TITLE
Towards Gene Therapy for Cystic Fibrosis Using a Lentivirus Pseudotyped With Sendai Virus Envelopes

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Short Title: Sendai-F/HN-pseudotyped SIV vector for CF therapy

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ABSTRACT

Gene therapy for cystic fibrosis (CF) is making encouraging progress into clinical trials. However, further improvements in transduction efficiency are desired. To develop a novel gene transfer vector that is improved and truly effective for CF gene therapy, a simian immunodeficiency virus (SIV) was pseudotyped with envelope proteins from Sendai virus (SeV), which is known to efficiently transduce unconditioned airway epithelial cells from the apical side. This novel vector was evaluated in mice in vivo and in vitro directed towards CF gene therapy. Here we show that a) we can produce relevant titres of a SIV vector pseudotyped with SeV envelope proteins for in vivo use, b) this vector can transduce the respiratory epithelium of the murine nose in vivo at levels that may be relevant for clinical benefit in CF, c) this can be achieved in a single formulation, and without the need for preconditioning, d) expression can last for 15 months, e) readministration is feasible, f) the vector can transduce human air-liquid interface cultures and g) functional CFTR chloride channels can be generated in vitro. Our data suggest that this lentiviral vector may provide a step change in airway transduction efficiency relevant to a clinical programme of gene therapy for CF.
INTRODUCTION

Cystic fibrosis (CF) is a fatal genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, one function of which is to act as a chloride channel in airway epithelial cells. CF is characterized by recurrent chest infections, increased airway secretions, and eventually respiratory failure [1]. Whilst symptomatic treatments have successfully increased median survival to approximately 36 years, definitive novel therapeutic approaches aimed at the basic defect are clearly needed.

Given the propensity of certain viruses for infection of the respiratory tract, CFTR gene transfer using these vectors has been extensively studied. However, to date no viral vector has met the requirements for clinical use [2]. Three major problems have been encountered. Gene transfer efficiency is generally poor, at least in part because the respective receptors for the viral vectors appear to be predominantly localized to the basolateral surface of the airway epithelium. Secondly, penetration of the augmented mucus layer typical of CF is generally poor. Finally, the ability to administer viral vectors repeatedly, mandatory for such transient expression systems in the treatment of a lifelong disease, is limited. With these limitations in mind, we describe a novel vector that is able to circumvent some of the difficulties described above.

We have previously described the use of Sendai virus (SeV) vectors for airway gene transfer [3, 4]. SeV is a single stranded RNA virus, belongs to the family of Paramyxoviridae, and is able to overcome the first two of the above noted difficulties. Firstly, gene transfer to the airway epithelium is highly efficient, because the sialic acid
and cholesterol receptors needed for transduction are present on the apical surface of airway epithelial cells. Further, SeV uses a cytoplasmic expression system, thereby removing the limitations imposed by the nuclear membrane. Secondly, whilst mucus does act as a partial barrier to SeV-mediated gene transfer, the very high expression levels generated allow this limitation to be readily tolerated. However, despite our best efforts to date, we have been unable to overcome the third impediment to clinical translation, namely repeated application of SeV.

A different solution to this problem would theoretically be provided by transduction of progenitor or stem cells, normally used to replenish the airway epithelium. If this was feasible, ‘single-hit’ gene therapy with a viral vector might overcome this remaining hurdle. However, a significant further issue is the identity and localization of these progenitor or stem cells. Existing data suggest the presence of specialized cells, whose identity and site varies with progression along the branching of the airways [5]. In addition, the ciliated epithelium may be able to renew or repair itself; the lifespan of these epithelial cells has historically been estimated to be around 3 months [6], but has been extended to 6 months in trachea and 17 months in lung in a most recent publication [7]. Irrespective of the appropriate target cell, an integrating vector would clearly be needed to achieve the goal of ‘single-hit’ gene therapy. Further, given our present lack of conclusive data regarding the identity of airway stem cells, a vector that could transduce cells irrespective of their level of turn-over, would be clearly advantageous.

In contrast to retroviral vectors that can only transduce proliferating cells [8], lentiviruses are able to produce gene expression in non-dividing cells, including those
of the airway epithelium. However, to allow for receptor-mediated cell entry these vectors require pseudotyping to allow display of appropriate ligands for their cognate receptors. The most common pseudotyping, with glycoproteins of the vesicular stomatitis virus (VSVG), produces efficient transduction of a broad range of cells, and the virus can be readily concentrated and purified by high-speed centrifugation [9, 10]. However, they are not able to transduce airway epithelial cells in vivo when delivered via the apical surface. This, in turn, relates both to the difficulties such vectors have in penetrating the overlying mucus, and the lack of apically localized receptors on the epithelial cells [11-13]. Thus, the use of detergents such as lysophosphatidylcholine (LPC) or ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA), help to breach these barriers, thereby allowing the vector to penetrate to the basolateral surface where the appropriate receptors reside. However, the suitability of such an approach in the bacteria-laden airways of CF patients is debatable. Thus, several groups have examined the use of a number of different pseudotyped lentiviral vectors for their airway epithelial transduction efficiency, including envelope glycoproteins of paramyxoviruses [14], filoviruses [15-17] and orthomyxoviruses [18]. Whilst encouraging data have been generated, we are not aware of a vector that fulfills the three key requirements of (1) efficient transduction of airway epithelial cells without the need for chemical pre-treatment, (2) long-term transgene expression and (3) the ability to be produced to high vector titres suitable for clinical application [19].

We describe here the development of a replication-defective lentiviral vector [20] derived from the simian immunodeficiency virus of the African green monkey (SIV) [21]. Using novel strategies we have been able to pseudotype this vector with the key Sendai virus envelope proteins, hemagglutinin-neuraminidase (HN) and fusion (F)
protein [14]. The HN and F proteins function respectively to attach to sialic acids, the receptor of SeV, and mediate cell fusion for vector entry to target cells. We have optimized vector production and transgene expression level of this F/HN-pseudotyped SIV vector by introducing the central polypurine tract (cPPT) [22] and the Woodchuck hepatitis virus posttranscriptional regulatory elements (WPRE) [23, 24]. We show that this F/HN-pseudotyped SIV vector can efficiently transduce nasal epithelial cells from the apical surface \textit{in vivo}, resulting in transgene expression sustained for periods far beyond the proposed lifespan of airway epithelial cells. Importantly, we show that readministration is feasible. Finally, we demonstrate that this vector can transduce a fully differentiated human airway epithelium and that functional CFTR chloride channels can be generated after transduction with F/HN-SIV carrying the human CFTR cDNA \textit{in vitro}. This vector may, therefore, be able to produce a step change in airway transduction efficiency relevant to a clinical programme of CF gene therapy.
RESULTS

F/HN-pseudotyped SIV vectors can be generated and produced at high titres

To accomplish pseudotyping of SIV vector with SeV envelope proteins, we have previously described that modifications of the F and HN proteins were needed [14]. Briefly, the cytoplasmic domain of the F protein was truncated to 4 amino acids, and the HN protein was fused with the cytoplasmic tail of the SIV transmembrane envelope protein. These modifications enabled the incorporation of F and HN-derived proteins into vector virions, generating an F/HN-pseudotyped SIV vector.

We next constructed a series of gene transfer vectors in which the cPPT and/or WPRE were inserted into the parent gene transfer vector [20]. Their function was first evaluated using VSV-G pseudotyped SIV vectors encoding GFP. As expected, insertion of cPPT and WPRE increased vector production, from $3.8 \times 10^6$ TU/ml (cPPT-/WPRE-) to $1.4 \times 10^7$ TU/ml (cPPT+/WPRE-) and $6.7 \times 10^6$ TU/ml (cPPT-/WPRE+), respectively. The insertion of both elements (cPPT+/WPRE+) led to the highest titre production ($3.9 \times 10^7$ TU/ml). Because the number of particles produced in the supernatant ($\sim 2 \times 10^8$/ml) was similar in all cases, the introduction of these elements increased the quality of the vector by improving the TU to particle ratio from 1:100 to 1:10. Importantly, consistent with these data, insertion of these sequences into the SeV-F/HN-pseudotyped vectors encoding GFP increased virus production from $1 \times 10^5$ TU/ml (cPPT-/WPRE-) to $5 \times 10^7$ TU/ml (cPPT+/WPRE+), leading to an improvement in TU to particle ratio from 1:100 to 1:20. Thus, the cPPT/WPRE-containing vector was used in all subsequent experiments. The F/HN-pseudotyped SIV vector could be further concentrated from $2.0 \pm 0.9 \times 10^7$ TU/ml to $6.1 \pm 2.0 \times 10^9$ TU/ml (n=3) through centrifugation, making high titre use in vivo
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feasible.

**F/HN-SIV-GFP transduces the nasal epithelium of mice**

We evaluated the *in vivo* gene transduction efficiency of the SeV-F/HN-pseudotyped SIV vector in the nasal airway epithelium of mice. This tissue was chosen because the characteristic pathophysiological abnormalities of CF are reproduced in the nasal, but not the lower airways of CF knockout mice. In addition, in contrast to the murine lung, cell composition in the murine nasal epithelium is more akin to the human lung and contains both submucosal glands, as well as mucous-secreting cells, thereby allowing evaluation of whether the vector is able to penetrate a mucus-enriched airway surface fluid layer. As part of this study approximately 100 mice were treated (for details see individual Figure legends). The overall survival over the course of the >15 months study was 100%.

To examine transduction efficiency of respiratory epithelial cells, we administered F/HN-pseudotyped SIV vector encoding GFP to the nose of mice at a dose of $4 \times 10^8$ TU/mouse. Thirty days after transfection, GFP positive respiratory epithelial cells were identified and quantified on histological sections collected 1 to 4 mm from the tip of the nasal bone (**Figure 1**). Transduction efficiency along the nasal septum was $4.5 \pm 0.7\%$ (mean $\pm$ SEM, n=8). This corresponds favorably with the approximately 5% of cells estimated to require transduction for generating clinical benefit.

**F/HN-SIV transduces cells in both the respiratory and olfactory epithelium**

Using high power microscopic analysis we were able directly to detect and identify GFP fluorescent cells 30 days after transfection. The murine nasal epithelium consists
of squamous cells in the anterior part of the nose, changing to respiratory epithelium in the mid-portion and neuroepithelial olfactory epithelium in the dorsoposterior regions [25]. The majority (approximately 69%) of GFP positive cells were ciliated respiratory epithelial cells (Figure 2A) followed by neuronal cells (21%) in the olfactory epithelium (Figure 2B) and squamous cells (7%) (Figure 2C). The number of neuronal cells transduced was very few in the anterior part of the nose and increased in the posterior region as expected. The remainder (3%) were mainly non-neuronal cells in the olfactory epithelium, most likely sustentacular cells (Figure 2D), a major constituent of the olfactory epithelium. Interestingly, we did not detect GFP-expressing goblet cells, a cell type commonly transduced in VSVG-SIV perfused LPC-pre-conditioned nasal epithelium (data not shown).

**GFP expression persists for more than 1 year after F/HN-SIV-GFP transduction**

To determine the duration of gene expression mice were perfused with F/HN-SIV-GFP (4x10^8 TU/mouse) or PBS and harvested 3 to 449 days after transfection (Figure 3A+B). F/HN-SIV-mediated GFP expression was visible in the nasal cavity using in situ imaging as early as 10 days post transduction and was still detectable in 7 out of 10 and 3 out of 10 mice 360 and 449 days after a single administration of the vector, respectively. At this time-point GFP positive cells appeared to be randomly distributed within the tissue similar to the distribution observed 30 days after transfection (Figure 3C). GFP was undetectable in LPC-preconditoned VSVG-SIV-GFP transduced tissue (data not shown). Histological sections were used to quantify the number of GFP positive cells over time. As a whole, the number of GFP positive cells gradually declined over time by approximately 70% (Figure 3D). In some mice marked decline of GFP positive cells were observed after
90 days post-transduction, however, in the other mice the number of transduced cells were maintained well even at 360 days post-transduction to the similar level of that at 30 days post-transduction. In addition, it is important to note that GFP positive cells were still detectable in 16 out of 17 mice 12 months after transduction.

We also used in vivo bioluminescence imaging after nasal perfusion with F/HN-SIV-Lux to assess gene expression over time in the same animal (Figure 3E). Figure 3F shows quantification of photon emission after intraperitoneal injection of luciferin substrate from one to eight months after transduction. Although persistence of gene expression far exceeds the expected life-span of airway epithelial cells of 100 days, possibly indicating progenitor cell integration, repeated bioluminescence in vivo imaging (BLI) after F/HN-SIV-lux transduction shows a gradual decline in photon emission over a 8 months period (month 1: $2.2\times10^{6} \pm 4.9\times10^{5}$, month 8: $2.7\times10^{5} \pm 8.8\times10^{4}$, n=10/group, p<0.005), but was still significantly (p<0.01) higher than the PBS control ($4.5\times10^{4}\pm2.0\times10^{3}$, n=5) This result is consistent with the decline in GFP positive cells described above. In addition we analyzed all data using a repeat measure test to determine if the decline in bioluminescence stabilizes during the 8 months study period. The analysis showed that gene expression significantly (p<0.05) declined for the first 4 months but then stabilized with expression levels from 5 to 8 months not being different compared to the 4 months levels.

The SIV vector-transduced cells show clustering after induced regeneration of the epithelium

To ascertain further whether F/HN-SIV induced chromosomal integration into nasal respiratory progenitor or stem cells, we artificially induced cell division after SIV vector
transduction by damaging the nasal tissue with the detergent (polidocanol) [6], which has previously been shown to strip the surface epithelium within a few hours, while retaining basal cells able to regenerate the epithelium within 7 days (Figures 4A-D). Seven and 28 days after vector transduction (4x10^8 TU/mouse, n=3) the nasal tissue was perfused with 2% polidocanol (10 µl/mouse) and gene expression analyzed 4 weeks after the last detergent treatment. Importantly, GFP-expressing cells now showed clustering after polidocanol treatment (Figure 4E and supplementary Figure S2), possibly indicating origination from a common progenitor.

SIV-mediated gene transfer can be achieved after three applications of the vector

Whilst the above data are encouraging, gene therapy for CF will require life-long treatment. We therefore, assessed the feasibility of readministering this vector, and compared transduction efficiency to the current optimal non-viral formulation for airway gene transfer in vivo. Figure 5 shows that following two administrations of F/HN-SIV-GFP separated by one month, a third administration of F/HN-SIV-lux (to prevent an immune response against the transgene) produced gene expression of approximately 40% of that seen following a single challenge with F/HN-SIV-lux. Further, these levels after three challenges with the SIV vector remained approximately 500-fold greater (p<0.01) than seen with an optimal non-viral formulation, previously used in a CF clinical trial.

F/HN-SIV transduces differentiated human airway epithelium

Differentiated human airway epithelium is in general difficult to transduce. In preliminary experiments we have shown that F/HN-pseudotyped lentivirus transduced
human airway cells grown as air-liquid interphase (ALI) cultures. ALIs were transfected with F/HN-SIV carrying a luciferase reporter gene at an approximate MOI of 100 and luciferase expression was detectable 10 and 26 days after gene transfer (day 10: 53.0 ± 6.3 RLU/mg protein, day 26: 7.9 ± 3.4 RLU/mg protein, untransfected: 0.01 RLU/mg protein, n=3/group) and Figure 6. Importantly, gene transfer occurred without the need for pre-conditioning.

Transduction with F/HN-SIV carrying the CFTR cDNA leads to expression of cAMP-dependent chloride channels

Iodide efflux is commonly used to demonstrate the presence of forskolin-activated chloride channels in vitro. In this assay iodide is used as a surrogate for chloride due to the shorter half-life of the radioactive material. We have constructed a F/HN-SIV carrying a GFP-CFTR fusion cDNA construct and transduced HEK293T cells to assess whether functional CFTR chloride channels are generated. Fusion of GFP to the N-terminus of CFTR has previously been shown not to effect CFTR function [26, 27]. Figure 7 shows that cells transduced with F/HN-SIV-GFP-CFTR (MOI 50) significantly (p<0.001) increased cAMP-mediated efflux compared to cells treated with a F/HN-SIV-GFP control virus. We, therefore, conclude that transduction with F/HN-SIV-GFP-CFTR generates functional CFTR chloride channels in vitro.
DISCUSSION

Here we show that a) we can produce titres of a novel SIV vector pseudotyped with Sendai virus envelope proteins appropriate for in vivo use, b) the vector can transduce the respiratory epithelium of the murine nose in vivo at levels that may be relevant for clinical benefit in CF, as previously suggested by in vitro mixing experiments [28], c) this can be achieved in a single formulation, and without the need for preconditioning, d) expression can last for at least half the lifespan of a mouse, e) the vector can produce levels of gene expression approximately 500-fold greater than the current optimal non-viral formulation after three repeated administrations, f) the vector is able to transduce a fully differentiated human airway epithelium and g) can produce functional cAMP-dependent CFTR chloride channels in vitro.

We inserted cPPT and WPRE sequences into the SIV vector. Both elements have previously been reported to increase gene transduction efficiency possibly due to acceleration of the movement of the pre-integration complex of the vector into the nucleus [22, 29, 30], or other mechanisms [23, 31]. Interestingly, a synergistic effect of these two elements has been observed for HIV-based lentiviral vectors [24]. Our data are in keeping with these observations, with simultaneous insertion of cPPT and WPRE increasing productivity of both the VSVG and SeV-F/HN-pseudotyped SIV vectors. Using these methods we were able to reach titers of the SeV-F/HN-pseudotyped SIV vector of 5x10⁷ TU/ml. Thus, this vector may be able to overcome one previously encountered important translational hurdle.

Lentiviral vectors pseudotyped with a variety of envelope proteins other than VSV-G have been described, including those from Ebola, Zaire [15, 16], influenza
hemagglutinin (HA) from fowl plague virus [18] and baculovirus GP64 envelopes [17]. Amongst these, arguably it is the transduction efficiency of the baculovirus GP64-pseudotyped vectors which is most impressive, when applied in a viscoelastic gel formulation (1% methylcellulose) as a vector solvent. However, the regulatory complexities of moving two new agents into the clinic simultaneously, underline the encouraging transduction efficiency and duration we report here without the need for additions to the formulation.

The likely target for CF gene therapy are the ciliated epithelial cells, and more than 70% of the cells transduced by the F/HN SIV vector were of this type. This is in keeping with transduction of these cells by the ‘parent’ SeV vector, and overcomes a second hurdle in the translation of these vectors towards the clinic. The number of cells requiring transduction for clinical benefit is a vexed, and unresolved question. In part this may depend on which of the many functions of CFTR requires correction. Thus, if the chloride channel function predominates, *in vitro* data suggest that as few as 5% of cells may be sufficient [32]. These values are in reach of the F/HN SIV vector described here.

Using a human cytomegalovirus (CMV) promoter we saw expression of GFP for more than 360 days in 16 out of 17 mice, although gene expression gradually decreased over time when quantified as number of cells expressing the GFP reporter gene or longitudinal assessment of bioluminescent imaging. The onset of transgene expression was typically delayed, with no GFP fluorescence detected at day 3, but clearly visible by day 10. This has also been reported for an Ebola virus Z protein (EboZ)-pseudotyped HIV vector [15]. Possible explanations include the delayed
movement of the pre-integration complex to the nucleus, or the shutting off of
promoter activity by concomitant inflammation consequent upon transduction [33, 34],
with subsequent expression following the resolution of inflammation. Irrespective,
transgene expression was still apparent up to 449 days after transduction the longest
timepoint assessed. Because the life span of terminally differentiated airway epithelial
cells has been estimated at around 90 days [6], both in mice and in man, we
considered whether this vector may have transduced progenitor or stem cells within
the airway epithelium. We would predict that in this case we would observe clonal
expansion, with clustering of transgene positive cells, and this was seen following
induced regeneration of epithelial cells after polidocanol treatment. Cells derived from
progenitor or stem cells thought to reside near the basement membrane, have
previously been shown to move laterally during differentiation [35]. Thus, in the
absence of epithelial damage, SIV vector-transduced progenitor or stem cell-derived
cells should be observed in a scattered pattern without clustering (see schematic
presentation in Figure 8A). In contrast, following epithelial stripping, a different
pattern would be predicted to occur, with clusters of transduced cells becoming visible
(see schematic presentation in Figure 8B). This hypothesis may explain, why we
observed clusters of GFP positive cells in damaged but not in undamaged epithelium.
However, more extensive studies will be necessary to understand and more
conclusively prove stem or progenitor cell transduction. Other explanations for the
unexpectedly long duration of expression include (1) an alteration in the cell cycle of
transduced respiratory epithelial cells, although to our knowledge this has not
previously been reported, (2) expression from -resident airway inflammatory or
immune cells, for which we saw no evidence or (3) a longer than 3 months half-life of
respiratory epithelial cells, which has recently been suggested by Rawlins et al [36].
The treatment of CF will require lifelong expression of the normal CFTR protein. Thus, despite these encouraging data showing long-lasting expression from a single administration, we assessed whether repeated application of this vector could sustain gene expression. We show that repeated mucosal administration of F/HN pseudotyped lentivirus, when given monthly over a 3 months period, is feasible and led to gene expression approximately 40% of that seen following a single administration. This dosing interval may be of subsequent clinical relevance. Sinn et al [36] have recently shown that seven weekly administrations of a GP64-pseudotyped FIV given in tandem with a ciliastatic agent, are able to produce repeatable expression. Further, each study was undertaken in a different inbred mouse strain. The significant differences between the studies, yet with similar outcome, provide a growing body of evidence that such vectors can be readministered.

Differentiated human airway epithelium is in general difficult to transduce. However, we have shown here that F/HN-SIV transduced fully differentiated human airway epithelium successfully, and that reporter gene expression could be detected for at least 26 days after transduction. Importantly, gene transfer occurred without the need for pre-conditioning with tight junction openers or cilia static agents that are often required with other viral vectors. This provides encouraging support for its use in human trial.

In addition to demonstrating that F/HN-SIV carrying the CFTR cDNA was able to generate cAMP-dependent chloride channels in vitro, we also attempted to correct
nasal potential difference in CF knockout mice. However, we did not detect any changes in ion transport (data not shown). Importantly, the suitability of the CF mouse nasal epithelium as a model has been put into question by two recent publications showing that the nasal bioelectrics are dominated by the olfactory rather than the respiratory epithelium [37, 38]. Our experience is in keeping with this observation. Transduction with Sendai virus, which transduces respiratory and olfactory epithelium led to significant increases in chloride transport [39], whereas lentivirus and non-viral gene transfer agents (unpublished data), which predominantly transduce ciliated respiratory epithelial cells were unable to alter ion transport in the mouse nose. Until more appropriate animal models become widely available, analysis of CFTR function after gene transfer may, for certain gene transfer agents, be restricted to in vitro models.

Clearly, at least one remaining crucial hurdle is the risk-benefit ratio of these integrating vectors. The cases of leukemia in the SCID trial using a retroviral vector have been well documented, but lentiviral vectors are considered by many to be less susceptible to these problems. Further, the slowly dividing airway epithelium may represent a very different risk to the rapid turnover of bone marrow stem cells. Encouragingly, in our study of approximately 100 mice, over a one year period we saw no adverse events attributable to the vector. However, the encouraging increase in median survival of CF patients to the current approximately 36 years suggests that extensive toxicology studies will be needed before clinical trials can begin.

In conclusion, we suggest that the SeV-F/HN-pseudotyped SIV vector reported here
may represent a further step towards translating such integrating viral vectors into clinical use. Several key hurdles have been potentially overcome, pushing these vectors into the arena as candidates for clinical trials.
MATERIALS AND METHODS

Cell culture. Human embryonic kidney 293T and 293T/17 cells (CRL-11268, ATCC, Manassas, VA) were maintained in Dulbecco’s minimal Eagle’s medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml).

Plasmid Construction. pCAGGS-Fct4 and pCAGGS-SIVct+HN were constructed as previously described [14]. The cPPT and WPRE sequences [40] were inserted in the SIV-derived gene transfer plasmid [21].

Production of SIV vector. Replication-defective self-inactivating SIV vector was constructed as previously described [20] with minor modifications. Briefly, the SeV-F/HN-pseudotyped SIV vector was produced by transfecting 293T/17 cells (15 cm diameter culture dishes) with four plasmids complexed to Lipofectamine/Plus reagents (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations [Plasmid-1: 10 µg SIV-derived transfer plasmid carrying a green fluorescent protein (GFP), a luciferase (lux) reporter gene or a GFP-CFTR fusion construct [26], Plasmid-2: 3 µg packaging plasmid, Plasmid-3: 2 µg pCAGGS-Fct4, Plasmid 4: 2 µg pCAGGS-SIVct+HN]. The VSV-G pseudotyped SIV vector was produced using a similar protocol, but a pVSV-G plasmid (2 µg, Clontech, Mountain View, CA) was used instead of pCAGGS-Fct4 and pCAGGS-SIVct+HN. Twelve hours after transfection the culture medium was replaced with 30 ml serum-free DMEM containing 5 mM sodium butyrate. Sodium butyrate stimulates the vector production to inhibit histone deacetylase [41, 42]. The culture supernatant containing the SIV vector was harvested 48 hours after transfection, filtered through a 0.45 µm filter membrane.
and further concentrated by high-speed centrifugation (20,000 g for 4 hours at 4°C, Beckman Avanti JA18 rotor). The vector pellets were suspended in phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA) to 100 ~ 200-fold concentration and stored at –80°C.

**Vector titration.** The particle titer was determined using real-time RT-PCR. Virus RNA was purified using a QIAamp viral RNA mini-kit (QIAGEN, Strasse, Germany), and reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA). The QuantiTect probe PCR system (QIAGEN, Strasse, Germany) and primers for amplifying 131 nucleotides (bp) spanning the WPRE sequence (forward primer: 5'-ggatacgctgctttaatgcc-3', reverse primer: 5'-acgccacgttgcttgacaac-3') were used according to the manufacturer’s protocol in an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). SIV gene transfer plasmid DNA (3x10^4 to 2x10^6 molecules) was used as standard.

Transduction units (TU/ml) were determined by transducing 293T cells with serial dilutions of vector stock and quantification of transduced cells by GFP fluorescence (for F/HN-SIV-GFP and VSVG-SIV-GFP) or staining with anti-luciferase antibody (for F/HN-SIV-lux). To work with a consistent virus concentration throughout the study virus stocks were all adjusted to a final volume of 4x10^9 TU/ml. A titre of 4x10^9 TU/ml was used for all animal studies.

**In vivo administration to the mouse nose.** All animal studies had been approved by the DNAVEC Animal Care Committee and the Imperial College Animal Ethics Committee and were carried out according to Home Office regulations. C57BL/6N
mice (female, 6-8 weeks) were used. Mice were anesthetized, placed horizontally on their backs onto a heated board and a thin catheter (<0.5 mm outer diameter) was inserted approximately 2.5 mm from the tip of nose into the left nostril. Using a syringe pump (Cole-Palmer, Illinois, USA) vector (100 μl) was then slowly perfused onto the nasal epithelium (1.3 μl/min) for 75 min. Despite, perfusion of virus into the left nostril, we routinely observe transfection in both left and right nostrils, which is due to dispersion of the solutions throughout the entire nasal cavity. PBS and VSVG-SIV transduced mice preconditioned with 1% lysophosphatidylcholine (LPC) as described by Limberis et al were used as controls [43]. At indicated time-points (3 to 360 days after transduction) mice were culled to visualize GFP expression.

In the repeat administration experiments groups of mice were transduced with either one dose of F/HN-SIV-lux (single dose group), or two doses of F/HN-SIV-GFP (day 0, day 28), followed by F/HN-SIV-lux on day 56 (repeat dose group). Importantly, mice receiving F/HN-SIV-lux (single dose group) and F/HN-SIV-lux on day 56 (repeat dose group) were of similar age and were transduced at the same time. Gene expression was analyzed 30 days after F/HN-SIV-lux administration. For comparison mice were transfected with the cationic lipid GL67A complexed to a luciferase reporter gene as previously described [44] and luciferase expression was measured 2 days after transfection.

**Induced regeneration of nasal epithelial cells by polidocanol treatment.** Nasal epithelial cells were stripped by polidocanol treatment according to the method described by Borthwick et al [6] with some modification. In brief, mice were anaesthetized and 10 μl Polidocanol (2%) were administered to the nose as a bolus
by “nasal sniffing”. To confirm the stripping and regeneration of nasal epithelial cells, nasal tissue was perfused with 10 µl of 2% (v/v in PBS) polidocanol (Nonaethylene glycol mono-dodecyl ether, SIGMA, St. Louis, MO) and histological analysis undertaken 24 hours and 7 days after treatment (n=3 per group). To analyze transduction of possible progenitor or stem cells, we first administered F/HN-SIV-GFP (4 x 10^8 TU/mouse) vector to mouse nasal epithelium. Seven days after transduction, nasal tissue was perfused with 10 µl of 2% (v/v in PBS) polidocanol, and this treatment was repeated again 3 weeks later. Histological sections were analyzed 58 days after vector administration (30 days after the last polidocanol treatment).

**Bioluminescent imaging.** Mice were injected intraperitoneally with 150 mg/kg of D-Luciferin (Xenogen Corporation, Alameda, CA) 10 min before imaging and were anaesthetised with isofluorane. Bioluminescence (photons s^-1 cm^-2 sr^-1) from living mice was measured using an IVIS50 system (Xenogen Corporation) at a binning of 4 for 10 minutes, using the software programme Living Image (Xenogen). For anatomical localisation a pseudocolour image representing light intensity (blue least intense, red most intense) was generated using Living Image software and superimposed over the greyscale reference image. To quantify bioluminescence in the nose photon emission in a defined area (red box) was measured by marking a standardized area for quantification. The size of the red box was kept constant and was placed over the heads of the animals as indicated in the Figure. Importantly the areas were marked using the greyscale reference image to avoid bias.

**Tissue preparation for histological assessment of GFP expression.** Mice were culled and the skin was removed. The head was cut at eye level and skin, jaw, tongue
and the soft tip of the nose were carefully removed. For in situ imaging of GFP expression in the nasal cavity, GFP fluorescence was detected using fluorescence stereoscopic microscopy (Leica, Ernst Leitz Optische Werke, Germany). Subsequently, the tissue was fixed in 4% paraformaldehyde (pH 7.4) overnight at room temperature and was then submerged in 20% EDTA (pH 7.5 for 5 days) for decalcification. The EDTA solution was changed at least every second day. After decalcification the tissue was incubated in 15% sucrose overnight at room temperature and was then embedded in Tissue Mount (Chiba Medical, Soka, Saitama, Japan). Ten µm sections were cut at 6 different positions in each mouse head (approximately 0 to 6 mm from the tip of nasal bone). GFP expression was observed using a fluorescent microscope (Leica, Ernst Leitz Optische Werke, Germany). Quantification and identification of cell types was carried out on 6 levels/mouse using a 40x or 63x objective. Prolonged image exposure was necessary to capture the structure of the nasal epithelium using fluorescent microscopy. This led to pixel saturation of GFP positive cells and causes GFP positive cells to appear almost white rather than the common green appearance that we, and others, observe under higher magnification.

**Transduction of air-liquid interphase cultures.** Fully differentiated airway epithelial cells grown as air-liquid interphase (ALIs) cultures were purchased from Epithelix (Geneva, Switzerland). ALIs were transfected with F/HN-SIV-Lux at an MOI ranging from approximately 25 to approximately 300. The virus was dissolved in 50 µl PBS and applied to the apical surface. After 6 hrs the virus was removed and ALIs were incubated for 10 to 26 days. The basolateral medium was changed every 48 hrs during this incubation period. At specified time-points the ALIs were lysed in 100 µl
RLB buffer and luciferase expression was quantified using the Luciferase Assay System (Promega, Southampton, UK) according to the manufacturer's instructions. The total protein content of the cultures was quantified using the BioRad protein assay kit (BioRad, Hemel Hempstead, UK). Each sample was assayed in duplicate. Luciferase expression was then presented as Relative Light Units (RLU)/mg total protein. For bioluminescence imaging 100 μg luciferin in PBS were added to the apical membrane.

**Iodide efflux assay.** HEK293T cells were transfected with F/HN-SIV-GFP-CFTR or a F/HN-SIV-GFP control virus at an MOI of 500 and cultured for 2 days. CFTR chloride channel activity was assayed by measuring the rate of $^{125}$iodide efflux as previously described [45]. The $^{125}$iodide efflux rates were normalized to the time of forskolin/IBMX addition (time 0). Curves were constructed by plotting rates of $^{125}$iodide efflux against time. To reflect the cumulative levels of $^{125}$iodide efflux following agonist-stimulation, all comparisons are based on areas under the time-$^{125}$iodide efflux curves. The area under the curve was calculated by the trapezium rule. Experiments were carried out in duplicate (n=6 wells/group/experiment).

**Statistical analysis.** Normal distribution was assessed for all data and parametric or non-parametric statistical analysis was performed as appropriate. Data in Figure 3f were analyzed using the Mann-Whitney U-test to compare bioluminescence at month 1 and 8, as appropriate for non-parametric data. In 2 out 10 mice bioluminescence had returned to baseline levels before month 8 and these mice did not undergo additional BLI. For the final quantification of gene expression at 8 months the mean bioluminescence of the PBS control cohort was used for these 2 mice. In addition data
in Figure 3f was analyzed using a Friedman repeat measure test followed by Dunn’s multiple comparison post-hoc test, as appropriate for non-parametric data.

Data in Figure 5 was analyzed by Kruskal-Wallis followed by Dunn’s Multiple Comparison test, as appropriate for non-parametric data. Data in Figure 6 were analyzed by ANOVA followed by Bonferroni’s Multiple Comparison Post-hoc test, as appropriate for normal distributed data. The null hypothesis was rejected at p<0.05.

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FIGURES

Figure 1 Transduction of mouse nasal epithelium with F/HN-SIV-GFP.
The murine nose was perfused *in vivo* with F/HN-SIV-GFP ($4 \times 10^8$ TU/mouse) vector and gene expression analyzed 30 days after transduction (n=8). Top panels: In situ imaging of GFP expression in the nasal cavity. Bottom panel: Microscopic imaging of GFP in histological sections. The sections were collected (A) 1 mm, (B) 2 mm, (C) 3 mm and (D) 4 mm into the nasal tissue (vertical white lines). GFP positive cells appear as small white punctuate signals.
Figure 2 Determination of cell types transduced.

The transduced GFP positive cells were identified using fluorescent microscopy (original magnification: x63) 30 days after administration of F/HN-SIV-GFP (4x10^8 TU/mouse) vector to the mouse nose. (A) Ciliated respiratory epithelial cell, (B) Neuronal cell in olfactory epithelium, (C) Squamous epithelial cell, (D) Non-neuronal cell in olfactory epithelium. The central image shows a cross section through the mouse nose and white boxes indicate regions in mouse nasal epithelium where respective transduced cell types were found. Panels A, B and D were rotated approximately 45, 130 and 180 degrees counterclockwise, respectively, to improve clarity of the figure.
Figure 3 Duration of GFP expression after transduction with F/HN-SIV-GFP.

Mouse nasal tissue was perfused with F/HN-SIV-GFP (4x10^8 TU/mouse or PBS and
gene expression was analyzed at indicated time-points after transduction. (A) Representative in situ images of GFP expression in the nasal cavity from mice analyzed 3 to 449 days after transduction. (B) In situ imaging of GFP expression in the nasal cavity from 10 mice analyzed 360 days after transduction. (C) Representative microscopic images of GFP expression in histological sections 360 and 449 days after transduction. GFP positive cells appear as small white punctuate signals. (D) Quantification of transduced cells. GFP positive cells were quantified on histological sections taken 2 mm into the nasal tissue of the nose. Data from 30 to 360 days after transduction are represented both by mean±SEM and individual values (ratio to GFP cells positive on day 30). The n number per group are 13 (day 30), 3 (day 50), 12 (day 90), 14 (day 160-180), 10 (day 220-270) and 17 (day 360). (E) Bioluminescence in vivo imaging one (1 M) to eight months (8 M) after transduction with F/HN-SIV-lux. Representative images of 2 out of 6 mice are shown. Red box indicates area chosen for quantification of photon emission. (F) Quantification of in vivo bioluminescence over time after transduction with F/HN-SIV-lux (black lines) or PBS (red lines). Each line represents photon emission over time in one animal. *** = p<0.005 compared to bioluminescence one month after gene transfer.
Figure 4: Clustering of transduced cells after the polidocanol-mediated stripping of epithelial cells followed by rapid regeneration.

Mouse nasal tissue was perfused with 10 μl of 2% (v/v) polidocanol (n=3) (A) Representative low power view (x 50 original magnification) of the nasal cavity 24 hrs after perfusion. Respiratory epithelium, marked by a white box was further magnified (x 200 original magnification). The respiratory epithelium prior to the treatment is shown in (B). Arrow indicates basal cells. The respiratory epithelium was completely stripped 24 hrs after polidocanol perfusion, whereas the basal cell layer was retained (C) and regenerated 7 days after treatment (D). This treatment was done after
transduction with F/HN-SIV vector (E). Seven days after transduction of nasal epithelial cells with F/HN-SIV-GFP (4x10^8 TU/100 µl/mouse), the nasal epithelium was stripped via perfusion with 10 µl of 2% (v/v) polidocanol. Polidocanol treatment was repeated again 3 weeks later. Histological sections were analyzed 58 days after vector administration (30 days after the last polidocanol treatment). In situ imaging of GFP expression in the nasal cavity of untreated mice (top panel in E) or mice treated with polidocanol (bottom panel in E). Clusters of GFP positive cells were seen in the polidocanol treated mice.
Mice were transduced with F/HN-SIV-lux (1 dose) or two doses of F/HN-SIV-GFP (day 0 and day 28) followed by F/HN-SIV-lux 4 weeks later (day 56=3 doses). Luciferase expression was measured 30 days after F/HN-SIV-lux transduction and compared to levels achieved with the non-viral gene transfer agent GL67A complexed to a luciferase reporter gene plasmid (pCIKLux). Each dot represents one mouse. Horizontal bars indicate the median per group (**p<0.01) compared to mice receiving GL67A/plasmid DNA).
Figure 6: Transduction of human air-liquid interface (ALIs) cultures with F/HN-SIV-lux

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ALIs were transduced with F/HN-SIV-lux at an approximate MOI of 25 (1-3) and 250 (4-6) or treated with PBS (7-9). 5 days after transduction ALIs were treated with luciferin and bioluminescent imaging performed.
HEK293T cells were transfected with F/HN-SIV-GFP-CFTR or a control virus carrying GFP (F/HN-SIV-GFP) at an MOI of 500. The iodide efflux assay was performed 2 days after transduction. Cells transfected with a eukaryotic expression plasmid carrying the CFTR cDNA under the control of a CMV promoter complexed to Lipofectamine 2000 were used as positive control. Data are presented as mean ± SEM. ***=p<0.001 compared to the control virus, n=6/group.
Figure 8 Schematic representation of epithelial cell migration in intact and damaged epithelium

A. Scattered pattern with regeneration in normal condition

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<td>Differentiated and Migrated Cells (Transduced)</td>
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<td>Differentiated and Migrated Cells (Non-Transduced)</td>
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E. Clustered formation after rapid & forced regeneration

We speculate that under normal physiological (undamaged) conditions, turnover may be comparatively slow and that newly generated epithelial cells may move laterally away from the stem or progenitor cell that they originated from (A). In contrast, if rapid regeneration is forced (after tissue damage with polidocanol) stem or progenitor cells have to divide rapidly and newly generated epithelial cells may (transiently?) stay in closer proximity to the cell that they originated from (B). This hypothesis may explain, why we observed clusters of GFP positive cells in damaged, but not in undamaged epithelium.