Secreted Gaussia luciferase as a sensitive reporter gene for in vivo and ex vivo studies of airway gene transfer

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The cationic lipid GL67A is one of the more efficient non-viral gene transfer agents (GTAs) for the lungs, and is currently being evaluated in an extensive clinical trial programme for cystic fibrosis gene therapy. Despite conferring significant expression of vector-specific mRNA following transfection of differentiated human airway cells cultured on air liquid interfaces (ALI) cultures and nebulisation into sheep lung in vivo we were unable to detect robust levels of the standard reporter gene Firefly luciferase (Fluc). Recently a novel secreted luciferase isolated from Gaussia princeps (GLuc) has been described. Here, we show that (1) GLuc is a more sensitive reporter gene and offers significant advantages over the traditionally used Fluc in pre-clinical models for lung gene transfer that are difficult to transfect. (2) GL67A-mediated gene transfection leads to significant production of recombinant protein in these models, (3) promoter activity in ALI cultures mimics published in vivo data and these cultures may, therefore, be suitable to characterise promoter activity in a human ex vivo airway model and (4) detection of GLuc in large animal broncho-alveolar lavage fluid and serum facilitates assessment of duration of gene expression after gene transfer to the lungs. In summary, we have shown here that GLuc is a sensitive reporter gene and is particularly useful for monitoring gene transfer in difficult to transfect models of the airway and lung. This has allowed us to validate that GL67A, which is currently in clinical use, can generate significant amounts of recombinant protein in fully differentiated human air liquid interface cultures and the ovine lung in vivo.

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1. Introduction

Cystic fibrosis (CF) is a single gene disorder with insufficient treatment options and a target organ, the lung, which is relatively easily accessible. Thus, gene therapy-based treatments may hold great promise for CF. The selection of appropriate gene transfer agents for chronic diseases such as CF, which require life-long treatment, is important. A variety of viral and non-viral vectors have been assessed in pre-clinical and clinical studies over the last 20 years since cloning the gene [1]. Although generally viral vectors are more efficient in transducing airway epithelial cells (the target cells in CF), than non-viral vectors, they (i.e. adenovirus, adenovirus-associated virus and Sendai virus) invariably induce immune responses, which negatively impact on protein expression upon repeated administration [2] Interestingly, lentiviruses, which have recently been assessed for airway gene transfer, overcome this problem in pre-clinical studies [3,4], but have not yet been evaluated in clinical trials.

A variety of non-viral vectors have been developed for airway gene transfer and assessed in clinical trials [1]. Although efficacy is generally lower than with virus-mediated gene transfer, proof-of-principle studies in both the nose [5–9] and the lung [10], as measured by vector-specific mRNA expression or partial correction
of the CFTR-specific chloride transport defect, has been established in clinical trials. However, the level of correction of the CF defect necessary to produce meaningful clinical outcome is unknown. Thus, the UK Cystic Fibrosis Gene Therapy Consortium has initiated a clinical trial programme to assess whether repeated administration of GL67A complexed to plasmid DNA encoding cystic fibrosis transmembrane conductance regulator (CFTR) cDNA can improve clinically relevant endpoints in the CF lung. Following extensive pre-clinical studies in mice [11] and sheep (manuscript submitted) the cationic lipid GL67A (Genzyme Corporation), which has already been used in two previous CF gene therapy clinical studies [10,12] was selected for this programme.

Human air liquid interface (ALI) cultures are grown on semi-permeable membranes exposing the basolateral surface to growth medium and the apical surface to air and, when fully differentiated, have a cell composition similar to human airways [13,14]. ALI cultures remain viable for prolonged periods of time (months) and have been used for a variety of applications including investigation of respiratory pathogens [15] and toxicology studies [16,17]. However, similar to human airways, differentiated ALI cultures are relatively difficult to transfect, a feature which arguably makes them a particularly suitable model of human airways. The low transfection efficiency is likely due to a combination of factors including active muco-ciliary clearance due to cilia beating, mucus production and the fact that the cells are non-dividing. Despite these extra- and intra-cellular barriers several studies have shown that viral vectors are able to transduce fully differentiated human ALI cultures [18–20]. In contrast, we and others have been unsuccessful in demonstrating recombinant protein expression in this model after non-viral gene transfer using a Firefly luciferase (Fluc) reporter gene (see data in this manuscript), which is generally regarded as a very sensitive reporter gene [21,22]. This represents a significant limitation in the development of these vectors for airway diseases such as CF.

Recently, a secreted luciferase reporter gene isolated from Gaussia princeps (Gluc) has been described [23]. The enzyme catalyzes the oxidation of the substrate coelenterazine leading to emission of light. Gluc is a secreted, thermostable and pH resistant protein [24]. In vitro studies have shown that Gluc is orders of magnitude more sensitive than Firefly or Renilla luciferase [23] and secreted alkaline phosphatase, a commonly used secreted reporter, in mammalian cells [25,26]. Gluc has most commonly been used to study a variety of biological processes including quantification of tumour growth [27,23] and monitoring of microbial infections [28] as well as screens for small interference (si)RNA [29]. The Gluc reporter has not been extensively used in the context of non-viral gene transfer studies. Nakanishi et al. injected a transposon carrying Gluc intravenously and documented sustained Gluc expression in blood for at least 2 months [30], but to the best of our knowledge there have not been any reports on using Gluc as a reporter for in vitro or in vivo gene transfer to airway epithelium. Here, we assessed if Gluc allows detection of GL67A-mediated gene transfer in fully differentiated human ALI cultures, as well as lungs of mouse and sheep.

2. Material and methods

2.1. Plasmids

Plasmid pCMV-Gluc1 expressing Gluc was purchased from Nanolights (Pinetop, Arizona, USA). Utilising a strategy described previously [31], the Firefly luciferase (Fluc) cDNA from plasmid pCIKFluc, in which Fluc is expressed under the control of the human immediate/early cytomegalovirus (CMV) promoter/enhancer [31], was replaced with the Gluc cDNA from pCMV-Gluc1 to generate the new plasmid, pCIKGluc. A CpG-depleted, codon-optimised version of the Gluc cDNA sequence was designed (soGluc), and after synthesis (GENEART AG, Regensburg, Germany) was also used to replace the Fluc cDNA in pCIKFluc to generate plasmid pCIKsoGluc. Both pCIKGluc and pCIKsoGluc express an identical Gluc protein under the control of the CMV promoter/enhancer, with similar transfection efficiencies in 293T human embryonic kidney cells and ALIs (data not shown). Both plasmids were used in ALI experiments but only pCMV-Gluc was used in in vivo studies. The soGluc cDNA was also sub-cloned into a fourth generation, CpG-free plasmid backbone [32] under the control of the synthetic hCEFI promoter (combining a CMV enhancer with a CpG-free version of the human elongation factor 1a promoter [32]), to generate plasmid pG4-hCEFIsoGluc (DNA manufactured by Cayla-Invo-Gen, Toulouse, France). An “empty” plasmid (pCI) carrying no reporter gene was used as a negative control in some experiments.

2.2. Gene transfer agents

2.2.1. GL67A

The cationic lipid GL67A [33] consists of the cationic lipid GL67 [27] ([3-5-en-3-ol]-3-[3-amino-indolyl]4-[4-(3-amino-indolylamino)butyl carbamato]) (Genzyme Haverhill, UK), DOPE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine) and DMP2-PEG5000 [1,2-Dimyristoyl-sn-Glycerol-3-Phosphoethanolamine-N-[methoxy(polyethylene glycol)5000]] (Avanti, Alabaster, Alabama, USA). To generate GL67A the three lipids are formulated at 1:2:0.05 (GL67:DOPE:DMP2-PEG5000) molar ratios and freeze-dried by OctoPus (Leiden, The Netherlands). Lipid:pDNA complexes for mouse nasal “sniffing” experiments were generated as previously described at 1:4 lipid:pDNA molar ratio [33] containing approximately 0.8 mg pDNA/ml final formulation. Lipid:pDNA complexes for ALI cultures and sheep nebulisation experiments were generated as previously described at 6:8 lipid:pDNA molar ratio [32] containing approximately 2.65 mg pDNA/ml final formulation; this formulation is similar to that used in previous clinical studies [10].

2.2.2. cPEI

cPEI cPEI was prepared as described previously [34]. Briefly, complexes were prepared at a PEI nitrogen to DNA phosphate (N:P) molar ratio of 10:1 by slowly adding equal volumes of appropriately diluted aqueous pDNA to aqueous PEI (4.3 mg/ml, pH 7.4) whilst vortexing, and allowing the mixture to stand at room temperature for 15–20 min to facilitate complex formation. Concentration of PEI:pDNA complexes was performed using a Model 8200 stirred ultrafiltration cell (Millipore, Watford, UK) fitted with a regenerated cellulose filter with a nominal molecular weight limit (NMWL) of 100 kDa and operating under an applied pressure of 10 psi nitrogen gas. Concentration of material was performed using initial formulation prepared at 0.4 mg/ml. A 50 ml volume of 0.4 mg/ml (36 mg) was concentrated until OD260 reading was equivalent to a concentration of 16 mg/ml ± 10%. DNA concentration in the PEI:pDNA complexes was quantified by absorbance measurements at 260 nm, using 1 OD260 = 45 μg/ml for DNA bound to 25 kDa branched PEI [35].

2.2.3. Lipofectamine

Lipofectamine (LF) 2000 (Invitrogen, Paisley, UK), was complexed at a 2.5 μg LF2000: 1 μg pDNA ratio (w:w) according to manufacturer’s recommendation.

2.3. Gene transfer

2.3.1. ALI

Fully differentiated human ALI cultures (MucilAir) were purchased from Epi-thelix SRL (Geneva, Switzerland) and cultured at 37 °C and 5% CO2 in a humidified atmosphere in a standard tissue culture incubator according to manufacturer’s recommendation. The basolateral culture medium (MucilAir culture medium, Epi-thelix) was replaced every 2–3 days. Appropriate quantities of lipid:pDNA complexes were diluted in 100 μl Opti-Mem/ALI. The complexes were removed 6 h after transfection and the basolateral medium was replaced. At indicated time-points after transfection Gluc was collected from the apical surface by adding 100 μl MucilAir medium. After 1 h incubation at 37 °C the ALI cultures were “washed” by gently pipetting the medium up and down five times. Preliminary experiments indicated that this procedure removed most of the Gluc from the apical surface (data not shown). The medium was then completely removed and stored at −80 °C until further analysis. In some experiments basolateral medium was stored at −80 °C to assess Gluc expression. Untransfected ALI cultures were included as negative controls. In preliminary experiments we determined that background luciferase expression was identical in untransfected ALIs and ALIs transfected with pcKEmpty (data not shown).

For quantification of intra-cellular Fluc cell lysates were prepared by adding 100 μl and 500 μl reporter gene lysis buffer (RLB, Roche, Basel, Switzerland) to the apical and basolateral surface of the ALI cultures, respectively. ALI cultures were incubated at room temperature (RT) for 20 min and cells were scraped off the membrane using a pipette tip. Cells were then freeze/thawed three times to ensure complete lysis and spun at 13,000 g, at 4 °C for 10 min. The supernatant was frozen for analysis.

2.3.2. Mouse

All animal studies had been approved by local Animal Ethics Committees at Imperial College and Edinburgh University and were carried out according to
2.3. Sheep

Suffolk Cross ewes, 35–60 kg, were treated with antiemetic (5% Fasine, 1 ml/kg (Novartis Animal Health, Camberley, UK), 1°C ydecin, 0.02 ml/kg (Fort Dodge Animal Health, Southampton, UK)) and underwent a preliminary examination involving peripheral blood sampling and bronchoscopic visualisation and recovery of BALF under gaseous anaesthesia on a positive pressure ventilation system (Model 708, Harvard Apparatus, Holliston, MA), 1–3 weeks prior to treatment, to confirm absence of pre-existing pulmonary disease. Aliquots of serum were stored at −80°C as pre-treatment controls. Pre-treatment BALF was collected by bronchoscopy from the right apical lung segment of some animals by instillation and collection of 2–20 ml of sterile PBS pH 7.8. In addition BALF from uncontrolled animals was collected by instillation and collection of 2–20 ml of sterile PBS pH 7.8. Control BALF at pH 7.4 had slightly higher background light emission than BALF collected with PBS at pH 7.8 (pH 7.4: 1318 ± 8.1, pH 7.4: 1013 ± 2.0 RLU/μl, p < 0.05), but this small difference did not affect interpretation of the data. BALF was filtered through a gauze to remove mucus and large particulates and stored at −80°C until further analysis.

20 ml of pDNA complexes to either GL67A or PEI containing approximately 53 mg and 32 mg of pDNA, respectively were delivered as an aerosol to anaesthetised sheep using PARI LC Plus nebulisers as previously described [34,37]. Twenty-four hours after dose delivery a peripheral blood sample was taken. Sheep were euthanised and lungs removed and dissected free from surrounding tissue. Pulmonary circulation was perfused with 2–3 L of PBS (pH 7.8) to minimise hae-moglobin content of samples. BAL samples were collected by single instillation and recovery of 40 ml of PBS (pH 7.8) (segment). BAL fluid was filtered through a gauze to remove mucus and large particulates, stored on ice and then centrifuged for 20 min at 5000 rpm. Biopsies were taken from airway bifurcations of eight lung segments using Gerritsma nasal biopsy forceps and stored at −80°C (Chillingham et al. 2011). Tissue pieces were obtained by gently pipetting the PBS up and down 3 times. The liquid was collected into a 22G venflon catheter (Abbocath, Sligo, Ireland) into the trachea. 0.5 ml of PBS (pH 7.8, Sigma–Aldrich, Gillingham, UK) was then injected gently with a syringe and withdrawn; the procedure was repeated three times (3 ± 0.5 ml PBS). BALF was then centrifuged at 5000g, for 20 min. The supernatant was collected and stored at −80°C until further analysis. Lungs were harvested and snap-frozen in liquid nitrogen and subsequently homogenised in 300 μl of RLB using a Fast-Prep homogeniser (ThermoFisher Scientific, Waltham, USA) at 4.0 m/s for 45 s. After vortexing and incubation at room temperature for 15 min, the homogenate was transferred to a Qiashredder column (Qiagen, Crawley, UK) and centrifuged at 13 000g (g) for 1 min. Columns were then removed and the homogenate was centrifuged for an additional 5 min. The supernatant was collected and stored at −80°C until further analysis.

2.4. Spiking experiments

To determine the half-life of recombinant Fluc and GLuc proteins in apical fluid collected from ALI cultures 100 μl MucAir culture medium were added to untransfected cultures (n = 6). After 30 min incubation at 37°C the ALI cultures were “washed” with gently pipetting the PBS up and down 3 times. The liquid was collected and pooled. The apical liquid was spiked with known amounts of recombinant Fluc (Promega) and GLuc (Nanolight, Pinetop, AZ, USA) protein and samples were analysed for protein levels in an Applican plate reader (Thermofisher Scientific, Waltham, MA, USA) immediately after spiking (T0) and at indicated time-points (up to 5 days) after storage at −37°C, 5% CO2.

2.5. Assays

2.5.1. Histological processing of ALI cultures

Cells were fixed in osmium tetroxide (OsO4)-based fixative and embedded in araldite as previously described [38]. The araldite blocks were trimmed and semi-thin sections (1 μm) were cut (n = 12 ALIs, n = 6 levels/ALI at approximately 300 μm intervals). The sections were stained with toluidine blue using standard histological techniques. Light microscopic analysis was performed using a Zeiss Axioskop 2 Plus microscope and 100× oil objective (Carl Zeiss Ltd, Welwyn Garden City, UK). Images were taken using Axiosvision software (Axioskop 2 plus).

2.5.2. Electrical assessment of ALI cultures in Ussing chambers

ALI cultures were mounted in an Ussing chamber (Warner Instruments Hamden, CT, USA, dual chamber model UZ5000) according to manufacturer’s instructions (http://www.warnerinstruments.com/upload/10720199F.0707.pdf). The cells were maintained at 37°C and gassed with 95%/5% CO2/O2 in Krebs-Henseleit buffer (NaHCO3 24.8 mM, NaCl 118 mM, KCl 4.8 mM, MgSO4 7H2O 1.8 mM, KH2PO4 0.92 mM, CaCl2 6H2O 2.74 mM). After stabilisation for 15 min the buffer resistance was dialled-out with a DVC–1000 epithelial dual voltage clamp and short circuit current measurements were performed. After determining the baseline current, sodium absorption via epithelial sodium channels (ENaC) was inhibited through addition of 10 μM amiloride (Sigma, Gillingham, UK) to the mucosal surface. Subsequently forskolin (1 μM in ethanol, Sigma, Gillingham, UK) was added to the mucosal and serosal surface to increase intracellular cAMP concentration and activate G-protein dependent chloride secretion. All data was stored graphically via Chart software after analogue to digital conversion (AD Instruments, Lawerenceville, GA, USA).

2.5.3. Firefly luciferase (Fluc)

Fluc bioluminescence was measured using a Firefly Luciferase Assay Kit (Promega, Southampton, UK) according to manufacturer’s recommendation using an Applican plate reader (ThermoFisher Scientific, Waltham, MA, USA). Total protein was determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer’s recommendation and the data were expressed as relative light units (RLU)/mg total protein.

2.5.4. Gaussia luciferase (GLuc)

GLuc assays were performed using a Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, USA) according to manufacturer’s recommendation and an Applican plate reader. For lung tissue homogenates total protein was determined using a Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer’s recommendation and the data was expressed as RLU/mg total protein. For ALI cultures, serum and BALF samples, GLuc expression was expressed as RLU/μl fluid.

2.5.5. Bioluminescent imaging

○ Luciferin (75 mg/kg in 100 μl, Xenogen Corporation, Alameda, CA) or coelenterazine [first dissolved in methanol (4 mg/ml) and then diluted to 0.67 mg/ml in PBS; a 150 μl were delivered per mouse, Nanolight Technology, Pinetop, AZ, USA] was administered topically using our “nasal snif” protocol described above, to mice 10 min before imaging. Bioluminescence (photons s−1 cm−2 sr−1) from living mice was measured using an IVIS50 system (Xenogen Corporation, Alameda, CA, USA) at a binning of 4, over 10 min, 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 lungs photon emission in the lung was measured by marking a standardized area for quantification.

2.5.6. RT-PCR assays

For RNA analyses, cells were scraped into 350 μl RLT/βME and extracted passed through a Qiashredder column. Total RNA was extracted from cleared cell lysates using RNeasy columns (Qiagen) with on-column DNase treatment (Qiagen) and in solution DNase (Ambion DNAfree, according to manufacturers’ instructions. Absolute levels of mRNA were quantified by two-step, real-time quantitative TaqMan RT-PCR using the ABI PRISM 7700 Sequence Detection System and Sequence Detector v1.6.3 software (Applied Biosystems, Warrington, Cheshire, UK), using plasmid or endogenous mRNA-specific primers and fluorogenic probes, on undiluted RNA. Sequences of the forward (F) and reverse (R) probe (P) and reverse transcriptase reaction priming (R2) oligonucleotides used for each mRNA target are listed below. Where no R2 oligonucleotide is indicated, the R primer was utilised in reverse transcriptase reactions. For pcKFLuc (also known as pCILuK), F: 5′-TGG AGC GGT CCG CAG GTC TTT-3′; R: 5′-GTC GTA TTA AGT AAG TCT ACC A-3′; P: 5′-FAM-CCA CTC CCA GGT CTA TTA CAC CAC-3′. R2: 5′-GCC CGT CCT ATG TAC TCT TCA GGT-3′. For endogenous ovine CFTR, F: 5′-GGG AAA GAC GGC CAG TGT C-3′; R: 5′-CCA GCC GGT TGC TGT ATC-3′; P: 5′-FAM-CCA AAC TTT TTT TCA GGT GCA CAC CAA-3′; for endogenous ovine CTR, F: 5′-GCC GAG GGC CAC CAC TCA-3′. A “no template control” and a “no-reverse transcriptase” (RT−) control were included, where total RNA, or MultiScript reverse transcriptase and RNase inhibitor were omitted from the reverse transcriptase reaction, respectively. The number of copies of plasmid-derived mRNA and endogenous mRNA were calculated from standard curves of in vitro transcribed RNA fragments that surrounded the amplified region as previously described [39]. The amount of plasmid...
derived mRNA was expressed as a percentage of the endogenous mRNA detected, such that samples containing equivalent amounts of each mRNA are expressed as 100% vector/endogenous mRNA. Samples for which the RT – reaction was within 3 Ct of the mean RT + Ct value are reported as ‘Not Determined’. Samples with quantifiable vector mRNA, and with endogenous mRNA levels below the lowest standard, are reported as ‘Positive But Not Quantifiable’. If no vector copies are detected and endogenous mRNA is quantifiable, samples are reported as ‘No Vector Detected’, otherwise they are reported as ‘Not Determined’.

2.6. Statistics

To assess normal distribution of the data the Kolmogorov–Smirnov normality test was performed. 2-group comparison of parametric data was performed using independent student t-tests (Figs. 5 and 8A) and paired t-test (Fig. 9), as appropriate. Non-parametric data were analysed using a Kruskal–Wallis test followed by Dunns multiple-comparison post-hoc test (Figs. 2 and 3) or Mann–Whitney (Figs. 1, 6 and 7 and 8B) as appropriate. The Spearman R correlation coefficient was used to analyse non-parametric data. All analysis was performed using GraphPad Prism4 and in all cases the null hypothesis was rejected at $p < 0.05$. 

Fig. 1. Characterisation and transfection of differentiated human air liquid interface cultures. (A) Differentiated air liquid interface (ALI) cultures (MucilAir) were fixed in osmium tetroxide-based fixative and embedded in araldite. Semi-thin sections (1 μm) were cut and stained with toluidine blue. A representative light microscopy image is shown. Original magnification ×100. Examples of ciliated epithelial, mucus and basal cells are indicated. (B) ALIs were mounted in Ussing chambers and short circuit current measurements were performed. Baseline current (BL), the response to perfusion with amiloride and forskolin are indicated. A representative trace of $n = 8$ independent ALIs is shown. ALIs were transfected with pCIKFLuc (pFLuc) (C) or pCIKGLuc (pGLuc) (D) complexed to GL67A or remained untransfected (negative controls) and Firefly luciferase (FLuc) or Gaussia luciferase (GLuc) were measured, respectively 48 h after transfection. Each symbol represents one ALI ($n = 6–12$)/group. Horizontal bars indicate group medians. *** $p < 0.001$ and * $p < 0.05$ when compared to control. (E) Quantification of vector-specific mRNA in the same ALIs 48 h after transfection. Each symbol represents one ALI ($n = 6$)/group. Horizontal bars indicate group medians. NVD = no vector-specific mRNA was detected, PBNQ = low levels of vector-specific mRNA were detectable, but not quantifiable, being outside the linear range of the standard curve. Data are expressed as % vector-specific mRNA ratioed to endogenous human CFTR mRNA as a denominator.
3. Results

3.1. Histological and electrical assessment of ALI cultures

To confirm that commercially available MucilAir human ALIs were of suitable quality to be used as a model for airway gene transfer we assessed the cultures histologically and measured their ion transport properties in Ussing chambers. Fully differentiated ALIs exhibited a pseudostratified epithelium of ciliated, mucus and basal cells that is indistinguishable from native human airways (Fig. 1A, image representative of $n = 12$ ALIs). Further, the ALIs expressed endogenous human CFTR mRNA ($123 \pm 25$ copies/ng total RNA, $n = 16$, two ALI batches assessed in independent experiments). Finally, their ion transport properties were similar to native human airways, with cultures responding to amiloride (reduction in potential difference (PD) due to inhibition of amiloride-sensitive sodium channels) and to perfusion with a low chloride solution containing forskolin which activates the cAMP-dependent CFTR chloride channel leading to hyperpolarisation across airway epithelium (Fig. 1B, trace representative of $n = 8$ ALIs).

3.2. Non-viral gene transfer into ALI cultures

Historically, others and we have struggled to detect robust levels of Fluc expression after non-viral gene transfer in ALIs. Transfection of ALIs with pCIKGLuc complexed to GL67A ($0.1, 1$ and $10 \mu g$ DNA/ALI) resulted only in a 1.6-fold increased in Fluc expression compared to controls ($n = 12$ ALIs/group, 2 independent experiments, $p < 0.05$) when assayed 48 h post-transfection (Fig. 1A). Transfection with pCIKGLuc complexed to GL67A ($0.1, 1$ and $10 \mu g$ DNA/ALI) resulted in a 10-fold increase in Gluc expression compared to controls ($n = 6$–12/group, two independent experiments, $p < 0.001$) when performed side-by-side with the same batch of ALIs (Fig. 1D). Quantification of vector-specific mRNA 48 h after transfection, showed that similar levels of pCIKFluc and pCIKGLuc mRNA were produced (Fig. 1E, $n = 6$), implying that the difference was related to the sensitivity of the assay as opposed differences in transfection efficiency. To address this point further...
we spiked apical ALI fluid with FLuc or GLuc protein and assessed decay over time. After a 2 h incubation only 5% of the FLuc activity was still detectable, whereas GLuc activity was still evident with 96% and 46% of protein activity remaining after 2 and 120 h, respectively. The increased stability of the GLuc protein likely contributed to the enhanced sensitivity of this reporter and, importantly, confirmed that the cationic lipid GL67A, which is currently used in CF clinical trials, transfects differentiated human airway epithelial cells. GLuc activity was also detectable in the basolateral compartment after non-viral gene transfer and there was a significant correlation \( r^2 = 0.7729, p < 0.0001, n = 82 \) samples) between gene expression in the apical and basolateral compartments. For this reason, only GLuc levels in the apical compartments were quantified for subsequent experiments.

### 3.3. Variable gene transfer into ALI cultures

During the course of our studies we noticed significant variability in transfection efficiency when performing independent experiments. We hypothesised that the age of the ALIs may contribute to the observed variability. Fig. 2A and B shows transfection efficiency of GL67A and LF2000 as a function of ALI age. These data imply that older ALIs are more refractory to transfection. To address this point further we compared the transfection activity of GL67A:pCMV-GLuc complexes in younger (70 days post-seeding) and older cultures (> 120 days, \( n = 4 \)) (Fig. 2C) and showed that GLuc levels were higher in younger than older cultures (\( p < 0.01 \)). Subsequent experiments, therefore, were performed on cultures that were 40–70 days post-seeding.

### 3.4. GLuc protein detection and RT-PCR

Identifying the most sensitive assay to measure the transfection activity of non-viral gene transfer vectors in ALI cultures is important. Real-time quantitative RT-PCR is generally regarded as a very sensitive assay for the detection of vector-specific mRNA. Here, we compared the relative sensitivities of assays to measure GLuc protein and mRNA levels. ALI cultures were transfected with varying amounts of pCIKsoGluc complexed to GL67A (0.1, 1 and 10 \( \mu \)g DNA/ALI) and GLuc and mRNA levels were quantified in the same ALI cultures. As shown in Fig. 3A and B robust levels of GLuc protein (\( p < 0.005, n = 6/group \)) and mRNA (\( p < 0.01, n = 6/group \)) could be detected in ALI cultures transfected with 10 \( \mu \)g DNA but not in ALIs transfected with lower amounts of plasmid DNA. We, therefore, concluded that the sensitivity of assays to detect GLuc protein and mRNA levels are similar, but importantly GLuc can be repeatedly quantified in the same ALIs whereas mRNA quantification is a terminal endpoint.

### 3.5. Assessment of promoter activity in human ALI cultures

We recently developed a synthetic, hybrid promoter (hCEFI), combining a CMV enhancer with a Cpg-free version of the human elongation factor 1\( \alpha \) promoter (hCEFI) which shows persistent expression in mouse lung [32] and is currently being used in our CF gene therapy trial. The ability to measure GLuc in transfected human ALI cultures provides us with a tool to assess the activity of this promoter in a human airway model. Fig. 4 shows that the hCEFI promoter in plasmid pG4-hCEFIsoGLuc can support persistent expression for at least 1 month (\( p < 0.05 \) when compared to negative control or pCIKsoLuc, \( n = 6/group \)), whereas the CMV promoter, consistent with published in vivo data, is shorter-acting with high levels of expression detected within 48 h of transfection followed by a rapid decline to baseline levels by day 8 after transfection.

### 3.6. GLuc expression in mouse lung tissue, BALF and serum

We have successfully used FLuc for many years to quantify GL67A-mediated gene transfer in the murine lungs in vivo. However, in our in vivo sheep model FLuc expression was not detectable (data not shown), despite robust levels of mRNA expression (McLachlan, manuscript submitted). Given the improved sensitivity of GLuc in ALIs we wished to assess this reporter in the sheep lung. However, before embarking on expensive large animal studies, we first assessed if GLuc was a robust reporter of non-viral gene transfer in mice.

pCMV-GLuc/GL67A was administered to mice by nasal snifing and GLuc expression was quantified in lung homogenate, BALF and serum 24 h after transfection. Robust levels of GLuc expression (\( p < 0.01 \) compared to negative controls, \( n = 5–6/group \)) were detectable in lung homogenate, BALF and serum (Fig. 5A–C), indicating that GLuc is a suitable reporter for the assessment of lung gene transfer. These results were reproduced in an independent experiment and although absolute levels of gene expression were lower (data not shown) there was good correlation between gene expression in lung homogenate and BALF (\( r^2 = 0.84, p < 0.005 \)).

We previously showed that non-viral mediated expression of FLuc expression in the lung can be detected using bioluminescence in vivo imaging (BLI) [40]. We, therefore, also assessed if BLI is suitable for detecting GLuc expression. Fig. 6A–C shows that although GLuc can be detected in the lung, administration of the substrate coelenterazine to the airways leads to significant levels of background light emission in untreated control animal, which was seen after luciferin administration [40,43]. Thus, whilst GLuc is a sensitive reporter gene for non-viral lung transfection in mice, we caution against the use of GLuc for in vivo BLI after topical administration of its substrate coelenterazine.

### 3.7. GLuc protein expression in sheep lung

Sheep were nebulised with pCIKGLuc complexed to GL67A and GLuc expression quantified in lung tissue 24 h after transfection. Significant (\( p < 0.005 \)) levels of GLuc were readily detectable in lung [11-fold over untransfected controls (\( n = 6/group \))] (Fig. 7A) indicating successful GL67A-mediated protein expression in a large animal.
Detection of GLuc in lung tissue homogenate is a post-mortem procedure that does not easily lend itself to monitoring duration of gene expression over time. We, therefore, also assessed GLuc expression in BALF, bronchial biopsies (BBx) and bronchial brushings (BBr) that can be repeatedly collected from anaesthetised sheep. Significant \((p < 0.0001)\) levels of GLuc were detectable in BALF [9-fold over untransfected controls \((n = 6/\text{group})\)] (Fig. 7B) but not in BBx and BBr. In addition, there was a significant correlation between GLuc levels in tissue homogenate and BALF (Spearman \(r = 0.62, p < 0.01\)). Thus, analysis of BALF may allow for monitoring of gene expression in the lung over time.

3.8. GLuc expression in sheep serum

Although BALF can be collected repeatedly in sheep to monitor the duration of gene expression, the procedure requires the sheep to be anaesthetised. In contrast collection of blood can be undertaken without anaesthesia; we, therefore, also assessed if GLuc can be detected in serum. However, we were unable to detect significant increases in GLuc expression 24 h after GL67A/pCIKGLuc transfection \((n = 9)\). Since our mouse experiments indicated that detection of GLuc in serum was feasible if gene transfer in the lung is sufficiently high, we delivered pCIKGLuc in a concentrated PEI (cPEI) formulation which we previously showed is capable of mediating robust levels of Firefly luciferase expression in the sheep lung [41]. cPEI-mediated gene transfer increased GLuc expression significantly by approximately 2–3-fold compared to GL67A-mediated expression (Fig. 8A and B), which led to a small but significant \((p < 0.05)\) increase in serum GLuc levels (Fig. 8C).

3.9. GLuc expression profile in sheep lung

Although GLuc expression in BALF may be suitable to determine duration of gene expression after non-viral gene transfer in sheep, we were concerned that the expression profile may be affected by the enhanced stability of GLuc that we and others observed in ex vivo models (see above). To address this point we collected BALF 1, 4, 7, 14 and 21 days following aerosol delivery to sheep with GL67A/pCIKGLuc \((n = 3)\). The expression profile mimicked the predicted activity of the short-acting CMV promoter, with high levels of GLuc expression 24 h after transfection returning to baseline levels by day 4 (Fig. 9). Thus, GLuc is a suitable reporter gene to follow expression in the sheep lung. In addition, this experiment showed that GL67A-mediated GLuc expression levels 24 h after gene transfer to the ovine lung was reproducible in independent experiments (compare levels in Figs. 8A and 9), further supporting GL67A as a robust transfection agent in large animals.

4. Discussion

Gene transfer agents are frequently assessed in ‘easy-to-transfect’ cell lines, potentially leading to over-expectations as to their performance in an in vivo setting. It seems more logical to test these agents in model systems that more closely mimic the clinical setting for which they are often intended for. Here we generated data that suggests human ALIs may provide a more appropriate in vitro model, more akin to the situation in vivo. We provide evidence to suggest that the difficulty of previous efforts to score transfection activity of non-viral vectors was likely related to the sensitivity of the reporter gene. Gaussia-derived luciferase provides significantly increased sensitivity via reduced protein breakdown as well as higher quantum yield and being secreted allows for

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**Fig. 5.** GLuc expression after non-viral gene transfer to mouse lungs. pCMV-GLuc/GL67A complexes were administered to mice by nasal sniffing and GLuc expression was quantified in lung homogenate (A), broncho-alveolar lavage fluid (BALF) (B), and serum (C) 24 h after transfection (n = 5–6/group). Negative control mice remained untransfected. Each symbol represents an individual animal. The horizontal bar indicates group medians. \(* * = p < 0.01\) compared to negative control.
assessment of gene transfer at multiple time-points in the same tissue or animal. We suggest this reporter gene deserves consideration as the standard for gene transfer studies to the respiratory epithelium.

Assessment of the histological and ion transport properties of the commercially available ALI cultures showed they closely resembled normal human airway epithelium. The ALI cultures consisted of a pseudostratified epithelium composed of ciliated, mucus and basal cells similar to reported by Wiszniewski et al.[13]. In addition the cultures were electrically tight and displayed ion transport properties characteristic of non-CF airway epithelium [13], including forskolin-mediated chloride secretion via cAMP-dependent chloride channels. MucilAir ALI cultures were, therefore, considered a suitable model for human airways. Significant inter-experimental variability in gene expression remains a problem for transfection of the MucilAir human ALI cultures. The cause is not completely understood, but our data indicate that the age of cultures, rather than donor variability, may in part determine transfection efficiency. We hypothesise that the generally lower transfection efficiency observed in older cultures, may relate to an increase in the volume of the mucus barrier, which accumulates over time. We now perform all our experiments in 40–70 day old cultures. Cultures younger than 40 days may not always be fully differentiated (Epithelix, personal communication, March 2008) and are, therefore, less useful.

Both the pCIKGLuc and pCIKFLuc expression plasmids carry a CMV promoter/enhancer and are, except for the reporter gene, identical. Thus, it is unsurprising that vector-specific mRNA levels after GL67A-mediated gene transfer were similar. Despite this finding, FLuc protein expression was barely detectable whereas secreted GLuc on the apical surface of ALIs was approximately 10-fold greater than in controls. We speculated that the increased signal-to-noise ratio of GLuc might be due to the increased stability of the protein in the context of airway surface liquid. Although apical liquid from ALIs was collected by washing the surface of the cultures with medium, which led to dilution of the airway surface liquid and proteases therein, the data showed that FLuc activity, consistent with previous reports, decreased rapidly[42]. In contrast the GLuc activity, consistent with published stability data (6 day half-life at 4 °C [24]) was maintained for prolonged periods of time (46% after 120 h at 37 °C), likely leading to accumulation of the protein over time. In addition to the prolonged half-life, the higher quantum yield, defined as the probability of photon emission per substrate molecule, of GLuc compared to FLuc contributes to the

Fig. 6. In vivo imaging of GLuc expression. Mice were transfected with pCMV-GLuc complexed to GL67A by nasal sniffing (80 μg DNA in 100 μl). Negative control mice remained untransfected. 24 h after transfection coelenterazine was administrated by nasal sniffing and photon emission captured in a bioluminescence imager. (A) Mice transfected with pCMV-GLuc (GLuc), (B) Negative controls, (C) Quantification of photon emission in lung. Representative images of n = 5–6 mice/group are shown. Each symbol represents an individual animal. The horizontal bar indicates group medians. *** = p < 0.005 compared to negative control.
increased sensitivity of this assay. Consistent with our data, Tannous et al. have previously shown that GLuc plasmids produces more than 1000-fold higher bioluminescence compared to FLuc plasmids after non-viral transfection of 293T cells [23].

In addition to the apical surface GLuc was also detectable in the basolateral medium and the relative concentrations in the two compartments correlated well. It is unclear if the comparatively small protein (19.9 kDa) was secreted via the basolateral membrane or moved from the apical surface to the basolateral side via paracellular routes. Importantly, sampling the basolateral medium of ALI cultures avoids the need for "washing" and, therefore, perturbing the apical surface and may be more suitable to follow gene expression in ALIs over time.

Despite its prolonged half-life, GLuc is suitable to assess promoter activity in human ALIs over time. "Washing" of the apical surface with medium removes most of the accumulated GLuc and, thereby, allows accurate quantification of newly produced GLuc at later time-points. In addition quantification of GLuc in the basolateral medium, which can be completely removed and changed, is a suitable way to follow gene expression over time. A comparison of CMV- and hCEFI-mediated GLuc expression not only showed that the hybrid hCEFI promoter/enhancer supports long lasting expression in a human airway model compared to the CMV promoter/enhancer, but also demonstrated that human ALIs mimic the in vivo performance of these regulatory elements in mouse models [32]. Studies in human ALIs may, therefore, be useful to assess promoter function and duration of expression in the context of human cells.

GL67A-mediated CFTR gene transfer into lungs of CF patients has been shown to partially correct the CF chloride transport defect [10]. In addition, we can detect robust levels of vector-specific mRNA (copy number equivalent to approximately 50% of ovine CFTR mRNA, median from n = 8 sheep, manuscript submitted) in lung tissue. However, we have not been able to detect expression of FLuc at these levels of gene transfer (data not available).
Imaging (BLI) and have also shown that photon emission is gene transfer to the lung can be detected using bioluminescence means exist. We have previously shown that FLuc expression after non-viral gene transfer in the sheep lung. However, before embarking on studies in large animals models we assessed if GLuc is a suitable reporter gene for non-viral lung transfections in mice. Encouragingly we were able to detect robust levels of GLuc not only in lung homogenate, but also in BALF and serum of treated mice. In pre-clinical and clinical CF gene therapy studies we, and others, often rely on detection of vector-specific mRNA by quantitative RT-PCR, generally regarded as a very sensitive assay, to provide evidence of successful gene transfer. Interestingly, the relative sensitivity of GLuc and QRT-PCR were comparable. In other studies, we are beginning to accumulate data suggesting that CFTR protein function may be a more sensitive assay than RT-PCR mRNA detection (manuscript in preparation). Thus, we question whether the latter should be considered the gold standard for molecular detection of gene transfer, where alternative means exist.

We have previously shown that FLuc expression after non-viral gene transfer to the lung can be detected using bioluminescence in vivo imaging (BLI) and have also shown that photon emission is enhanced if the substrate luciferin is delivered topically by nasal instillation rather than intraperitoneal administration, likely due to higher substrate concentrations reaching the lung. Here, we assessed GLuc expression combined with topical administration of coelenterazine for BLI in the lung and showed that although GLuc expression can be detected in the lung, topical administration of the substrate leads to significant background photon emission in untransfected mice. Background photon emission in vitro has previously been reported for coelenterazine and has been attributed to auto-oxidation of the substrate, but interestingly did not appear to interfere with GLuc detection in various organs after systemic administration of the substrate. It is likely that the high local concentration of coelenterazine in the airways achieved after topical administration leads to a particular enhancement of the background signal and may be reduced if the substrate is administered systemically. In addition, various coelenterazine analogues are being developed which may be less prone to background photon emission. In summary, GLuc is a suitable reporter for non-viral gene transfer to the mouse lung, and detection of GLuc in the blood may allow us to follow gene expression over time.

Confirmation of GLuc expression in the mouse lung enabled us to assess this reporter in sheep. Importantly, and in contrast to our experience with FLuc, robust levels of GLuc were detectable in lung tissue and BALF, showing, for the first time, that significant and reproducible levels of recombinant protein can be produced after aerosolisation of GL67A/pDNA complexes to the ovine lung in all treated animals. Detection of GLuc expression in BALF allowed us to monitor gene expression in the same sheep over time. Despite the prolonged half-life of GLuc in vitro the gene expression profile of pCMV-GLuc was transient (highest levels at day 1 after transfection, returning to baseline by day 4 after transfection), which follows the expected profile for the shorter-acting CMV promoter/enhancer. This expression profile is consistent with a comparatively short half-life of GLuc in vivo (approximately 20 min). The protein is rapidly removed from the systemic circulation by excretion via the kidneys, but it is less clear how it is cleared from the lung.

Although repeated collection of BALF is feasible the procedure requires sheep to be anaesthetised, whereas blood can be easily and frequently collected without the need for general anaesthesia. Although GL67A-mediated GLuc expression was not detectable in blood, transfection with concentrated PEI (cPEI) led to approximately 3-fold higher expression in the lung and allowed detection of GLuc in serum. We are currently developing a lentiviral vector for CF gene therapy, which is more efficient than non-viral gene transfer in the airway. We predict that sampling of GLuc in serum may allow us to easily monitor persistence of lentivirus-mediated gene expression in the airways.

We also attempted to quantify GLuc in exhaled breath condensate (EBC), but were unable to detect significant increases after non-viral gene transfer. However, considering the small size of the protein (19.9 kDa) detection of GLuc in EBC may be feasible when more efficient GTAs are used.

5. Conclusions

We have shown here that GLuc is a sensitive reporter gene and is particularly useful for monitoring gene transfer in difficult to transf ect models of the airway and lung. Specifically, we show that (1) GLuc is a more sensitive reporter gene and offers significant advantages over the traditionally used FLuc in pre-clinical models for lung gene transfer that are difficult to transf ect, (2) GL67A-mediated gene transfection leads to significant production of recombinant protein in these models, (3) promoter activity in ALI cultures mimics published in vivo data and these cultures may, therefore, be suitable to characterise promoter activity in a human ex vivo airway model and (4) detection of GLuc in large animal broncho-alveolar lavage fluid and serum facilitates assessment of duration of gene expression after gene transfer to the lungs. Importantly, this study has allowed us to validate that GL67A, which is currently in clinical use, can generate significant amounts of recombinant protein in fully differentiated human air liquid interface cultures and the ovine lung in vivo.

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Appendix

Figure with essential colour discrimination. Figs. 1 and 6 of this article may be difficult to interpret in black and white. The full colour image can be found in the online version at doi:10.1016/j.biomaterials.2010.12.001.

References