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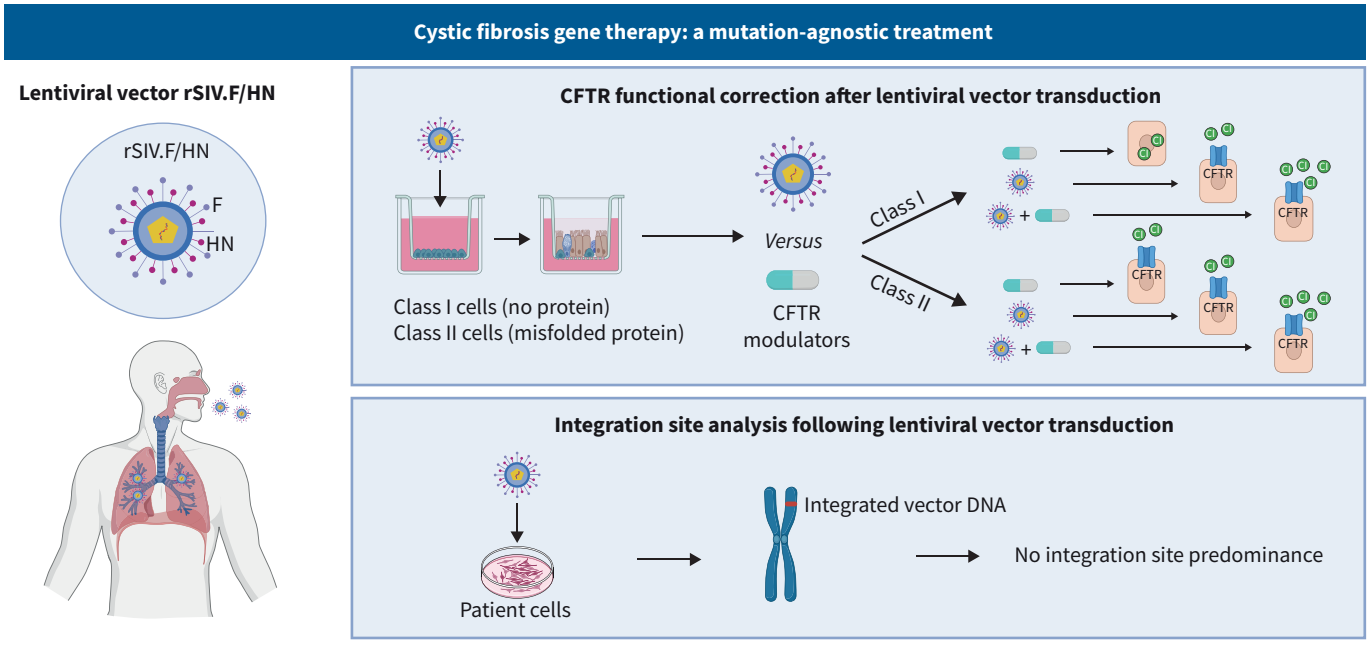

GRAPHICAL ABSTRACT Summary of the main study findings. CFTR: cystic fibrosis transmembrane conductance regulator. Created with BioRender.com.

https://doi.org/10.1183/13993003.01683-2023 Eur Respir J 2025; 65: 2301683

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Pharmacological and pre-clinical safety profile of rSIV.F/HN, a hybrid lentiviral vector for cystic fibrosis gene therapy

Alena Moiseenko[1], Anthony Sinadinos[2,3], Ana Sergijenko[2,3], Kyriel Pineault[2,3], Aarash Saleh[2,3], Konradin Nekola[1], Nathalie Strang[1], Anastasia Eleftheraki[1], A. Christopher Boyd[2,4], Jane C. Davies[2,3,5], Deborah R. Gill[2,6], Stephen C. Hyde[2,6], Gerry McLachlan[2,7], Tim Rath[8], Michael Rothe[9], Axel Schambach[9,10], Silke Hobbie[1], Michael Schuler[1], Udo Maier[1], Matthew J. Thomas[1], Detlev Mennerich[1], Manfred Schmidt[8,11,12], Uta Griesenbach[2,3,13], Eric W.F.W. Alton[2,3,5,13] and Sebastian Kreuz[1,13]

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The functional characterisation of the lentiviral gene therapy vector rSIV.F/HN supports its potential for a mutation-agnostic therapy of cystic fibrosis. In addition, a novel assay for quantification of CFTR protein expression is described. https://bit.ly/3WBN4mm

Cite this article as: Moiseenko A, Sinadinos A, Sergijenko A, *et al*. Pharmacological and pre-clinical safety profile of rSIV.F/HN, a hybrid lentiviral vector for cystic fibrosis gene therapy. *Eur Respir J* 2025; 65: 2301683 [DOI: 10.1183/13993003.01683-2023].

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Received: 2 Oct 2023
Accepted: 2 Aug 2024

Abstract

Rationale and objective Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. CFTR modulators offer significant improvements, but ∼10% of patients remain nonresponsive or are intolerant. This study provides an analysis of rSIV.F/HN, a lentiviral vector optimised for lung delivery, including CFTR protein expression, functional correction of CFTR defects and genomic integration site analysis in preparation for a first-in-human clinical trial.

Methods Air–liquid interface cultures of primary human bronchial epithelial cells (HBECs) from CF patients (F508del/F508del), as well as a CFTR-deficient immortalised human lung epithelial cell line mimicking class I (CFTR-null) homozygous mutations, were used to assess transduction efficiency. Quantification methods included a novel proximity ligation assay for CFTR protein expression. For assessment of CFTR channel activity, Ussing chamber studies were conducted. The safety profile was assessed using integration site analysis and *in vitro* insertional mutagenesis studies.

Results rSIV.F/HN expressed CFTR and restored CFTR-mediated chloride currents to physiological levels in primary F508del/F508del HBECs as well as in a class I cells. In contrast, the latter could not be achieved by small-molecule CFTR modulators, underscoring the potential of gene therapy for this mutation class. Combination of rSIV.F/HN-CFTR with the potentiator ivacaftor showed a greater than additive effect. The genomic integration pattern showed no site predominance (frequency of occurrence ⩽10%), and a low risk of insertional mutagenesis was observed in an *in vitro* immortalisation assay.

Conclusions The results underscore rSIV.F/HN as a promising gene therapy vector for CF, providing a mutation-agnostic treatment option.

Introduction

Despite the recent successes of cystic fibrosis transmembrane conductance regulator (CFTR) channel modulators [1], an unmet need remains for those patients unable to tolerate side-effects of ion channel

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modulator therapy, or subsets still lacking disease-modifying treatment options such as patients affected by two class I mutations.

To date, several gene therapy approaches for cystic fibrosis (CF), including adenovirus, adeno-associated virus (AAV) and non-viral vectors, have been investigated in clinical trials, but none have progressed to market authorisation due to limited efficacy [2–8]. A clinical trial (Aerow study) using an improved AAV capsid is currently underway [9]. Lentiviruses have been used widely as gene therapy vectors, combining a large packaging capacity with stable long-term expression and the potential for repeated dosing due to their favourable immunological properties [10–12]. However, so far lentiviral vectors have not been tested clinically in the context of CF.

To optimise lentiviral gene delivery to the lung epithelium, a self-inactivating (SIN) simian immunodeficiency virus (SIV) vector was pseudotyped with the Sendai virus (SeV) derived envelope fusion (F) and hemagglutinin/neuraminidase (HN) proteins [13]. The resulting rSIV.F/HN vector showed high efficiency and stable transduction of airway epithelium in the mouse lung, *in vivo* [14]. Previous studies also showed stable expression following repeated dosing [15], thereby pointing towards an important advantage of rSIV.F/HN compared to other viral vectors such as AAV, where repeated dosing is inefficient due to the generation of neutralising antibodies [10, 16]. Furthermore, rSIV.F/HN-Lux expressing luciferase was able efficiently to transduce *ex vivo* human and sheep lung slices, as well as primary human airway epithelial cells [15, 17]. Additionally, rSIV.F/HN showed no evidence of chronic toxicity in mice over a 2-year period [15, 17].

The lead candidate derived from these studies, rSIV.F/HN driving expression of a codon-optimised and cytosine guanine dinucleotide-depleted CFTR cDNA (coCFTR) via the hCEF promoter, was able to transduce CF relevant airway epithelial cells in the murine lung *in vivo*, including bronchial ciliated, goblet and club cells. Further, it restored CFTR function in a swelling assay based on human intestinal organoids derived from CF patient cells [18].

Here we have built on this previous work by establishing a correlation between transduction levels, CFTR protein expression, and the restoration of CFTR chloride channel function to help guide pre-clinical safety studies and subsequent clinical trials. As a model system, fully differentiated air–liquid interface (ALI) cultures derived from human bronchial epithelial cells (HBECs) carrying the most prevalent class II mutation F508del/F508del [1], or from primary immortalised HBECs mimicking class I CFTR-knockout (KO) mutations, were used to quantify CFTR mRNA and protein expression and to conduct electrophysiological measurements of CFTR chloride current following transduction of the basal progenitor cells with CFTR expressing rSIV.F/HN. To investigate the downstream functional consequences of coCFTR expression, ciliary beat frequency (CBF) was measured as a surrogate readout of mucociliary clearance. Further, to assess genotoxicity risk, integration patterns of rSIV.F/HN were analysed in primary human airway basal cells by shearing extension primer tag selection ligation-mediated PCR [19], combined with functional analyses of transformation events in a standardised *in vitro* insertional mutagenesis assay [20].

Materials and methods

Methods are presented in the supplementary material.

Results

Transduction efficiency of rSIV.F/HN in HBECs grown at an ALI culture

To analyse the transduction efficiency of HBECs (basal cells) subsequently grown at an ALI culture, cells were transduced in submerged culture with rSIV.F/HN, expressing green fluorescent protein (GFP) (rSIV.F/HN-GFP) at multiplicities of infection (MOI) of 3, 10, 30 and 90, followed by transition from submerged to ALI at 2 days post-transduction (figure 1a). 3–4 weeks after transitioning to ALI, cells were analysed in their fully differentiated state. At day 21 after transitioning to ALI, the percentage of GFP-positive cells was measured by flow cytometry. Cells transduced at MOIs 3, 10, 30 and 90 resulted in significant dose-related increases in transduction efficiency (figure 1b).

Next, we examined the cellular profile of the ALI cultures derived from the transduced basal cells by applying immunofluorescence staining for different epithelial cell markers at day 28 post-transduction. Co-localisation of GFP with ACTUB (ciliated cells), KRT5 (basal cells), SCGB1A1 (club cells) and MUC5AC (goblet cells) was detectable, confirming that rSIV.F/HN produced expression in multiple cell types subsequent to basal cell transduction (figure 1c). These findings were further confirmed by flow cytometry measurement of GFP^+ cell percentages of $ACTUB^+$, $KRT5^+$, $SCGB1A1^+$ and $MUC5AC^+$ cells (figure 1d).

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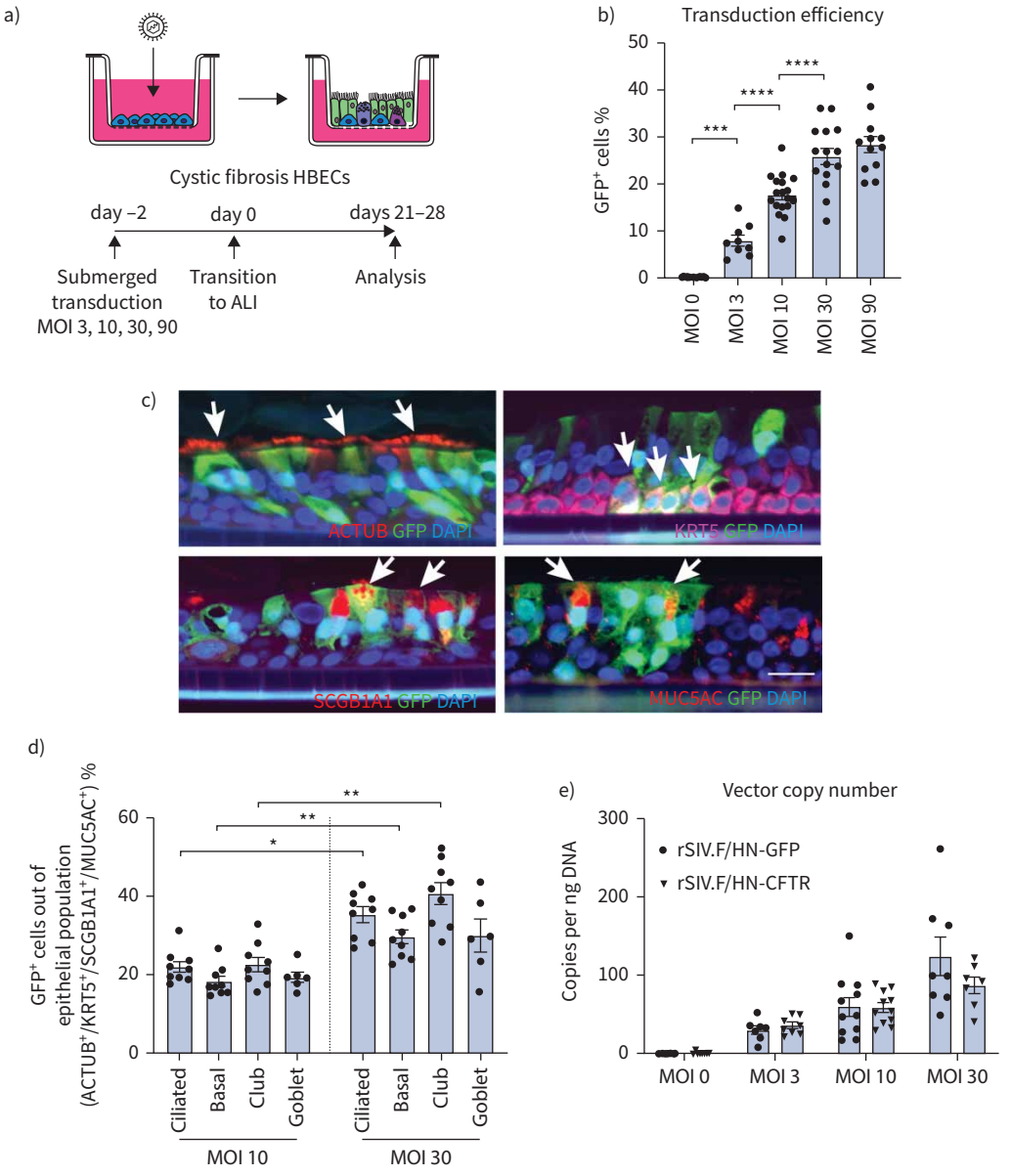

FIGURE 1 Transduction efficiency and transduced cell types in human bronchial epithelial cells (HBEC) air–liquid interface (ALI). For the presented experiments samples from three cystic fibrosis donors were used; n indicates the number of samples assessed, derived from these donors. a) Experimental setup. b) Quantification of transduced green fluorescent protein-positive (GFP^+) cells at day 21 post-transduction (n=9–20). c) Immunofluorescence for ciliated (ACTUB), basal (KRT5), club (SCGB1A1) and goblet (MUC5AC) cells and co-localisation with transduced GFP^+ cells (arrows indicate co-localisation). Scale bar=20 µm. d) Flow cytometry quantification of percentage of transduced cells in specific epithelial cell populations (n=6–9). e) Vector copy number quantitative PCR analysis in bulk samples transduced either with GFP- or cystic fibrosis transmembrane conductance regulator (CFTR)-expressing rSIV.F/HN (n=7–11). MOI: multiplicity of infection; DAPI: 4′,6-diamidino-2-phenylindole. Data were analysed using b) one-way ANOVA followed by Holm–Šídák's multiple comparison test and d, e) Kruskal–Wallis test followed by Dunn's multiple comparison tests. *: p<0.05, **: p<0.005, ***: p<0.001, ****: p<0.0001.

Average integrations in genome (vector copy number (VCN)) were analysed in DNA samples from ALI cultures, which were independently transduced with either GFP- or CFTR-expressing rSIV.F/HN. Bulk DNA analysis showed a dose-related increase in VCN for both GFP- and CFTR-expressing rSIV.F/HN (figure 1e). No difference in VCN was observed between GFP- and CFTR-expressing rSIV.F/HN at any of the MOIs analysed (figure 1e). This analysis suggests that the transduction efficiencies of GFP-expressing

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rSIV.F/HN and CFTR-expressing rSIV.F/HN are comparable, and the percentage of GFP-expressing cells measured by flow cytometry can be used as a surrogate readout to estimate percentage of CFTR-expressing cells, thereby enabling correlative analyses between expression levels and the degree of functional CFTR restoration.

Transduction of CF ALI cultures (F508del/F508del) with rSIV.F/HN-CFTR results in transgenic CFTR expression, restoration of CFTR chloride current and increased ciliary beat frequency

To determine whether rSIV.F/HN-CFTR transduced CF ALI cultures can efficiently produce vector-derived codon-optimised CFTR mRNA (coCFTR), quantitative droplet digital PCR analysis was performed. Dose-related coCFTR expression was observed in cells transduced with rSIV.F/HN-CFTR at MOIs 3 and 10, while no expression was observed in cells transduced with rSIV.F/HN-GFP (figure 2a). Expression of endogenous CFTR was also analysed and, as expected, no differences between nontransduced samples, samples transduced with rSIV.F/HN-GFP and samples transduced with rSIV.F/HN-CFTR at MOI 10 were observed (figure 2b). mRNA expression of coCFTR 10 times higher than expression of endogenous CFTR was achieved (figure 2c), suggesting that a low number of transduced CFTR high-expressing cells (17% for MOI 10) are enough to restore CFTR expression to levels higher than endogenous values.

To analyse the functionality of rSIV.F/HN-CFTR-expressed channels, CFTR-mediated chloride current was measured in Ussing chambers using rSIV.F/HN-CFTR-transduced ALI cultures. First, sodium channels were blocked using amiloride, followed by stimulation of CFTR using forskolin and the change in short-circuit current was calculated (both peak and steady-state (plateau) values). In some experiments, ivacaftor, a small-molecule CFTR potentiator which increases channel open probability [21] was added for additional stimulation of CFTR current. Finally, the chloride current was blocked with CFTR-inhibitor 172 (figure 3a, schematic drawing of the measurement; examples of original measurements are included in supplementary figures S1–S4) [22]. As expected, nontransduced cells (MOI 0), or cells transduced with rSIV.F/HN-GFP, did not respond to forskolin or the CFTR-inhibitor, confirming the absence of functional CFTR channels. In contrast, a dose-related increase in CFTR chloride current was observed in ALI cultures transduced with rSIV.F/HN-CFTR. The data indicate that the effect of virus-mediated protein expression on CFTR functional correction is not transient (peak), but also present in a sustained phase (plateau). At an MOI of 3, there was restoration of 49±6% (peak) and 38±4% (plateau) of the non-CF chloride current (measured in non-CF HBEC ALIs). When the rSIV.F/HN-CFTR (MOI 3) was combined with the potentiator ivacaftor, there was a significant increase in stimulation of chloride current (61±4% for both peak and plateau). With an increased MOI of 10, we observed a higher restoration (94±11% peak, 66±6% plateau) of the non-CF chloride current; a combination of MOI 10 and ivacaftor led to further increase in restoration (121±11% for both peak and plateau). Higher MOIs (30 and 90) resulted in further increases. Thus, rSIV.F/HN-CFTR is able to completely restore the CFTR-related chloride current to non-CF values

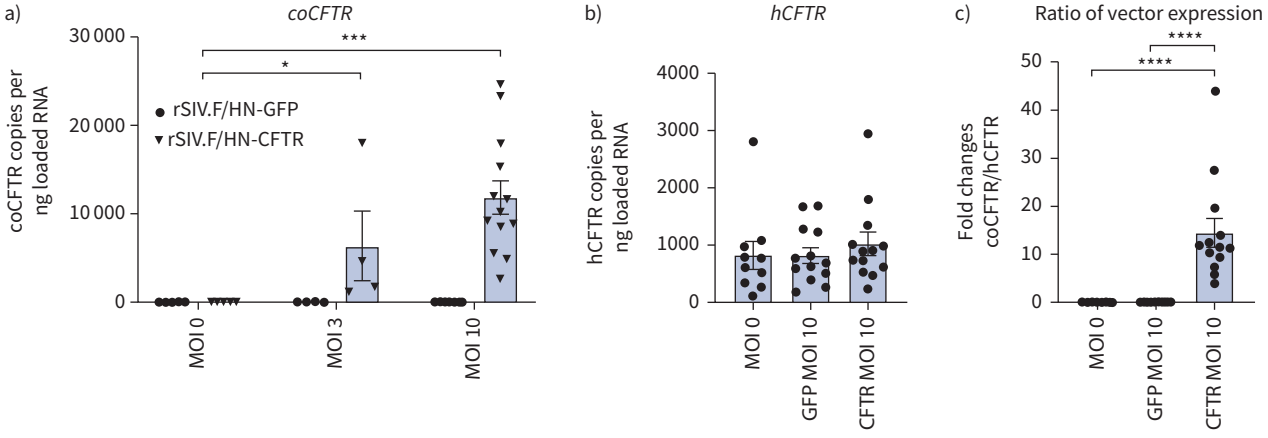

FIGURE 2 Transduction with rSIV.F/HN-CFTR (cystic fibrosis transmembrane conductance regulator) results in expression of codon-optimised CFTR (coCFTR) in primary cystic fibrosis human bronchial epithelial cells (HBECs). For the presented experiments samples from three cystic fibrosis donors were used; n indicates the number of samples assessed, derived from these donors. a) CoCFTR transgene gene expression showing high expression of transgene in CFTR-transduced cells, but not in the negative control green fluorescent protein (GFP)-transduced cells (n=4–13). b) Endogenous human (h) CFTR gene expression (n=4–13). c) Gene expression ratio between transgene coCFTR and hCFTR (n=11–13). Data were analysed using Kruskal–Wallis test followed by Dunn's multiple comparison tests. *: p<0.05, ***: p<0.001, ****: p<0.0001.

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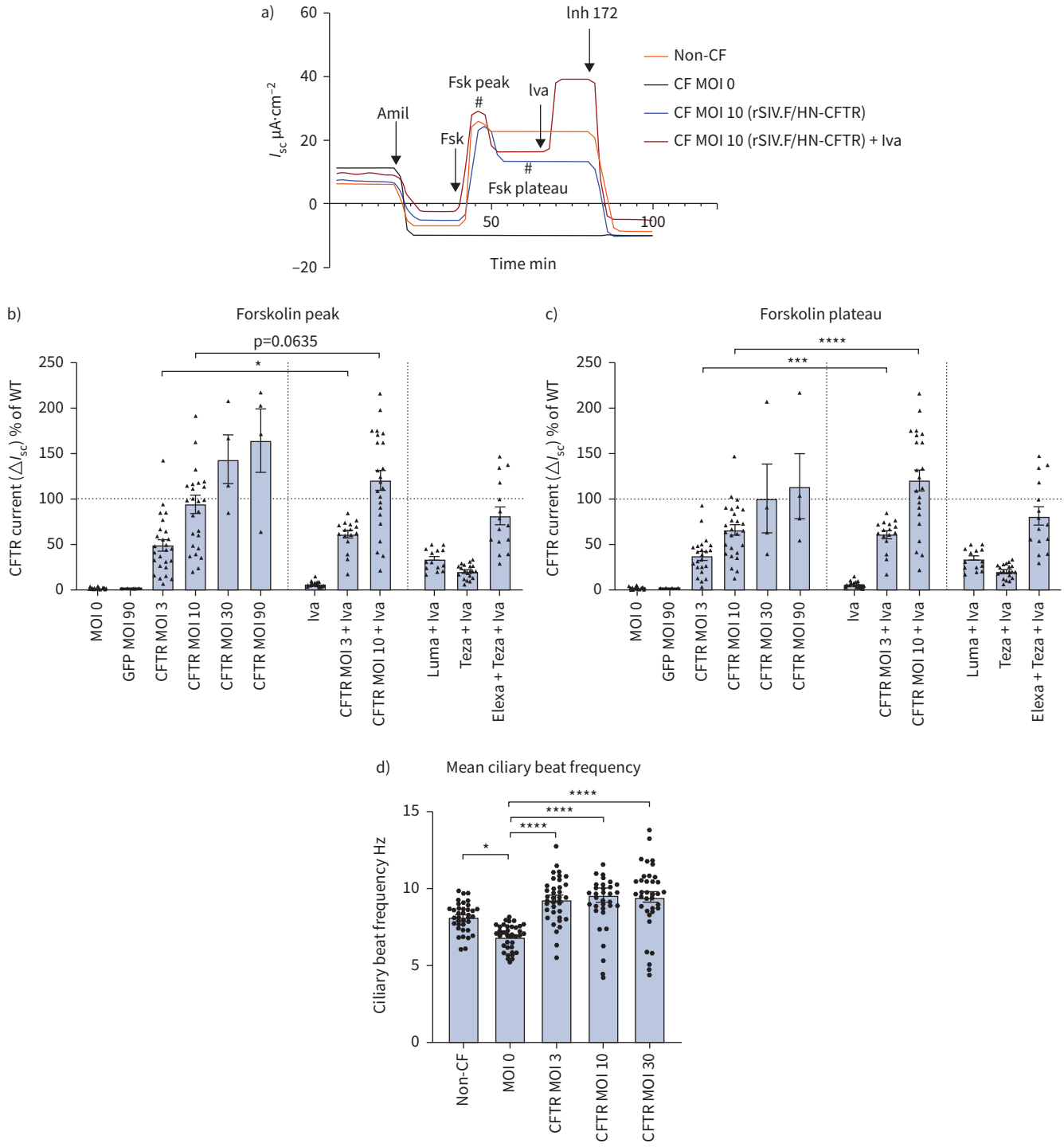

FIGURE 3 Functional data demonstrating that rSIV.F/HN restores cystic fibrosis transmembrane conductance regulator (CFTR) chloride current in primary cystic fibrosis (CF) human bronchial epithelial cells (HBECs). For the presented experiments, samples from three non-CF and three CF donors were used; n indicates the number of samples assessed, derived from these donors. a) Schematic drawing of Ussing chamber measurements. #: forskolin peak and forskolin plateau. b) Ussing chamber data represented as the percentage of wild-type (WT) CFTR current; difference between maximum forskolin peak current and current after CFTR-inhibition is calculated (n=14–26 for all conditions except for multiplicity of infection (MOI) 30 and 90 (n=4)). c) Ussing chamber data represented as percentage of WT CFTR current; difference between forskolin plateau and CFTR-inhibited current is calculated (n=14–26 for all conditions except for MOI 30 and 90 (n=4)). d) Mean ciliary beat frequency analysis in primary non-CF, CF and transduced CF HBECs (n=36). ΔI_{sc}: short-circuit current change; Amil: amiloride; Fsk: forskolin; Iva: ivacaftor; GFP: green fluorescent protein; Luma: lumacaftor; Teza: tezacaftor; Elexa: elexacaftor. Data were analysed using b, c) Mann–Whitney test or d) Kruskal–Wallis test followed by Dunn's multiple comparison tests. *: p<0.05, ***: p<0.001, ****: p<0.0001.

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and a combination of rSIV.F/HN-CFTR with ivacaftor amplifies this effect of gene therapy by 1.3–1.8-fold. These data compare favourably with current approved modulator therapies, which were also assessed. Thus, treatment with lumacaftor+ivacaftor and tezacaftor+ivacaftor produced 34±3% and 21±2% of CFTR current restoration respectively, while treatment with elexacaftor+tezacaftor+ivacaftor resulted in 81±10% of restoration (figure 3b, c and supplementary table S2).

Ciliary beat frequency measurement demonstrated a significant reduction in the CF ALIs. Transduction with rSIV.F/HN-CFTR was able to restore CBF to non-CF values (figure 3d).

Transduction of CFTR-KO cells (class I mutation model) with rSIV.F/HN

Class I CFTR-null mutations result in complete absence of full-length CFTR protein and are thus not amenable to functional correction by current CFTR modulators [23]. Gene therapy provides an opportunity to establish a disease-modifying treatment for patients with all mutation types, including those homozygous for these null mutations. To enable functional characterisation of rSIV.F/HN-CFTR in a class I mutation background, a mutation model (CFTR-KO) was generated using the previously described immortalised human small airway basal cell line hSABCi-NS1.1 (hSABCi) [24, 25] (supplementary material) and the efficiency of lentiviral transduction in CFTR-KO cells grown in ALI cultures was analysed. CFTR-KO cells were transduced and analysed as for primary F508del/F508del cells. Flow cytometry analysis showed that transduction with rSIV.F/HN-GFP at MOIs 3, 10, 30 and 90 resulted in a dose-related increases (figure 4a). Additionally, DNA VCN analysis of CFTR-KO ALI cultures independently transduced with either rSIV.F/HN-GFP or rSIV.F/HN-CFTR showed no difference in VCN at any MOIs analysed (figure 4b). These data suggest that transduction efficiencies of rSIV.F/HN-GFP and rSIV.F/HN-CFTR are similar. Finally, rSIV.F/HN-GFP-mediated transduction of CFTR-KO cells was analysed at the cellular level by immunofluorescence staining for different epithelial cell markers at day 28 post transduction. Co-localisation of GFP with ACTUB, KRT5, SCGB1A1 and MUC5AC was observed, confirming that rSIV.F/HN produced expression in multiple cell types subsequent to basal cell transduction (figure 4c). Additionally, flow cytometry measurement of GFP^+ cell percentages in $ACTUB^+$, $KRT5^+$, $SCGB1A1^+$ and $MUC5AC^+$ cells confirmed our previous findings in class II cells (figure 4d). In addition, we have shown that the vector can transduce primary human nasal epithelial cells *ex vivo* using RNAscope *in situ* hybridisation (supplementary figure S6).

It is widely acknowledged that many commonly used CFTR antibodies lack specificity [26]. To quantify CFTR protein expression, we developed a CFTR immunodetection method based on the proximity ligation assay (PLA) [27]. Application of this technique to hSABCi-KO cells transduced with rSIV.F/HN-CFTR was quantified (figure 5a) using a custom image analysis enabling simultaneous quantification of CFTR signal over tens of thousands of cells (supplementary figure S9) and showing ∼42% and ∼53% of cells as $CFTR^+$ at MOI 10 and 30, respectively (figure 5b). At the same vector MOIs, this expression efficiency was qualitatively similar to the earlier GFP result (figure 4a), further indicating that GFP expression analysed by flow cytometry is a good proxy for CFTR expression under these experimental conditions.

Transduction of CFTR-KO ALI cultures with rSIV.F/HN-CFTR results in expression of coCFTR mRNA and CFTR current restoration, while ion channel modulators fail to restore CFTR current

Codon-optimised CFTR mRNA expression was analysed in CFTR-KO ALIs transduced with rSIV.F/HN-GFP and rSIV.F/HN-CFTR. Dose-related increases in coCFTR expression were observed in cells transduced with rSIV.F/HN-CFTR in comparison to nontransduced cells, while no coCFTR expression was observed in cells transduced with rSIV.F/HN-GFP (figure 6a). Functional analysis via Ussing chamber measurements (figure 6b; examples of original measurements are included in supplementary figures S10 and S11) showed a dose-related increase in CFTR current in CFTR-KO ALIs transduced with rSIV.F/HN-CFTR (figure 6c, d). Combination of rSIV.F/HN-CFTR with ivacaftor, which increases CFTR channel open probability, further increased the effect of gene therapy. In addition, clinically used CFTR modulators were analysed and as expected showed no evidence of CFTR restoration (figure 6c,d and supplementary table S3).

Insertion site analysis of rSIV.F/H reveals no preferred locus for integration in primary human airway basal cells

The integration profile of the SIV.F/HN-GFP was analysed by non-restriction-based linear amplification mediated PCR and sequencing of unknown genomic regions flanking integrated vector DNA [19]. HBECs were transduced with SIV.F/HN-GFP at MOIs 1, 3 and 10 and harvested for analysis on day 7 (submerged) or day 28 post-transduction (ALI) (figure 7a). The composition of all samples was polyclonal with a high number of individual insertion sites for each sample. In total, ∼4.7 million, ∼15.1 million and ∼13.6 million demultiplexed sequencing reads were obtained from three individual sequencing runs, resulting in a total of

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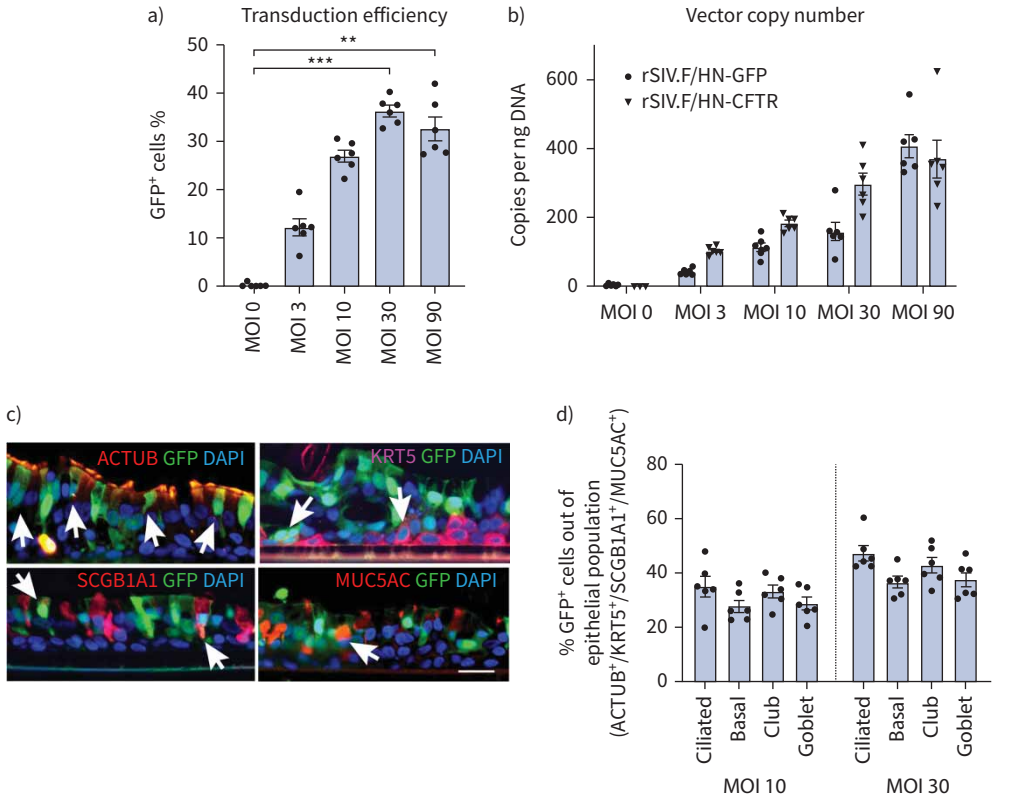

FIGURE 4 Assessment of transduction with rSIV.F/HN-GFP (green fluorescent protein) and rSIV.F/HN-CFTR (cystic fibrosis transmembrane conductance regulator) in CFTR-knockout (KO) (CFTR-null, model of class I mutation) immortalised human small airway basal cell line (hSABCi). a) Flow cytometry quantification of transduced GFP$^+$ CFTR-KO cells at day 21 post-transduction (n=6). b) Vector copy number quantitative PCR analysis in bulk CFTR-KO samples transduced with GFP- and CFTR-expressing vectors (n=6). c) Immunofluorescence for ciliated (ACTUB), basal (KRT5), club (SCGB1A1) and goblet (MUC5AC) cells and co-localisation with transduced GFP$^+$ cells. Arrow indicates co-localisation. Scale bar=20 µm. d) Flow cytometry quantification of percentage of transduced cells in specific epithelial cell populations (n=6). MOI: multiplicity of infection. Data were analysed using Kruskal–Wallis test followed by Dunn's multiple comparison tests. **: p<0.01, ***: p<0.001.

94 122 unique exactly mappable insertion sites. MOI 1 resulted in an average of 81 unique mappable insertion sites per sample at day 7 and 216 insertion sites at day 28 post-transduction. For MOI 3, these numbers were 213 and 502, respectively, and for MOI 10, 672 and 1241, respectively (figure 7b). For each sample the 10 most prominent insertion sites were identified and the frequency of their occurrence among all other insertion sites of that sample was quantified (figure 7b). The higher the insertion site number, the lower the frequency for the top 10 most often occurring insertion sites, suggesting that there is no preferred locus for integration (figure 7b, c). No insertion sites accounted for >10% of the total integration events.

Common insertion sites (CIS) between samples of each MOI were identified (figure 7d and supplementary tables S4 and S5). None of the top 10 CIS regions detected in samples transduced at any MOI has been associated with genomic regions previously identified as insertion sites associated with adverse events such as clonal outgrowth or malignant transformation in clinical gene therapy trials reported to date (*LMO2*, *MECOM*, *MN1*, *CCND2/CCND2-AS1*, *HMGA2*) [28–36]. No preferred integration was detected in, or near to genes associated with cancer or genes that have previously been involved in severe adverse events associated with gene therapy (supplementary figures S12–S14 and supplementary table S6).

Discussion

This study provides a functional characterisation of rSIV.F/HN to support further pre-clinical and clinical development activities. Specifically, we show in human bronchial cells that rSIV.F/HN is capable of expressing CFTR protein and correcting the CF chloride defect to non-CF levels. Additionally, a

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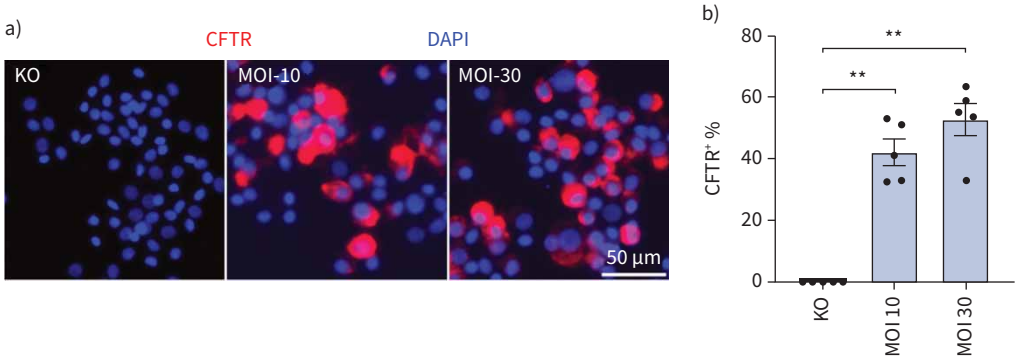

FIGURE 5 Cystic fibrosis transmembrane conductance regulator (CFTR) protein detection and quantification of percentage of positive cells following lentiviral vector transduction in CFTR-knockout (KO) immortalised human small airway basal cell line (hSABCi). a) Representative micrographs of cytospun CFTR- (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue: nuclei)-stained hSABCi cells, showing untransduced CFTR-KO cells along with CFTR-KO cells transduced at multiplicities of infection (MOI) 10 and 30. Scale bar=50 µm. b) Quantification of CFTR percentage-positive CFTR-KO cells with and without rSIV.F/HN transduction, showing an average of 42.25% and 52.86% CFTR-positive cells in the MOI 10 and MOI 30 samples, respectively (n=5). Data were analysed using the Brown–Forsythe and Welch multiple comparison test. **: p<0.01.

potentiation of the newly expressed CFTR was observed by co-treatment with ivacaftor. These data are of potential clinical relevance given the large number of CF patients taking modulators which include ivacaftor, thereby providing the potential for a synergistic increase in CFTR function. Moreover, we established a relationship between transduced cell numbers and the degree of functional CFTR correction and assessed the integration profile of rSIV.F/HN in lung-derived cells. Overall, these studies provide further support for the translation of rSIV.F/HN into first-in-human trials.

Previous studies have suggested that a range of 5–25% corrected cells should be sufficient to restore the CFTR chloride current to normal values [37–40]. This is supported by individuals with certain residual function mutations retaining 10% of normal CFTR expression per cell and generally maintaining lung function [41]. In the present study an MOI of 10 produced 10 times higher mRNA levels than endogenous CFTR expression. Protein expression in rSIV.F/HN-transduced cells is regulated by an optimised hCEF promoter/enhancer. This hybrid promoter is probably stronger than the endogenous CFTR promoter (most cell types express only low levels of CFTR protein) and transduced cells will therefore express higher than normal levels of CFTR. This supports the idea that a low percentage of transduced cells, combined with high levels of CFTR expression per cell, is sufficient to provide significant restoration of the CFTR-related chloride current. While Farmen *et al.* [38] reported CFTR mislocalisation to the basolateral membrane following strongly elevated CFTR expression levels, we are not aware of any pathology that has been noted as a result, including the ongoing Aerow AAV trial, which has reported supraphysiological CFTR expression, but well-tolerated treatment [42].

One limitation of the applied ALI model is the fact that nondifferentiated basal cells are transduced instead of fully differentiated airway cells, which in our hands show a very limited permissiveness for viral transduction. This observation is in contrast to efficient transduction of lung epithelium *in vivo* across varies species. However, the reasons for this discrepancy are not fully understood. The described model system cannot be considered as an assay format for predicting *in vivo* transduction efficiencies, but rather as a mechanistic model to allow for the establishment of transduction level and functional CFTR restoration correlations, which was the main intention of this work.

We show for the first time that lentiviral vector-mediated CFTR function can be further enhanced with the clinically approved CFTR potentiator ivacaftor, which acts by increasing channel open probability. The combination of gene therapy plus the potentiator reduced the number of transduced cells needed to achieve full restoration of the chloride current and decreased the required MOI by ∼1.5-fold. This is potentially beneficial both in terms of safety profile and applicability to a broad range of the CF population given that ivacaftor is a constituent of currently used modulators. We have also performed experiments with triple therapy modulators (ivacaftor, tezacaftor, elexacaftor) and as expected detected a greater than additive effect in F508del/F508del cells, but not in class I CFTR-KO cells.

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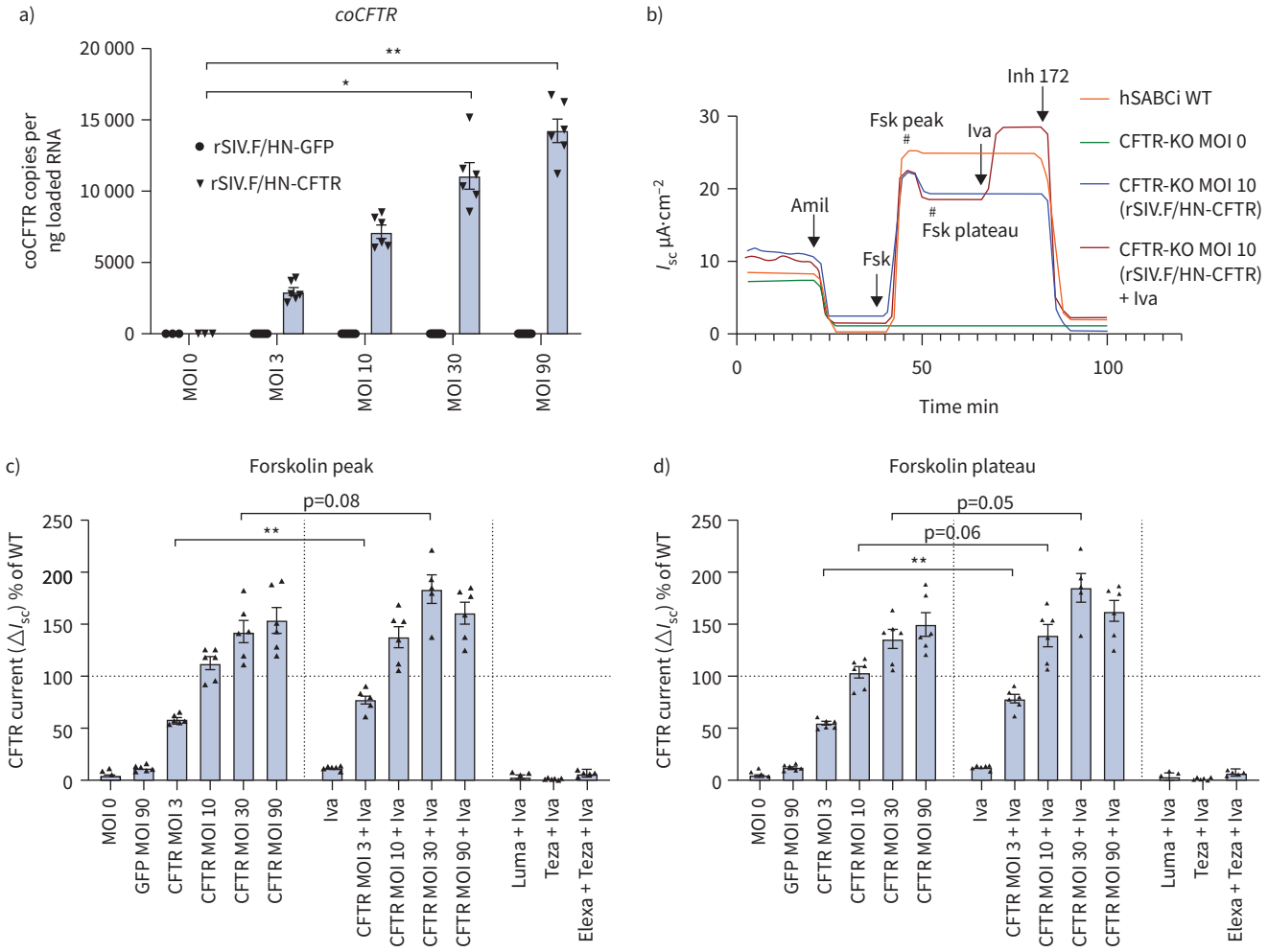

FIGURE 6 Functional data demonstrating that rSIV.F/HN restores cystic fibrosis transmembrane conductance regulator (CFTR) chloride current in CFTR-knockout (KO) cells in contrast to modulators. a) Codon-optimised CFTR (coCFTR) transgene gene expression showing high expression of transgene in CFTR-transduced cells, but not in green fluorescent protein (GFP)-transduced cells (n=6). b) Schematic drawing of Ussing chamber measurements. #: forskolin peak and forskolin plateau. c) Ussing chamber data represented as percentage of wild-type (WT) CFTR current; difference between maximum forskolin peak current and current after CFTR-inhibition is calculated (n=6–11). Of note, there was no CFTR chloride current activation after treatment with modulators (Luma+Iva, Teza+Iva, Elexa+Teza+Iva). d) Ussing chamber data represented as percentage of WT CFTR current; difference between forskolin plateau and CFTR-inhibited current is calculated (n=6–11). Of note, there was no CFTR chloride current activation after treatment with modulators (Luma+Iva, Teza+Iva, Elexa+Teza+Iva). ΔI_{sc}: short-circuit current change; Iva: ivacaftor; Luma: lumacaftor; Teza: tezacaftor; Elexa: elexacaftor. Data were analysed using a) Kruskal–Wallis test followed by Dunn's multiple comparison tests or c, d) Mann–Whitney test. *: p<0.05, **: p<0.01.

As part of this study, we also developed a novel proximity ligation assay for CFTR protein detection and quantification. By applying this assay, we were able to detect human CFTR in a stably transduced cell line and in human primary CF epithelial cells following rSIV.F/HN-CFTR transduction. In these studies we also observed a good correlation with vector genome copy numbers. It has recently been reported that a majority of widely utilised CFTR monoclonal antibodies nonspecifically bind to non-CFTR antigens, in addition to their expected CFTR interactions, under typical epithelial staining conditions [26]. The described PLA-based method may help to overcome existing challenges of CFTR immunodetection in future studies.

While the optimal target cell type for successful CF gene therapy remains unclear, recent studies have provided further insights to address this question. Utilising single-cell RNA-sequencing technology it has been shown that apart from ionocytes as a rare cell type, CFTR is mainly expressed by SCGB1A1^{+} club cells and to a lesser extent by basal cells, collectively accounting for ~80% of all CFTR^{+} cells [43]. We have previously shown that rSIV.F/HN is able to transduce the majority of these cells in the murine lung

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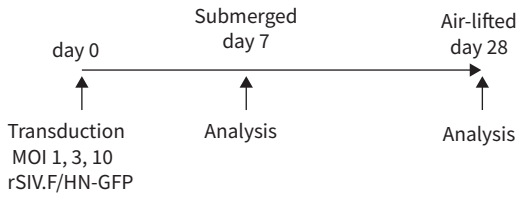
a)



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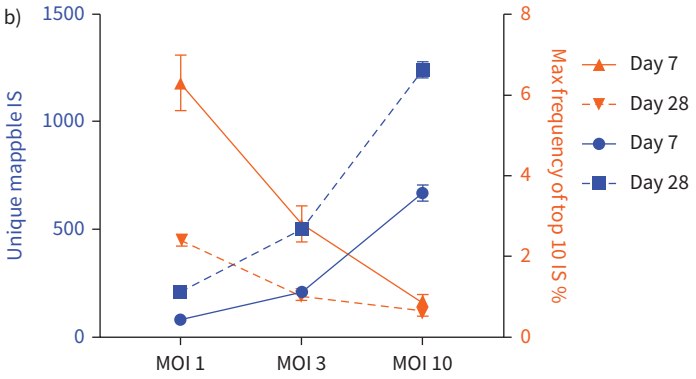

c)

MOI	MOI 1	MOI 1	MOI 10	MOI 10
Time Point	Day 7	Day 28	Day 7	Day 28

Rank	Gene name	Frequency %	Gene name	Frequency %	Gene name	Frequency %	Gene name	Frequency %
Top1	SUFU	4.806	MIR548G	1.694	TANGO6	0.466	IQGAP1	0.357
Top2	WASF2	4.062	FNTA	1.456	SRP68	0.424	LIPE-AS1	0.339
Top3	HIPK3	3.724	PRKAR2A	1.385	DGKE	0.421	SLC20A2	0.325
Top4	FOXG1-AS1	3.666	ANKRD13D	1.311	ARPC4-TTLL3	0.376	HORMAD2	0.32
Top5	N4BP2	3.252	C11orf49	1.303	EYA3	0.361	CCDC57	0.302
Top6	GNS	3.171	CUX2	1.247	NIPAL2	0.34	ASH1L	0.296
Top7	SH3PXD2A	3.164	PCED1B	1.227	TNFRSF10A	0.335	RNF13	0.296
Top8	GNA15	2.895	USP48	1.215	SMARCA4	0.326	RNF34	0.289
Top9	CYP24A1	2.753	MGAT4C	1.175	CYTH1	0.325	LCORL	0.273
Top10	PPP3CC	2.602	DVL1	1.171	HSPA9	0.318	PCNXL2	0.258
#All other mappable IS	253	65.905	550	86.816	2513	96.308	3834	96.945

Sequence count 10 strongest	85147	23252	6204	6281
Sequence count all other mappable IS	164587	153116	161834	199302
Total sequence counts used	249734	176368	168038	205583

d)

Subject ID	Top 10 CIS	CIS order	Chromo-some	Integration locus	Dimension (nucleotide)	Proximity genes
MOI 1	Top 1	58	8	144064768	459674	ADCK5, BOP1, CPSF1, CYC1, CYHR1, DGAT1, FBXL6, FOXH1, HGH1, HSF1, LRRC14, LRRC24, MROH1, OPLAH, PPP1R16A, RECQL4, TONSL, VPS28
	Top 2	41	1	1256366	315463	ANKRD65, ATAD3A, ATAD3B, CCNL2, CPSF3L, DVL1, MRPL20, MXRA8, SSU72, UBE2J2, VWA1
	Top 3	41	11	64999597	440166	ARL2, BATF2, CAPN1, CDC42EP2, CDCA5, DPF2, FRMD8, MIR612, POLA2, SLC22A20, SNX15, SPDYC, SYVN1, VPS51
	Top 4	25	9	136379479	221808	LINC01573, NOTCH1, PMPCA, SEC16A, SNAPC4
	Top 5	24	19	49770448	114955	AP2A1, MED25, PNKP, PTOV1, TBC1D17
	Top 6	23	16	89683304	141477	CDK10, FANCA, SPIRE2, VPS9D1, ZNF276
	Top 7	20	16	1547776	288027	CRAMP1L, FAHD1, HN1L, IFT140, MAPK81P3, MEIOB, SPSB3
	Top 8	20	11	67121410	305371	ADRBK1, ANKRD13D, CARNS1, KDM2A, LOC100130987, RAD9A
	Top 9	19	17	4225396	108560	ANKFY1, UBE2G1
	Top 10	19	9	136811011	132069	FBXW5, MAMDC4, RABL6, TRAF2

FIGURE 7 Insertion site (IS) analysis in primary human bronchial epithelial cells (HBECs). For the presented experiments, samples from three cystic fibrosis donors were used; n indicates the number of samples assessed, that were derived from these three donors. a) Study design. b) Number of retrieved unique IS from each sample and maximum frequency (%) of top 10 IS among all mappable IS (n=8–27). c) Frequencies of the 10 most prominent IS detected in samples transduced with multiplicity of infection (MOI) 1 and 10 and analysed at days 7 and 28. IS location and RefSeq names of genes located closest to the respective IS are given. Relative sequence count contributions of the 10 most prominent IS and all remaining mappable IS are shown. d) Top 10 common integration sites (CIS) detected in all samples transduced with MOI 1. Nearest genes for all IS within the CIS are listed. GFP: green fluorescent protein.

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in vivo [18] and here further confirm high expression in HBECs. In addition, we show that the vector can transduce primary human nasal epithelial cells (nasal brushings) *ex vivo*. However, in these cells the basolateral membrane is exposed and the vector may enter the cells through the apical as well as basolateral membrane. It has also been shown that lentiviral vectors are able to transduce lung stem cells without disrupting cell plasticity [44].

Apart from providing a novel and sustainable treatment option for a broad range of CF patients including those affected by the most common F508del/F508del mutation, gene therapy is especially attractive for those carrying class I mutations, which result in complete absence of CFTR protein and for whom there is currently no modulator treatment available. In order to analyse the functional effects of rSIV.F/HN in cells with a class I mutation, a novel CFTR-KO cell line was generated. Transduction of CFTR-KO cells with rSIV.F/HN again resulted in a high expression in all relevant cell types. As expected, none of the modulators produced an effect on the CFTR chloride current. In contrast rSIV.F/HN was able to fully restore CFTR function in a dose-related manner, as was the case for the F508del/F508del cells. Furthermore, we again saw a greater than additive effect of ivacaftor. These data suggest that rSIV.F/HN may be relevant for the treatment of a broad spectrum of CF patients.

Additionally, we assessed the downstream consequences of chloride current restoration through assessment of CBF as a surrogate readout of mucociliary clearance (MCC). Clinical data suggest that MCC becomes impaired with increasing disease severity, probably related to lack of adequate hydration of both the mucus and the underlying airway surface liquid [45]. The CF ALI cultures used in this study demonstrated a reduced CBF, probably secondary to these two parameters, and rSIV.F/HN-CFTR restored CBF in F508del/F508del ALI cultures to wild-type levels. These findings provide a further indication that rSIV.F/HN CFTR could improve lung physiology in CF patients *in vivo*, through a positive effect on MCC.

Lentiviral vectors integrate into the genome of transduced cells, thereby supporting long-lasting gene expression. However, integration also presents a potential mutagenic risk by de-regulating the expression of neighbouring genes and consequent development of adverse events. In previous gene therapy trials involving γ-retroviral vectors, a number of severe adverse events (SAE) have been observed [28–36] and have been attributed to integration events activating nearby proto-oncogenes leading to the development of leukaemia. Deletion of the long terminal repeat promoter/enhancer elements in self-inactivating (SIN) lentiviral vectors has significantly improved the biosafety of this class of vectors [46]. In this study for the first time the integration pattern of a SIN lentiviral vector has been analysed in HBECs. Insertion site analysis of rSIV.F/HN into HBECs revealed a highly polyclonal integration profile, no preferred locus for insertion and no integration hotspots adjacent to proto-oncogenes or other SAE-associated genes. This is consistent with long-term (18 months) toxicology studies performed in mice which did not show any evidence of insertional mutagenesis [15]. Previously reported IS analyses of lentiviruses were mostly performed in haematopoietic stem cells or immune cells and frequencies of the occurrence of the top 10 IS were much higher in comparison with the top 10 frequencies from the current study [47–49]. Haematopoietic cells have higher chromatin accessibility in comparison to fully differentiated cells [50], thus increasing the number of potential insertion sites and therefore the occurrence of possibly genotoxic integrations. Moreover, despite the fact that basal stem cells of the airways are also characterised by a more open chromatin structure, no evidence for preferred insertion sites in this cell type was found. The data presented are consistent with rSIV.F/HN possessing a low genotoxic risk in the lung. Recent studies and United States Food and Drug Administration recommendations suggest that results of vector-integration site analysis should be considered abnormal when a single site constitutes >30% [51] or >10% [52–54] of the total integrations. In our case no IS accounted for >10% of the total integration events.

In conclusion, this study demonstrates that rSIV.F/HN can achieve sufficient CFTR expression levels in human cells to completely correct the chloride defect, both in primary HBE cells from F508del/F508del patients as well as in a novel CFTR-KO hSABCi cell line as a model of class I homozygous null mutations. The potentiator ivacaftor showed a greater than additive effect in combination with rSIV.F/HN-CFTR. These data, coupled with the lack of evidence for insertional hotspots, suggest that this lentiviral vector is a strong candidate for the treatment of CF patients independent of the underlying mutation class, with an initial focus on patients being nonresponsive to modulator therapy. Supported by these data, rSIV.F/HN-CFTR has entered a phase I/II clinical trial in November 2024 (NCT06515002).

Acknowledgements: We thank James Garnett, Megan Webster and Benjamin Strobel for their scientific input in the study, as well as Nadine Rehm, Eva Martin, Philipp Rechtsteiner and Dragica Blazevic for technical support (all from Boehringer Ingelheim Pharma GmbH, Biberach an der Riss, Germany).

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Ethics statement: Animal experiments were approved according to the German animal safety law (§4 2015/92 and §4 2020/254).

Author contributions: Conceived the study: A. Moiseenko, A. Sinadinos, A.C. Boyd, A. Schambach, U. Griesenbach, S. Hobbie, E.W.F.W. Alton and S. Kreuz. Acquired and analysed the data: A. Moiseenko, A. Sinadinos, A. Sergijenko, A. Saleh, K. Pineault, K. Nekola, N. Strang, A. Eleftheraki, A.C. Boyd, T. Rath, M. Rothe, U. Maier and S. Kreuz. Contributed to methodology: A. Moiseenko, A. Sinadinos, K. Nekola, M. Rothe, A. Schambach, M. Schuler and M. Schmidt. Drafted the manuscript: A. Moiseenko. Edited the manuscript: A. Sergijenko, A.C. Boyd, J.C. Davies, D.R. Gill, U. Griesenbach, S.C. Hyde, G. McLachlan, T. Rath, M.J. Thomas, D. Mennerich, E.W.F.W. Alton and S. Kreuz.

Conflict of interest: A. Moiseenko reports support for the present study from Boehringer Ingelheim and UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC), and patents planned, issued or pending (02-0580-GB-1). A. Sinadinos reports support for the present study from Boehringer Ingelheim International GmbH. A. Saleh reports support for the present study from Boehringer Ingelheim International GmbH. K. Nekola reports support for the present study from Boehringer Ingelheim Pharma GmbH & Co. KG. N. Strang reports support for the present study from Boehringer Ingelheim. A.C. Boyd reports support for the present study from Boehringer Ingelheim International GmbH. J.C. Davies reports support for the present study from ArticulateScience LLC, grants from UK Cystic Fibrosis Trust, Cystic Fibrosis Foundation, Cystic Fibrosis Ireland and EPSRC, payment or honoraria for lectures, presentations, manuscript writing or educational events from Vertex Pharmaceuticals, Boehringer Ingelheim, Eloxx, Algipharma, Abbvie, Arcturus, Enterprise Therapeutics, Recode, LifeArc, and Genentech, and a leadership role with *Journal of Cystic Fibrosis* (Deputy Editor). D.R. Gill reports support for the present study from Boehringer Ingelheim International GmbH, and patents planned, issued or pending (lentiviral gene therapy for CF) with Boehringer Ingelheim International GmbH. S.C. Hyde reports support for the present study from Boehringer Ingelheim International GmbH, and patents planned, issued or pending (lentiviral gene therapy for CF) with Boehringer Ingelheim International GmbH. G. McLachlan reports support for the present study from Boehringer Ingelheim International GmbH. T. Rath reports support for the present study from ProtaGene CGT GmbH (former name GeneWerk GmbH). M. Schuler reports support for the present study from Boehringer Ingelheim Pharma GmbH & Co. KG. U. Maier reports support for the present study from Boehringer Ingelheim Pharma GmbH & Co. KG. D. Mennerich reports support for the present study from Boehringer Ingelheim. U. Griesenbach reports support for the present study from Boehringer Ingelheim International GmbH, patents planned, issued or pending (lentiviral gene therapy for CF) with Boehringer Ingelheim International GmbH, and leadership roles with Cell and Gene Therapy Catapult (nonexecutive director) and AlveoGene (director). E.W.F.W. Alton reports support for the present study from Boehringer Ingelheim International GmbH. S. Kreuz reports support for the present study from Boehringer Ingelheim and UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC), and patents planned, issued or pending (02-0580-GB-1). The remaining authors have no potential conflicts of interest to disclose.

Support statement: The study was funded by Boehringer Ingelheim Pharma GmbH. Additional funding was obtained through a Wellcome/Department of Health Health Innovation Challenge Fund award and the Medicor Foundation. E.W.F.W. Alton, U. Griesenbach and J.C. Davies are supported by the National Institutes of Health and Care Research (NIHR) through the Imperial Biomedical Research Centre, the Royal Brompton Clinical Research Facility. E.W.F.W. Alton and J.C. Davies are supported by a NIHR Senior Investigator award. Funding information for this article has been deposited with the Crossref Funder Registry.

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