Preparation for a first-in-man lentivirus trial in cystic fibrosis patients

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Materials and Methods

Generation of pharmacopoeia-compliant producer plasmid

Five producer plasmids were required to generate recombinant SIV vector. The sequences were as described(1), except that the ampicillin antibiotic-resistance gene was replaced with the CpG-free kanamycin antibiotic resistance gene from plasmid pGM169(2). For preparation of lentiviral vectors pseudotyped with Vesicular Stomatitis Virus G glycoprotein (VSV-G), the two plasmids expressing the F and HN proteins were replaced with one plasmid expressing VSV-G; full details of the construction and sequences of the pharmacopoeia-compliant producer plasmids will be published elsewhere (Virus Production paper; In Preparation).

In brief, multiple vector genome plasmids were constructed containing a variety of transgenes and transcription elements. DNA fragments encoding reporter transgenes such as Luciferase (lux), secreted Gaussia luciferase (GLux), Enhanced Green Fluorescent Protein (EGFP), and a fusion of EGFP and Luciferase (EGFPLux) were inserted into unique NheI and ApaI restriction sites in the vector genome plasmid. In addition, a vector genome plasmid expressing Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein was constructed by insertion of the CpG-free NheI-ApaI DNA fragment encoding codon-optimised soCFTR2 from plasmid pGM169(2).

Promoter/enhancer sequences, including CMV(3), hCEF(2) and EF1α(3), were incorporated into the vector genome plasmid via BglII and NheI restriction sites, following removal of the intron, or synthetic intronic sequence. To generate integrase-defective (ID) vectors, a D64V point mutation in the integrase gene(4) was incorporated into the gag/pol packaging plasmid.

Vector production and titration

Production of recombinant SIV vector expressing a variety of transgenes was performed using the five-plasmid transient transfection method(1), except that 25K branched Polyethylenimine (PEI)(5) was used for transfection of producer plasmids into HEK293T producer cells grown in suspension culture in Freestyle media (Life Technologies, Paisley, UK). Virus supernatant was harvested at 72 hours post-transfection and purified essentially as described by Merten *et al*(6) except that Mustang QXT Anion Exchange membranes (Pall, Life Sciences, Portsmouth, UK) were used instead of DEAE columns (Virus Production paper; In Preparation). The virus was formulated in Freestyle medium aliquoted and stored at -80^0C.

The viral particle titre (VP/ml) was determined essentially as described by Mitomo *et al*(1), using Real-Time Quantitative PCR (Q-PCR) with primers spanning the WPRE sequence (Forward: TGGCGTGGTGTGCACTGT; Reverse: CCCGGAAAGGAGCTGACA; Probe: 6FAM-TTGCTGACGCAACCCCCACTGG-TAMRA). Virus RNA was prepared using QIAamp Viral RNA kit including carrier RNA (QIAGEN, Crawley, UK), followed by in-solution DNAse (Ambion, DNAfree) and quantified by one-step RT-qPCR using QuantiTect (QIAGEN) against a standard curve of RNA mimics containing the WPRE sequence (Virus Production paper; In preparation). This assay was also used to measure vector-specific RNA expression following transduction.

Functional titre, reported as transducing units per ml (TU/ml) was calculated following transduction of HEK293F cells with serial dilutions of viral vector and extraction of DNA using QIAamp blood DNA kit (QIAGEN) (Virus Production paper; In preparation). Viral DNA genomes were quantified by Q-PCR (same WPRE primers as above) against a standard curve of plasmid DNA containing the WPRE sequence, using TaqMan Universal Mastermix (Life Technologies), then normalised to total ng DNA using PicoGreen (LifeTechnologies) or Nanodrop ND2000 (Thermo Scientific, MA, USA). Titre was calculated from the slope of the

best-fit line on a plot of WPRE copies per well of cells against volume of virus per well of cells. This assay was also used to calculate Vector Copy Number (VCN) in transduced cells and tissues.

Vector Transduction:

All animal procedures were performed in accordance with the conditions and limitation of the UK Home Office Project and Personal licence regulations under the Animal Scientific Procedure Act (1986). Female C57BL/6N mice (6–8 wk old, Charles River Laboratories, UK) were used for most experiments. Male and female gut-corrected CF knockout mice (~6-12 weeks)(7) were used for assessment of CFTR expression and function. Mouse nose and lungs and air liquid interface (ALI) cultures were transduced with the vector as previously described(1) (see RESULTS and FIGURES for details about vector titers used) and transduction efficiency was quantified using bioluminescent imaging (BLI, IVIS, PerkinElmer, USA), luciferase expression in tissue homogenates as previously described(1) and immunohistochemistry (see below).

Integration site profiling

Genomic DNA was extracted (AllPrep, Life Technologies, Glasgow, UK) from transduced human air liquid interface (hALI) cultures and mouse nose and lung tissue samples. Viral copy number (VCN) was determined as described above. LAM-PCR was carried out according to Schmidt *et al*(8) with minor modifications. Two cocktails of enzymes, GC (HinPII, HpyCH4IV, HpaII, TaqI: NEB, UK) and AT (MseI, BfaI, CviQI: NEB, UK) were used to maximise flanking gDNA amplification. 25-150 ng of template gDNA was used for each LAM-PCR procedure. To increase the probability of IS retrieval, three separate linear amplification reactions of 300 cycles were pooled and then divided into three identical

triplicate aliquots. Products were size-fractionated after the nested PCR stage by using low melting point agarose gel extraction to remove DNA<100 bp. The length profile and band diversity of amplicons were visually assessed using a bioanalyser (Agilent Technologies, Stockport, UK) before proceeding. Purified PCR products were then prepared for Ion Torrent sequencing as recommended by the manufacturer (Life Technologies, Glasgow, UK) and sequenced. Sequencing reads were processed bioinformatically using a pipeline of custom Perl scripts to validate the input and remove superfluous sequences of the primers, linkers, remaining 32 bp of LTR and other vector sequences. The pipeline allows two levels of quality control based on degree of homology to the reference genomes termed *high* (≥ 90% match with no gaps) and *medium* (≥ 75% match with up to one gap occurring) stringency. The processed reads were compared to the reference mouse (GRCm38/mm10) or human (GRCh37/hg19) genome sequences using BLAT(9). IS in repeat sequence elements were discarded and only those IS with three or more reads of ≥ 35 bp, or two or more reads of ≥ 50 bp per BLAT hit were further analysed. For each IS, distance to transcription start sites (TSS) and transcription site residency were determined using GREAT(10), QuickMap(11) and the UCSC and ENSEMBL genome browsers.

Assessment of toxicity

Mice were transduced at monthly intervals by nasal instillation with one to four doses (1E8 TU/dose) of rSIV-F/HN-CMV carrying Lux or EGFP reporter gene. Controls included untreated and D-PBS treated mice, as well as mice treated with conventional (CpG containing) luciferase plasmid DNA/GL67A complexes or CpG-free CFTR plasmid pGM169/GL67A complexes which were used in our recently completed Phase IIb multi-dose trial(12). The non-viral formulation was prepared as previously described(13). All mice were culled 24 hr after the last dose. Lung tissue sections were stained with haematoxylin&eosin

and were scored semi-quantitatively. Scoring: 0=no inflammation; >0-0.5: =very few/few foci of inflammation in peribronchial or perivascular walls; 1: =patchy cell infiltrates in bronchial or vascular wall in <30% of the section; 2: = localised cell infiltrates in up to 60% of the section; 3: =cell infiltrates in >60% of the section.

Immunohistochemistry

Lungs were processed, cut and de-paraffinised using standard histological procedures. For antigen retrieval slides were treated with pre-heated 0.1M EDTA for 10 min at 100°C. Details of all antibodies and dilutions used are presented in Table E2. Primary and secondary antibodies were incubated at room temperature for 1hr, 0.1% Triton X-100 in 0.1M PBS was used for all dilutions and washing steps. After the final washes sections were mounted with ProLong Gold antifade medium with DAPI (Molecular Probes, Molecular Probes, Life technology, Eugene, Or, USA). Images were generated using a Zeiss LSM-510 inverted confocal microscope (Zeiss, Jena, Germany) using a 40x or 63x oil objective (1.4 NA). The AlexaFluor 594 was excited with the HeNe543 laser. The emission signal was filtered by a 595 nm long pass filter. AlexaFluor 488 was excited with 488 nm and detected with a 505-550nm band pass filter. DAPI was excited with a 405 nm laser and the detection range was 420-490 nm. EGFP expressing airway epithelial cells were quantified using a 63x objective and an Axioskop II fluorescent microscope (Zeiss, Germany) to allow a comparatively large number of airways and cells to be quantified. Twenty random airways and approximately 5000 airway cells were assessed per mouse. Airways were selected using DAPI staining rather than antibody staining to avoid bias for highly transduced regions, but quantification was performed visualising EGFP expression. At a 63x magnification individual cells were clearly visible.

Acquired and Pre-existing immunity

a. Neutralising antibodies in mouse serum

Serum of untreated mice and mice transduced with rSIV.F/HN-Lux (1E8 TU/mouse) was collected 28 days vector administration (n=5-6/group). Sample from each group were pooled and an *in vitro* transduction inhibition assay performed as described below.

b. Anti-Human Parainfluenza virus I immunity in mice (passive immunisation)

rSIV.F/HN-CMV-EGFP was first incubated with 1:2 to 1:32 serial dilutions (duplicate samples for each dilution) of purified anti-hPIV1-3 IgG and IgA antibodies (Abcam, Cambridge, UK) and an *in vitro* transduction inhibition assay was performed as described below.

Mice (n=5/group) received human immunoglobulins (Gamunex® 10% IVIg, (Grifols International, S.A., Barcelona, Spain) by intraperitoneal injection (400 µl, 40 mg IVIG) or intranasal instillation (100 µl, 10 mg IVIG) or remained untreated. 24 hr after passive immunisation human IgG levels were quantified in mouse serum using an anti-hPIV1 IgG Elisa kit according to the manufacturer's protocol (Abcam) to confirm that antibody titres are in a range relevant to what is detectable in human serum (see below). Separate cohorts of mice (n=6/group) were treated with IVIg as described above and transduced with rSIV.F/HN-hCEF-EGFPLux (1E8 TU/mice in 100 µl per/animal) by nasal instillation 24 hr after passive immunisation. Control animals received either vector but no IVIg or IVIg but no vector. Luciferase expression in nose and lung was quantified by using BLI 7 and 28 days after transduction. The mice were then culled and luciferase expression was also quantified in nose and lung tissue homogenate. In addition serum was collected to quantify residual levels of human IgG 29 days post IVIg injection. To assess vector toxicity in mice with pre-existing immunity to hPIV1 we also monitored gross behaviour, body temperature and

weight in all groups in the acute phase after vector administration (until day 5) and bodyweight at the end of the experiment (day 28).

c. Anti-Sendai virus immunity in mice

Mice were transduced with two doses (monthly interval) of transmission-incompetent F protein deleted Sendai virus (ΔF/SeV, 1E6 or 17 infectious units (IU) in 100 μl per mouse, n=8/group) by nasal instillation. The SeV virus did not carry a reporter gene (ΔF/SeV-empty) and was produced by DNAVEC Corporation, Tsukuba, Japan as previously described (14). Control animals remained untransduced. Prior to transduction with rSIV.F/HN-hCEF-EGFPLux (1E8 TU/mice in 100 μl per/animal) 1 month after the second SeV transduction we confirmed that SeV transduced mice had generated anti-SeV IgG antibodies in serum and BALF using an anti-SeV IgG Elisa kit according to the manufacturer's protocol (Alpha Diagnostic International Inc., distributed by Source Bioscience Life Sciences, Nottingham, UK). Before nasal instillation the lentivirus was nebulised through the eFlow® mesh nebuliser (Pari Medical Ltd., West Byfleet, UK) and the aerosol was collected to mimic clinical trial conditions as closely as possible. Control animals remained either (i) untransduced, (ii) received two doses of ΔF/SeV-empty and (iii) no lentivirus or (iv) received lentivirus only.

To assess vector toxicity in mice with pre-existing immunity to SeV animals were monitored daily and assessed using a semi-quantitative gross morphological scoring system monitoring activity, general appearance, posture, hydration and respiration over a 3 month period. Food and water consumption, body temperature and body weight were also monitored.

d. Pre-existing anti-Human Parainfluenza I immunity in serum and broncho-alveolar lavage fluid (BALF)

Serum samples were obtained from the Respiratory Biomedical Research Unit (BRU) biobank using the appropriate biobank ethical approval and consent processes. Adult BALF was obtained as previously described(15). Briefly, all samples were collected from subjects undergoing clinically indicated bronchoscopy. A total volume of 240 ml of warmed saline was instilled into a segment of the right middle lobe and fluid retrieved by gentle manual aspiration. Written informed consent was obtained from all subjects and the study was approved by the Local Research Ethics Committee (Ref 10/H0720/12). Parents of children undergoing a clinically indicated bronchoscopy and BAL (as previously described(16) provided informed consent for an aliquot of BALF to be used for research purposes (Ref 10/H0504/9). Post collection aliquots of unfiltered and unprocessed BALF was immediately placed on ice then stored at -80ºC.

An *in vitro* transduction inhibition assay was performed as previously published (17). Briefly, HEK 293T cells were seeded into 24-well plates (4E5 cells/well) and incubated overnight at 37ºC in 5% CO_2. Heat-inactivated serum (30 min at 56ºC) and BALF were serially diluted with D-MEM (Life Technologies, UK) in a total volume of 100 µl and incubated with 100 µl rSIV.F/HN-CMV-EGFP or rSIV.VSV-G-CMV-EGFP for 1 hour at 37ºC in 5% CO_2. The samples were then added to the HEK-293T cells (n=3 wells/sample) and incubated overnight after which 200 µl D-MEM containing 20% FBS (Sigma –Aldrich, UK) were added and incubated for a further 24 hrs. Cells were then trypsinised, resuspended in D-PBS+1% BSA and the % of EGFP positive cells was calculated using a BDTM LRS II flow cytometer (BD, Biosciences, Canada). A minimum of 10,000 cells per well were counted. Controls included untransduced HEK 293T cells (negative control) and cells transduced with virus not incubated with clinical samples (serum free and BALF free positive controls). All data were expressed as a % of serum and BALF free positive controls, as appropriate. The neutralising

antibody titre was defined as the lowest sample dilution where transduction efficiency reached at least 50% of the positive control sample.

Assessment of CFTR function

a. Iodide efflux measurements were carried out as previously described(1) except that A549 cells (adenocarcinoma human alveolar basal epithelial cells) were used instead of HEK293T cells, because preliminary experiments showed that the hCEF promoter leads to higher levels of gene expression in A549 cells (data not shown).

b. CF Organoids

The Ethics Committee of the Erasmus Medical Centre Rotterdam approved this study and informed consent was obtained. Organoids were generated from rectal biopsies after intestinal current measurements for standard care (subject with CF) or for diagnostic purpose (healthy control) and cultured as described previously(18;19). For viral transduction, organoids (passage 30–40) from a 7-day old culture were trypsinized (TrypLE, Gibco) for 5 min at 37 °C and seeded in 96-well culture plates (Nunc) in 4 μl matrigel (Corning) and virus (1:1 v/v virus:matrigel) containing 100-200 single cells and small organoid fragments as described previously(20). These cells were incubated at 37 °C 10 min and immersed in 150 μl medium. The medium was refreshed (250 μl) 2 days after viral transduction. Four days after viral transduction, organoids were incubated for 30 min with 3 μM calcein-green (Invitrogen), stimulated with forskolin (5 μM) and analyzed by confocal live cell microscopy at 37 °C for 120 min (LSM710, Zeiss). The total organoid area (xy plane) increase relative to t = 0 of forskolin treatment per well was quantified using Volocity imaging software (Improvision). Cell debris and unviable structures were manually excluded from image analysis based on criteria described in detail in a standard operating procedure. The area

under the curve (AUC; t =120 min; baseline =100%) was calculated using Graphpad Prism. After forskolin stimulation and confocal analysis, organoids were lysed in Laemmli buffer supplemented with complete protease inhibitor tablets (Roche). Lysates were analyzed by SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% milk protein in TBST (0.3% Tween, 10 mM Tris pH8 and 150 mM NaCl in H_2O) and probed 3 h at RT with mouse monoclonal E-cadherin-specific (1:10000; DB Biosciences) or CFTR-specific antibodies (450, 570 and 596; 1:3000; Cystic Fibrosis Folding consortium), followed by incubation with HRP-conjugated secondary antibodies and ECL development. Secreted Gaussia luciferase expression was quantified in the medium as previously described(21).

c. Nasal potential difference measurements in CF mice

The nasal epithelium of gut-corrected cystic fibrosis knockout mice(7) was transduced with rSIV.F/HN-hCEF-CFTR and nasal potential difference measurements were performed as previously described(22).

Vector stability in delivery devices

Virus stability was assessed in a range of delivery devices suitable for vector administration to the lung and nose: Polythylene endoscopic wash catheter (PEC, Olympus KeyMed, UK), Trudell AeroProbe ® catheter (Trudell Medical International, Ontario, Canada) and nasal spray devices (GSK parts No. AR5989 30mL bottle/AR9488 30 ml actuator). An rSIV.F/HN- vector expressing a EGFP or Lux reporter gene was passed through the delivery device and re-collected. HEK293T cells were transduced with the processed vector or with non-processed control virus and EGFP or Lux. Expression was quantified 48 hrs post transduction using routine FACS (on average 20,000 cells were counted for each well) or standard

luciferase assays, respectively. Untransduced cells served as negative control. Stability in each delivery device was assessed in at least two independent experiments. Data are expressed as % of non-passaged control.

Statistical Analysis

ANOVA followed by a Bonferroni post-hoc test or Kruskal-Wallis test followed by Dunns-multiple comparison post-hoc test was performed for multiple group comparison after assessing parametric and non-parametric data distribution with the Kolmogorov-Smirnov normality test, respectively. An independent student t-test or a Mann-Whitney test was performed for two group parametric and non-parametric data as appropriate. All analyses were performed using GraphPad Prism4 and the null hypothesis was rejected at $p<0.05$.

Results

Generation of pharmacopoeia-compliant cGMP vector

All producer plasmids were engineered to be pharmacopoeia-compliant by removal of unnecessary base pairs and replacing the ampicillin-resistance gene with the kanamycin-resistance gene (Virus Production paper; in preparation). To distinguish recombinant SIV vectors generated with the pharmacopoeia-compliant producer plasmids from vector configurations published previously, the vectors in this study are designated rSIV.F/HN or rSIV.VSV-G throughout.

Insertion site (IS) analysis

Samples of mouse lung transduced with rSIV.F/HN produced a total of 85 unique IS when filtered with a high stringency filter and 107 when filtered at medium stringency; the corresponding unique IS from murine nose samples were 14 (high stringency) and 12

(medium stringency) (see Table S3 in the online data supplement). The reason for the lower IS retrieval rate from nose compared with lung samples, despite the higher vector copy number (VCN) in the former is unknown; insufficient gDNA samples remained to further investigate this observation. Only two IS (at either stringency) were obtained from human ALI samples, an outcome likely attributable to the low DNA yield from this source. The rejection rate of reads through filtering and removal of repeat sequences was much higher than anticipated: this was mainly due to removal of unacceptably short (< 35 bp) sequences, implying that the size fractionation step to exclude short DNA fragments had been suboptimal (see Table S3 in the online data supplement).

IS from murine lung and nose samples transduced with rSIV.F/HN-hCEF-EGFPlux were mapped onto the mouse karyogram, and the distances to transcriptional start sites (TSS) determined (see Figure S2 in the online data supplement). Positional analysis showed that 73% of total IS were located in transcription units; six in exons and 73 in introns, of which 42% were integrated in intron 2 (data not shown). Frequency analyses calculated by GREAT using regions defined as IS ± 10 kb and IS ± 100 kb showed that 73% and 70% respectively of each were between 5 and 500 kb from TSS (see Figure S2 in the online data supplement). Although there are insufficient IS to draw definitive conclusions, an exploratory ontological survey revealed no preference for integration near oncogenic loci (data not shown).

Efficient IS retrieval was compromised because samples often produced an excess of short sequence reads. In addition, some samples failed to produce enough DNA to perform the LAM-PCR. Future work will concentrate on refining assay sensitivity to maximise retrieval, and systematically analysing transduced mouse airway samples. Using the accumulated IS data, we will also use gene ontological approaches(23) to further estimate the genotoxic risk by investigating to what extent IS appear in proximity to known oncogenic loci.

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