# Highly efficient genome editing in primary human bronchial epithelial cells differentiated at air–liquid interface

*To the Editor*:

The structure and composition of the bronchial epithelium is altered in respiratory diseases such as COPD and asthma, in which goblet cell hyperplasia and reduced numbers of ciliated cells impair mucociliary clearance. Current standard of care focuses on alleviating disease symptoms, such as airway obstruction and inflammation, using bronchodilators or steroids, respectively. Whilst these approaches are effective, they do not address the underlying pathogenic processes. An alternative way forward would be to identify and target the dysregulated cellular pathways responsible for impaired bronchial epithelial function. In this study we describe a single-step, highly efficient and easily scalable genome editing pipeline to aid the dissection of the molecular mechanisms underlying primary human bronchial epithelial cell (BEC) differentiation and function at air–liquid interface (ALI).

The primary BEC based ALI system is an established *in vitro* airway model that allows the study of differentiation and function of bronchial epithelia [1–3]. Following a 28-day differentiation at ALI, primary BECs form a pseudo-stratified epithelium containing ciliated and secretory cells [2–6]. To facilitate functional studies at ALI, rodent lung samples are predominantly used to source primary cells due to the availability of genetically engineered animals. However, species specific differences pose concerns when exploring therapeutic targets or interrogating complex mechanisms as the resulting observations have limited translatability to human. Furthermore, ethical, regulatory, cost and time considerations are making animal studies increasingly challenging.

Earlier attempts to genome edit human BECs employed viral transduction to create stable Cas9/sgRNA expressing lines [7–10]. These strategies resulted in relatively poor editing efficiencies in bulk populations and required subsequent sub-cloning and/or antibiotic selection to enrich for genome edited cells. However, stable Cas9/sgRNA expression over prolonged periods increases the risk of genomic instability and off target effects. This is especially relevant for cells cultured at ALI over 28 days. Furthermore, specialised culture manipulations were required to extend the life span of the Cas9/sgRNA expressing lines (throughout selection) in order to preserve multipotent differentiation capacity.

To allow the interrogation of modulators of primary human bronchial epithelial differentiation and function, we developed single step, robust and easily scalable genome editing pipelines in ALI differentiated primary human BECs (figure 1a). By employing transient, plasmid- and virus-free delivery of CRISPR/Cas9 to human BECs, we achieved efficient gene inactivation without the need for cloning or positive selection. We established both electroporation- and lipofection-based genome editing protocols and described detailed materials and methods elsewhere [11]. Briefly, passage 1 primary human BECs (Lonza) were thawed and expanded in T75 flasks for 3 days. Upon reaching 70% confluency, cells were trypsinised and 200 000 cells were resuspended in 8.6 µL P3 Primary Cell Nucleofector Solution (V4XP-3032, Lonza). Prior to detaching the cells, 90 pmoles of Cas9 protein (1081059, IDT) were assembled with 450 pmoles synthetic guide RNA (gRNA) to form the ribonucleoprotein (RNP) complex. 0.9 µL of electroporation enhancer (1075916, IDT) was added to the RNP solution which was subsequently mixed with the primary cell solution and transferred to Nucleocuvette Strips (Lonza).

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**A single-step, highly efficient CRISPR/Cas9-based genome editing pipeline allows the dissection of the molecular mechanisms underlying primary human bronchial epithelial cell differentiation and function at the air-liquid interface** http://bit.ly/2Oymgkw

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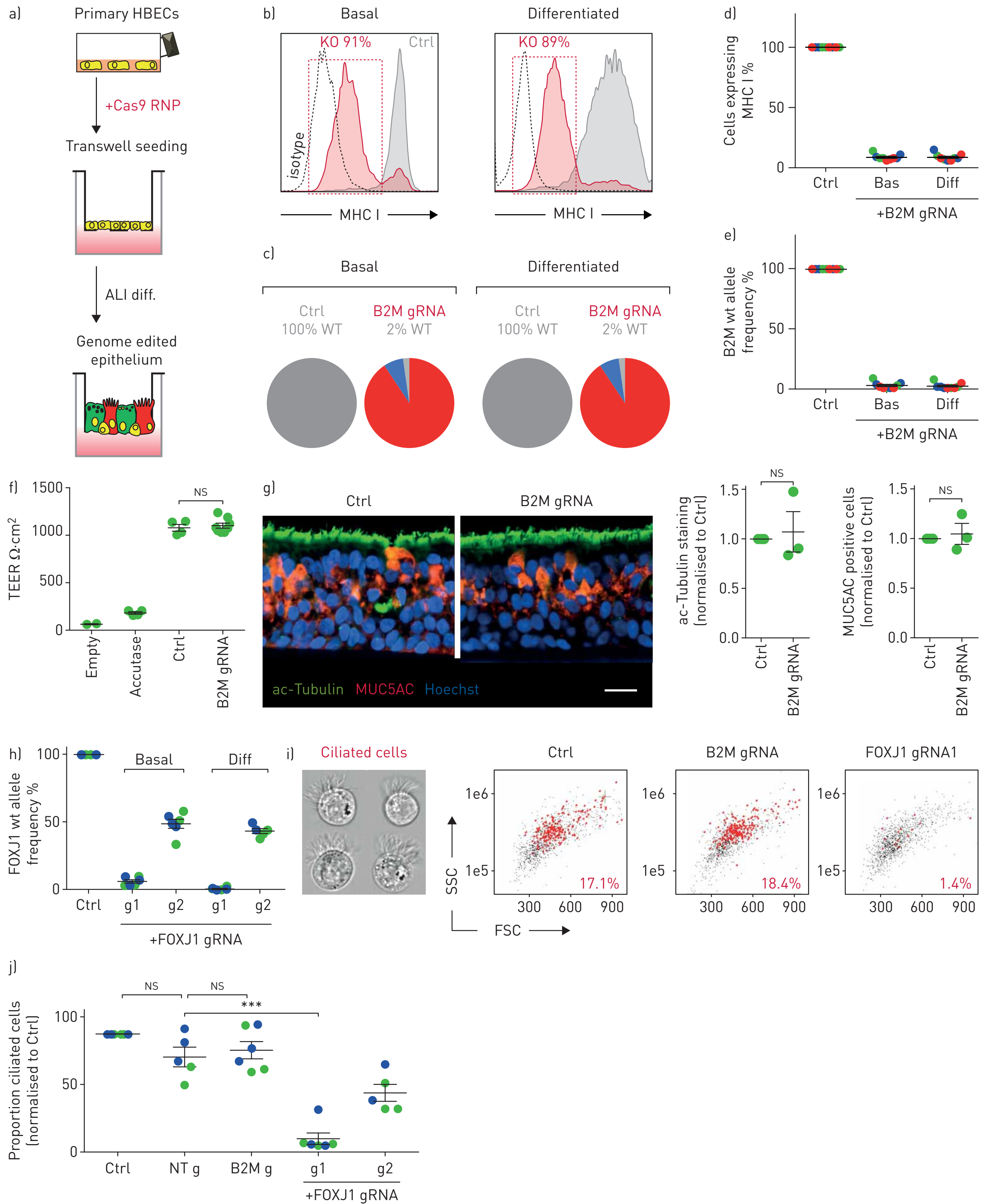


FIGURE 1 Legend overleaf.

FIGURE 1 a) Overview of genome editing in air–liquid interface (ALI) differentiated primary human bronchial epithelial cells (HBECs). b) Cytofluorometric analysis of major histocompatibility complex I (MHC I) expression in beta-2-microglobulin (B2M) depleted HBECs (red histogram) at 96 h (basal) and 36 days (differentiated) post-transfection. c) High-throughput sequencing of the B2M locus in basal and ALI differentiated cultures. Showing non-transfected (Ctrl) and B2M depleted cells. Pie charts represent the total number of sequenced alleles. Wild-type alleles are marked in grey whilst indels are coloured in red (out-of-frame mutations) and blue (in-frame). d) Percentage of cells expressing cell surface MHC I after B2M gene inactivation. Basal (Bas) and differentiated (Diff) cells were assayed by flow cytometry. Mean±SEM from experiments in three donors, across three technical repeats. Green data points: donor NS838; blue: NS955; red: C083. e) Frequency of wild-type B2M alleles after B2M gene editing, as assayed by high-throughput sequencing. f) Transepithelial electrical resistance (TEER) measurement of Ctrl (non-transfected) and genome edited ALI differentiated epithelia. Accutase: brief enzymatic treatment to disrupt tight junctions. NS: nonsignificant. g) Ctrl and B2M depleted ALI epithelia were embedded in paraffin wax and sectioned. Samples were colabelled for acetylated-$\alpha$-Tubulin (ac-Tubulin, green), MUC5AC (red), Hoechst (blue) and analysed by immunofluorescence microscopy. Scale bar: 20 µm. The area stained by the ac-Tubulin antibody relative to the tissue area was quantified using the HALO software (Indica Labs) in the middle panel. The number of MUC5AC positive cells per length of epithelium was quantified in the right panel. Both plots show data from three technical repeats. For each repeat, three immunofluorescence-labelled formalin-fixed paraffin-embedded sections were stained, analysed and the average value displayed on the plot. Data were normalised to Ctrl. Statistical significance was evaluated using paired, two-tailed t-tests. h) Frequency of wild-type FOXJ1 alleles after FOXJ1 gene inactivation, as assessed by high-throughput sequencing. Assaying basal and differentiated (Diff) cells. g1, 2: gRNA 1, 2. i) Imaging flow cytometric analysis of B2M and FOXJ1 depleted HBECs. Morphologically distinct ciliated cells (left panel) were marked in red on forward scatter/side scatter plots. The proportion of ciliated cells is shown in red. j) Proportion of ciliated cells in non-transfected, NT (non-targeting gRNA transfected), B2M depleted and FOXJ1 depleted HBECs. Assayed by imaging cytometry. Values were normalised to Ctrl. ***: $p \leqslant 0.001$.

The cell suspension was electroporated using the CM-113 program on the Amaxa 4D Nucleofector (Lonza) and cells were swiftly transferred to pre-warmed culture media and incubated at 37°C.

To demonstrate efficient genome editing, we targeted the beta-2-microglobulin (B2M) subunit of the major histocompatibility complex class I (MHC I) in primary human BECs originating from healthy non-smoker (NS838 and NS955) and diseased (C083) donors, across three technical repeats. We achieved MHC I depletion in 91.2%±0.8% of human BECs, as assayed by flow cytometric analysis (figure 1b and d), and 96.9%±0.9% gene editing efficiency, as assayed by high-throughput sequencing of the B2M locus (figure 1c and e) [11, 12]. B2M depleted cells were then differentiated at ALI and MHC I depletion was assessed post-differentiation. We found that high protein depletion and gene editing efficiencies were maintained throughout the 28-day differentiation at ALI (figure 1b–e).

We next wanted to prove that the genome editing protocol does not affect epithelial barrier formation and differentiation. To assess barrier integrity, we measured the transepithelial electrical resistance (TEER) of ALI differentiated cells. We obtained TEER values of 1079.7±36.35 $\Omega \cdot cm^2$ for control (non-transfected) epithelia (figure 1f) indicating adequate barrier formation [13]. Importantly, we observed no significant difference in TEER values between control and genome edited epithelia (figure 1f). To study the morphology and constitution of ALI epithelia, we employed immunofluorescence (IF) labelling of formalin-fixed paraffin-embedded (FFPE) ALI cultures (figure 1g). Both the control and genome edited epithelia were polarised and highly organised across 4–6 layers. Although the cells showed some variation in size and shape, the apical layers maintained a distinctive cuboidal to columnar shape, consistent with previous reports [14]. Ciliated and secretory cells were confirmed by immunostaining with anti-acetylated-alpha-Tubulin (T6793; Sigma) and anti-MUC5AC (MA5-12175; Thermo) antibodies, respectively. We observed no significant difference in morphology, ciliated or secretory cell distribution between control and genome edited epithelia (figure 1g). Taken together, the TEER and IF-FFPE data indicate that our genome editing protocol does not affect barrier formation and differentiation at ALI.

Having determined that the genome editing protocol does not hinder differentiation at ALI, we next sought to introduce targeted gene disruptions that modulate ciliation. As proof of concept, we targeted the transcription factor Forkhead box J1 (FOXJ1), which has been shown to regulate ciliation in animal models, as previously reviewed [15]. Cas9 RNP was transfected into basal human BECs derived from donors NS838 and NS955, in three technical replicates. Two FOXJ1 targeting gRNAs were utilised for this experiment and we achieved FOXJ1 editing efficiencies of 93.4%±1.2% using gRNA1 and 51.2%±3.4% by using gRNA2, as assayed by high-throughput sequencing of the FOXJ1 locus (figure 1h). FOXJ1 KO human BECs were then differentiated at ALI. We found that FOXJ1 gene inactivation was maintained throughout the 28-day differentiation, suggesting that FOXJ1 depletion is not negatively selected during ALI differentiation (figure 1h).

We hypothesised that depletion of FOXJ1 would preclude ciliation in ALI differentiated bronchial epithelia. To accurately quantify the number of ciliated cells, we employed image cytometry, which combines the speed and sensitivity of flow cytometry with the detailed imagery of microscopy. This allowed us to determine the number of morphologically distinct ciliated cells in ALI cultures (figure 1i). Indeed, efficient disruption of FOXJ1, but not B2M, decreased the proportion of ciliated cells in human BECs (figure 1i and j). As expected, the poorer editing efficiencies achieved with FOXJ1 gRNA 2 induced a weaker reduction in ciliated cells (figure 1h and j). This observation was consistent in differentiated BECs derived from both donor NS838 and NS955 (figure 1h and j), indicating that the strength of the

phenotype was dependent on genome editing efficiency and not due to CRISPR/Cas9 off target effects or transfection or culture artefacts.

Herein we established a genome editing pipeline that enables the identification of novel factors that modulate primary human BEC differentiation and function at air liquid interface. Our CRISPR/Cas9 RNP-based transfection protocols allowed for single-step, highly efficient, cloning- and selection-free, genome editing of ALI differentiated primary human BECs. After ALI differentiation, the effect of specific gene inactivation on BEC differentiation was assayed using IF-FFPE and imaging cytometry. This pipeline is easily scalable and immediately amenable to functional arrayed CRISPR screens with multi-parametric endpoint analyses. We demonstrated proof of concept by showing that depletion of FOXJ1 significantly reduced the proportion of ciliated cells in human ALI cultures. To our knowledge, this is the first study to show that FOXJ1 is essential for ciliation in ALI differentiated primary human BECs.

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## References

1. Bhowmick R, Gappa-Fahlenkamp H. Cells and culture systems used to model the small airway epithelium. *Lung* 2016; 194: 419–428.
2. Plasschaert LW, Zilionis R, Choo-Wing R, *et al.* A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. *Nature* 2018; 560: 377–381.
3. Montoro DT, Haber AL, Biton M, *et al.* A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* 2018; 560: 319–324.
4. Fulcher ML, Gabriel S, Burns KA, *et al.* Well-differentiated human airway epithelial cell cultures. *Methods Mol Med* 2005; 107: 183–206.
5. Whitcutt MJ, Adler KB, Wu R. A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells. *In Vitro Cell Dev Biol* 1988; 24: 420–428.
6. Yamaya M, Finkbeiner WE, Chun SY, *et al.* Differentiated structure and function of cultures from human tracheal epithelium. *Am J Physiol* 1992; 262: L713–L724.
7. Everman JL, Sajuthi S, Saef B, *et al.* Functional genomics of CDHR3 confirms its role in HRV-C infection and childhood asthma exacerbations. *J Allergy Clin Immunol* 2019; 144: 962–971.
8. Chu HW, Rios C, Huang C, *et al.* CRISPR-Cas9-mediated gene knockout in primary human airway epithelial cells reveals a proinflammatory role for MUC18. *Gene Ther* 2015; 22: 822–829.
9. Peters-Hall JR, Coquelin ML, Torres MJ, *et al.* Long-term culture and cloning of primary human bronchial basal cells that maintain multipotent differentiation capacity and CFTR channel function. *Am J Physiol Lung Cell Mol Physiol* 2018; 315: L313–L327.
10. Voets O, Tielen F, Elstak E, *et al.* Highly efficient gene inactivation by adenoviral CRISPR/Cas9 in human primary cells. *PLoS One* 2017; 12: e0182974.
11. Rapiteanu R, Karagyozova T, Zimmermann N, *et al.* Highly efficient genome editing in primary bronchial epithelial cells establishes FOXJ1 as essential for ciliation in human airways. *BioRxiv* 2019; preprint [https://doi.org/10.1101/533869]. .
12. Schmid-Burgk JL, Schmidt T, Gaidt MM, *et al.* OutKnocker: a web tool for rapid and simple genotyping of designer nuclease edited cell lines. *Genome Res* 2014; 24: 1719–1723.
13. Srinivasan B, Kolli AR, Esch MB, *et al.* TEER measurement techniques for *in vitro* barrier model systems. *J Lab Autom* 2015; 20: 107–126.
14. Ishikawa S, Ito S. Repeated whole cigarette smoke exposure alters cell differentiation and augments secretion of inflammatory mediators in air-liquid interface three-dimensional co-culture model of human bronchial tissue. *Toxicol In Vitro* 2017; 38: 170–178.
15. Thomas J, Morle L, Soulavie F, *et al.* Transcriptional control of genes involved in ciliogenesis: a first step in making cilia. *Biol Cell* 2010; 102: 499–513.

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