRISPR-treated cells (Figure 2a). In contrast melting behavior was highly similar between GFP control and MUC18 CRISPR–Cas9 treated cells for amplicons designed across the top four predicted off-target sites, indicating the gRNA was targeting specifically to the MUC18 exon (Supplementary Figure 2). To determine the base composition of the sequence changes and quantify their occurrence in the selected cell populations, we generated sequencing libraries from the PCR products. These libraries were examined by massively parallel sequencing technology. Strikingly, we found 99.6% of the 18,171 sequencing reads examined from the selected MUC18 CRISPR–Cas9 cell population contained indels, versus only 1.9% of the selected GFP-control cell population reads (Figure 2). These results indicate that only integrated cells survived selection, the CRISPR–Cas9 machinery had mediated cutting, and subsequent repair had occurred in nearly all cells in the batch-selected population. Consistent with the batch selection of cells and random nature of indel formation in nonhomologous repair, we observed five different deletions and one insertion with a frequency of >1% in the MUC18 CRISPR–Cas9-treated cells (Figure 2). However, we did observe the majority of reads contained a 5-bp deletion, suggesting a bias in the repair process to favor generation of this deletion (Figure 2).

Optimized generation of MUC18 KO nasal airway epithelial cells
We attempted to repeat MUC18 gene editing in nasal epithelial cells from a new donor (AEC-2) and also to reduce the time and passages necessary to generate the edited cell population (Figure 3a). In this experiment we also infected cells using a lentivirus carrying the CRISPR machinery with a scrambled MUC18 gRNA sequence, to generate a suitable control cell population for comparison in the characterization experiments of the MUC18 KO cells. Cells from this donor were infected at passage 2, and during selection passages cells were seeded at increased density (0.5–2.5 × 10^5 per 100-mm dish) relative to the first experiment to decrease the time between passages (Figure 3a). Again, high efficiency of transduction was observed by infection of cells with GFP expressing lentivirus (Figures 3b and c). Editing frequency was determined after each passage to establish when maximum

Figure 1. Lentiviral vector and MUC18 knockout targeting strategy. (a) Simplified schematic of the published plentiCRISPRv1 vector we used to target MUC18. We designed the MUC18 gRNA and ligated into the plentiCRISPRv1 vector. The plentiCRISPR vector coexpresses both the MUC18 gRNA and the Cas9 protein. (b) The gRNA and thus cutting site was chosen to be immediately downstream of the start codon for MUC18 so indels introduced by nonhomologous repair of the double-stranded break would likely disrupt the protein reading frame.
editing frequency was reached. After a single-passage MUC18 CRISPR–Cas9-treated cells displayed indels in 62% of sequenced reads (P3 cells), which increased to 95.6% of reads after an additional passage (P4 cells). The frequency of indels detected was 96.9% and 96.3% for P5 and P6 cells, respectively (Figure 2). On the basis of this stabilization of edited reads at 96% we suggest that all cells are likely edited at this point and the remaining % of unedited reads may derive from low-level feeder cell contamination. Comparison of HRM amplicons from scrambled gRNA control and the MUC18–TIRAP CRISPR–Cas9–treated and selected AECs. PCR was used to amplify a sequence crossing the MUC18 cut site from the two experiments. (a) High resolution melt (HRM) analysis of PCR amplicons, revealing a significant melting curve shift for the amplicons derived from MUC18 CRISPR–Cas9–treated versus GFP–virus–treated AEC–1 cells. (b) Results of next-generation sequencing analysis of the same PCR amplicons for indels over the MUC18 cut site. This sequence data reveals the presence of indels in nearly all reads originating from MUC18 CRISPR–Cas9–treated AECs from the two experiments. (c) We list the most common indels detected across the MUC18 CRISPR–Cas9 cut site from the two experiments.

Effects of MUC18 KO on proinflammatory cytokine production in TLR agonist-stimulated AECs

The function of MUC18 in airway epithelial cells remains unclear. In our previous studies in mouse lung macrophages, we found that MUC18 exerts a proinflammatory function by promoting TNF-α production.16 IL-8 is one of the major proinflammatory cytokines in the airways. Therefore, we determined if MUC18 KO affects airway epithelial cell IL-8 responses to stimulation by the agonists of three major TLRs. These agonists mimic microbial components of bacteria and viruses. Importantly, we observed that production of IL-8 protein by agonists of TLR2 (Pam2CSK4), TLR3 (poly(I:C)) and TLR4 (LPS) was significantly dampened in MUC18 KO cells as compared with the GFP control cells, in donor AEC-1 (Figure 5). These results were consistent with IL-8 mRNA expression (Figure 5). MUC18 KO cells from the second donor (AEC-2) had a similar decrement in IL-8 protein response for poly(I:C) and LPS stimulation, as compared with the cells expressing the scrambled MUC18 CRISPR–Cas9 (Figure 5). IL-8 mRNA expression response to all agonists was reduced in the MUC18 KO cells (Figure 5).

We were able to form polarized air–liquid interface (ALI) cultures from these MUC18 KO cells. We confirmed cell polarization by transepithelial electrical resistance (TEER) measurements (Figure 6a). These ALI cultures were used to test the TLR3 agonist response. As shown in Figure 6b when MUC18 KO cells (AEC-2) were stimulated with poly(I:C) they produced significantly lower levels of IL-8 than the control cells (scrambled CRISPR), consistent with submerged culture results. These results strongly suggest a
proinflammatory function of MUC18 in airway epithelial cells in the context of both bacterial and viral lung infections.

**DISCUSSION**

The ability to specifically edit the genome of human cells would permit gene/sequence variant functional analyses and allow determination of their contribution to human disease pathology. The emergence of CRISPR–Cas9 technology with its efficiency of editing, lower cost, and open-source availability of reagents has seemingly put the goal of generating cell lines harboring gene KOs within reach of most investigators. Lentiviral-delivered CRISPR–Cas9 technology has recently been used to successfully perform genome-wide gene KO screens and correct genetic sequence defects. However, most of these studies were performed in cells with an enhanced capacity to proliferate, namely iP5 cells or transformed cell lines. These studies are limited, in the case of transformed cells, by the degree to which the transformed cells represent the primary cells from which they were derived, and for iP5 cells by the requirement to differentiate these cells into the specific cell type important to the disease being studied. Therefore, the application of editing methods to the various primary cell types currently being used to study cellular mechanisms of disease is highly desirable. Traditional limitations to primary cell editing include low transfection efficiency, limited proliferative capacity, and sensitivity to selection methods.

To overcome these limitations in primary human nasal airway epithelial cells, we applied recently developed culture methods to conditionally reprogram epithelial cells. As previously reported, this reprogramming allows a large number of cell passages and extensive proliferation of primary epithelial cells, with retention of specific primary cell character. Importantly, this process does not involve cellular transformation or cancerous phenotypic changes (such as loss of contact inhibition and mutations). The application of these culture conditions resulted in low cell toxicity while achieving near complete lentiviral transduction, as has recently been reported in AECs. In addition, these methods allowed us to subculture cells at low density in selective media for multiple passages, aiding in the generation of edited cells. Importantly, we demonstrated that gene KO in primary cells can be maintained not only in undifferentiated cells, but can be achieved in polarized cells. Considering this conditional reprogramming method has been successful in multiple other epithelial cell types, we believe the methods we outline here may be broadly applicable to various other primary cells.

Supporting this, CRISPR–Cas9 gene targeting with homology directed repair was recently used to correct the CFTR del508 mutation in intestinal stem cells, which were then grown into organoids.

Our editing efficiency compares favorably with recent editing studies in other primary mammalian cells both in vitro and in vivo. Specifically, our second attempt at editing by this method resulted in 95.6% of cells edited after just 8 days of selection. In fact, this editing rate and selection timeframe is comparable to the 92%
Cas9 machinery may result in phenotypic changes unrelated to the gene KO. Again, these problems are partially cation of protein loss and agreement in functional consequences between selected populations. Second, the integration and continued expression of the CRISPR–Cas9 machinery may result in phenotypic changes unrelated to the gene KO. Again, these problems are partially addressed by generation of multiple KO cell populations. Alternative strategies to develop KO’s include ‘knock-in-knock-outs;’ that is gene interruption by homologous insertion of a selection cassette, which would directly address this issue. The efficiency of CRISPR machinery delivery and cutting we achieved here, increase the likelihood this more complex strategy will be fruitful. Despite these limitations we believe our study represents a substantial initial step toward the realization of other primary cell types.

**MATERIALS AND METHODS**

Nasal brushing, cell recovery, and expansion

Human nasal airway epithelial cells were collected from two healthy subjects, by brushing the region posterior to the inferior turbinate with a cell cytology brush using a nasal illuminator, as previously described. The epithelial cells from the brush were dispersed in PBS, washed twice and cultured using a modified Schlegel method. Briefly, cells were cultured on a monolayer of irradiated NIH 3T3 fibroblasts in F-Media plus 10-μm ROCK inhibitor (Y-27632). Double trypsinization was used to separate epithelial cells from fibroblasts at passage. Specifically, trypsin is added to the cultures and the fibroblasts typically release quickly, ~30 s to 1 min. Complete release of fibroblasts is monitored by microscope. The epithelial colonies are then washed and trypsinized a second time to remove epithelial cells. This trypsinization is longer; it takes 5–7 min depending on the size of the colonies. The study was approved by the Institutional Review Board at National Jewish Health. The two study subjects provided written informed consent.

Design of MUC18 gRNA and lentiviral CRISPR–Cas9 vector

We used the lentiviral expressing CRISPR–Cas9 vector generated by the Zhang lab, plentiCRISPRv1. This one vector system expresses the gRNA, Cas9 protein, and puromycin resistance gene from one virus. The MUC18 gRNA was designed using the Zhang lab software available at http://crispr.mit.edu. The gRNA sequence used was 5'-CTGCTGCTTCTCCTCGGTCCG-3'. According to Zhang lab protocols (http://www.genome-engineering.org/crispr?page_id=23), DNA oligonucleotides for the gRNA and reverse complement sequence plus adapters needed for ligation were synthesized and cloned into the plentiCRISPRv1 vector (forward oligo, 5'-CACCGCGTGC CGTCTTCTCCTCGGTCCG-3'; reverse oligo, 5'-AAAAACGAGCCGAGGACAGCAG CAGC-3'). We also scrambled the MUC18 gRNA sequence and cloned this ligated oligo (forward oligo, 5'-CTGCTGCTTCTCCTCGGTCCG-3'; reverse oligo 5'-AACGAGCCGAGGACAGCAGC-3') into the same vector to serve as a control for characterization experiments. Correct cloning and study as we recently found that MUC18 protein was increased in alveolar macrophages from both asthma and COPD patients. Moreover, study of MUC18 KO mice indicated a proinflammatory function of MUC18, as KO mice displayed reduced inflammation and neutrophil recruitment in response to lung bacterial infections. To date, the functional relevance of airway epithelial MUC18 expression to inflammation in the lung remains unclear. Our MUC18 KO of human AECs reveal this molecule has proinflammatory function under stimulation with various TLR agonists, mimicking bacterial and viral infections, and have brought these findings into the context of human cells. These results suggest the need for future studies using live respiratory bacteria or viruses to clearly demonstrate MUC18 function under these disease conditions. Given the importance of bacterial and viral infections in triggering asthma/COPD exacerbations and lung inflammation, MUC18 may have a significant role in these processes.

We also realize that further dissection of the molecular mechanisms whereby MUC18 exerts a broad spectrum of proinflammatory functions will be important. Nonetheless, in a recent paper by our group, we determined the mechanism by which MUC18 amplifies the proinflammatory response to the TLR3 agonist polyIC. Our previous data suggest that MUC18 seizes phosphorylation particularly at the cytoplasmic tail is critical to MUC18 proinflammatory function in response to various TLR agonists. In response to polyIC, increased phosphorylation of MUC18 serines was observed. Reduction of MUC18 serine phosphorylation by inhibiting ERK activity was associated with less production of IL-8 following polyIC stimulation.

In summary, our results indicate epithelial-derived MUC18 contributes to airway inflammation in response to important microbial innate immunity triggers. In addition, we suggest the methods outlined herein to achieve CRISPR–Cas9-mediated gene KO can be applied to the study of other genes in primary human airway epithelial cells and potentially other primary cell types.
sequence was confirmed by capillary resequencing of the plentiCRISPRv1 constructs.

Generation of MUC18 CRISPR–Cas9 and GFP lentiviral particles
Lentivirus particles were generated in the Lenti-X 293 T packaging cell line by transfecting the psPAX2 packaging plasmid (Addgene Plasmid #12260; Addgene, Cambridge, MA, USA), the pCMV-VSV-G pseudotyping plasmid (Addgene Plasmid #8454; Addgene), and either the plentiCRISPRv1-MUC18, plentiCRISPRv1-MUC18_Scr or pLJM1-EGFP (Addgene Plasmid #19319; Addgene) viral vector plasmids, to produce MUC18 CRISPR–Cas9, MUC18_Scr CRISPR–Cas9 and GFP-expressing viruses, respectively. Transfections were performed with lipofectamine 2000 (Life Tech, Carlsbad, CA, USA) in 100-mm dishes using 7 μg of the viral vector, 9 μg of the packaging

Figure 5. Proinflammatory response of MUC18 knockout AECs to TLR agonist stimulation. The two MUC18 knockout cell populations (AEC-1 and AEC-2) were incubated in triplicate with PBS (medium control) or agonists of TLR2 (Pam2CSK4), TLR3 (polyI:C) and TLR4 (LPS) for 24 h. Cell supernatants were collected for detection of IL-8 protein by ELISA (n = 3 replicates for each condition) and IL-8 mRNA by real-time PCR (n = 1). IL-8 protein data (median/interquartile range) are expressed as changes of IL-8 in TLR agonist-stimulated cells minus IL-8 in medium control cells. *P < 0.05 comparing KO supernatants with GFP virus or scrambled MUC18 CRISPR–Cas9. Src, scrambled MUC18 CRISPR–Cas9; Pam 2, Pam2CSK4; KO, MUC18 CRISPR–Cas9.

Figure 6. Air–liquid interface (ALI) polarized MUC18 knockout AECs have decreased IL-8 production in response to TLR3 agonist polyI:C. (a) MUC18 knockout and scrambled MUC18 CRISPR–Cas9-treated AECs were cultured at ALI for 10 days. Transepithelial electrical resistance (TEER) measurements are shown before and after polarization. Bars represent median and interquartile range for 8 ALI inserts at two different time points. A single empty insert was used to produce the blank measurement at both time points. *P < 0.001. (b) Polarized ALI day 10 cells were stimulated in triplicate with polyI:C for 24 h. IL-8 protein data (median/interquartile range) are expressed as changes of IL-8 in polyI:C stimulated cells minus IL-8 in medium control cells. Scr, scrambled MUC18 CRISPR–Cas9-treated cells; KO, MUC18 knockout cells.
plasmid and 0.9 μg of the pseudotyping vector. Virus-containing media was collected 72 h later and centrifuged. Adequate titer was determined by positive Lenti-X GoStix test, which indicates a titer >5 × 10^{12} IFU/ml. The pLJM1-EGFP viral vector also expresses a puromycin resistance gene.

Viral transduction and selection of AECs

Nasal AECs were passaged twice using the modified Schlegel culture method.17,18 Prior to transduction, the nasal AECs were subcultured on collagen-coated 100-mm dishes in BEGM growth media (CC-4175, Lonza) plus 10-μM ROCK inhibitor, 16 mM HEPES buffer and 12 μg ml^{−1} polybrene) by spin infection (920 g at 25 °C) for 1 h. At 24 h post-infection puromycin (1 μg ml^{−1} final concentration) was added to the culture media. In parallel, we transduced 3T3 fibroblasts with the empty plentiCRISPR vector and selected these cells (1 μg ml^{−1} puromycin) to generate feeder cells resistant to puromycin. The viral transduced nasal AECs were grown to 80–90% confluence and passaged using the modified Schlegel culture method and the puromycin-resistant 3T3 cells. These modified Schlegel culture conditions were maintained throughout the selection period to generate transduced GFP expressing, MUC18 scrambled gRNA expressing and MUC18 KO cells, as described in Figure 3 and Supplementary Figure 1.

High resolution melt analysis of MUC18 CRISPR–Cas9 target site and potential off-target site amplicons

PCR primers were designed to specifically amplify the chromosome 11 genome region 119187608–119187798 that spans the MUC18 CRISPR–Cas9 cut site (primer sequences listed below). The same CRISPR gRNA design tool used to generate the MUC18 gRNA sequence provides a list of the top potential off-target sites for the gRNA that it designs. We designed primers to amplify the regions containing the overall top three off-target sites and top two genic off-target sites (four in total) for cutting by HRM assay. The off-target locations and primers to amplify these regions are listed in Supplementary Table 1 and 2. These primers were used to perform a PCR reaction on the sequencing reads to subsequently analyze the amplicons using the MeltDoctor HRM Master Mix (Life Tech, Part #4415440) and standard protocol. PCR reaction and HRM analysis were performed on a Life Technologies QuantiStudio6 quantitative PCR instrument (Life Tech).

Sequence analysis of MUC18 CRISPR cut site

Library generation and sequencing. We generated Ion Torrent PCR amplicon libraries using a fusion method (Ion Torrent User Guide 468326 Rev. B). Namely, the primers used to perform HRM analysis were used to amplify the MUC18 CRISPR–Cas9 cut site. The forward primer contains a 5′ universal adapter sequence followed by the MUC18 priming sequence 5′-TCGTCGTACGACGCTTGGGATCCGGGAGCCGAATTACCGAGGAGGAC-3′. The reverse primer contains the 5′ tr1 primer sequencing adapter followed by the MUC18 priming sequence 5′-CTCTCTATGGGCAGTCGGTGATGGCT-3′. This primary PCR reaction was performed with Pyromark PCR reagents (Qiagen, Valencia, CA, USA; Cat#9798703) according to standard protocols. These PCR products were used to template a secondary PCR reaction, which introduces the A-sequencing adapter and barcodes specific to each sample through the forward primer, 5′-CATCTCCTCCGTCTGCAGCTGAGGGTGACATGACGAGGAGGAGGACGAGGT-3′ (X, Ion Torrent barcode sequences) and maintains the tr1 primer sequencing adapter with a tr1 reverse primer, 5′-CTCCCTATGGCCAGCCTGCT-3′. Secondary PCR reactions were completed with Phusion PCR reagents using HF buffer (New England Biolabs, Ipswich, MA, USA; Cat#M0530). Secondary PCR products representing the sequencing libraries were gel-purified and combined in equimolar amounts to form the sequencing library pool. Standard Ion Torrent templating of Ion Spheres was completed followed by sequencing on a 314 chip with the Ion Torrent Personal Genome Machine (PGM).

Bioinformatic analysis of sequencing reads. Sequencing reads were mapped to the PCR amplicon using the default settings of the Ion Torrent mapper, TMP. We interrogated the cut site (3–4 bp upstream of the gRNA PAM sequence) within sequencing reads for insertions and deletions (indels). The boundaries up- and downstream of the cut site defining the area within which indels were counted, were determined by the last amplicon position, which exhibited an indel frequency of at least 0.5% in the CRISPR–Cas9-treated sample. In addition, only indels of at least 2 bp in length were considered for this purpose, in order to eliminate possible sequencing error artifacts. The total indel rate for each sample was estimated as the number of reads harboring any ≥ 2-bp long indel within the defined cut site divided by the total number of reads spanning the entire cut site. All of these calculations were performed using custom written Perl scripts using mapped sam files as input, and confirmed by visual inspection using the Integrative Genomic Viewer.25 Using the above described criteria, in both samples we observed a significant number of reads (~8%), which were not scored as containing an indel. Investigating these reads we discovered that the majority harbored one of two 1-bp insertions at exactly the same positions in close proximity to the expected cut site. Frequency of these particular 1-bp insertions in the control samples were negligible; therefore, we believe this event to be a direct effect of the CRISPR treatment. Therefore indel frequencies listed in Figure 2 reflect this insertion.

Gene and protein expression analysis of MUC18 CRISPR–Cas9-treated AECs

MUC18 and IL-8 mRNA in epithelial cells was determined by real-time quantitative PCR and analyzed using the comparative threshold cycle method, normalizing to the housekeeping gene GAPDH. Western blots were used to examine epithelial MUC18 protein (Epitomics, Burlingame, CA, USA; Catalog #2505-1). Densitometry was performed to quantify the protein in relation to GAPDH. IL-8 protein levels in culture supernatants were determined by using the IL-8 enzyme-linked immunosorbent assay (ELISA) Duoset development kit (R&D Systems, Minneapolis, MN, USA; Cat#DY8000C).

TLR agonist treatment of submersed Nasal AECs

KO and MUC18 scrambled CRISPR/Cas9 nasal AECs were seeded in collagen-coated 12-well plates at 2 × 10{sup}5 cells per well in standard BEGM media on coated inserts at 1.2 × 10{sup}5 cells per insert. At 24 h post-transduction, the nasal AECs were subcultured on ALI as described in Karp et al.26,27 Specifically, 6.5-mm inserts (Costar 3470, Corning Inc., Corning, NY, USA) were precoated with 1 collagen (Purecol Bovine Collagen #5005-B, Advanced BioMatrix, Carlsbad, CA, USA). KO and MUC18 scrambled cells were plated in airway medium 1{sup}27 on coated inserts at 1.2 × 10{sup}5 cells per insert. At 24 h post-plating airway media 1 was replaced with airway medium 2{sup}27 (containing 2% Ultroser G, Pall Corporation, Port Washington, NY, USA). Air-lift (removal of apical media) was performed 72 h post-plating. Cells were maintained air-lifted for 10 days. Transepithelial electrical resistance (TEER) was determined using the Millicell ERS-2 Epithelial Volt-Ohm Meter (Millipore). Day 10 ALI cultures were stimulated at both apical and basolateral sides with polyI:C (2.5 μg ml^{−1}) for 24 h. The combined supernatants from both apical and basolateral sides were collected for measuring IL-8 protein.

Statistical analysis

Comparison of KO and control cells was performed by Wilcoxon signed rank test for IL-8 protein data and Mann–Whitney test for TEER measurements. Data are presented as medians ± interquartile ranges. Sample sizes were not chosen based on a prespecified effect, as this was an exploratory analysis. A P-value of <0.05 was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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