

NONVIRAL AND VIRAL VECTORS FOR GENE THERAPY OF CYSTIC FIBROSIS LUNG DISEASE

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Since the discovery of the CFTR gene in 1989, many efforts have been made to develop an efficacious and safe gene transfer system for gene therapy of Cystic Fibrosis (CF) directed to the airways. Here we review the most recent findings and development in nonviral and viral vectors used to transfer the CFTR cDNA into the airway epithelial cells of CF patients and animal models.

1) NONVIRAL VECTORS

Background

Many nonviral vectors are being explored for gene therapy of CF; those used clinically to date have mainly been cationic liposomes. With cationic liposomes, much of the DNA fails to reach the nucleus of target cells. Nevertheless, several early placebo-controlled clinical trials of liposome-mediated CFTR gene transfer to the upper (nasal) and lower (bronchi) airway epithelium of CF patients confirmed a degree of functional correction [1,2]. Importantly, the major benefit using this system is the relative lack of toxicity or immunogenicity. The enhancement of the delivery efficiency of existing nonviral vector and novel synthetic vectors is needed.

Gene transfer to the respiratory tissues has been achieved using nonviral vectors via either an intravenous or a direct airway route of administration. The pulmonary route allows delivery of the therapeutic agents directly to the lungs, bypassing several biological and physiological barriers encountered by intravenously administered agents. However, for a correct compliance by the patients, the most noninvasive approach to gene delivery should be preferred.

Results recently achieved

The efficient transfer of DNA to the nucleus by means of nonviral vectors is the major focus of recent studies. A nonviral 'DNA nanoparticle' has been recently developed: it consists of polyethyleneglycol-substituted 30-mer lysine peptides. This vector mediates very high gene transfer levels into the airway epithelium of mice with minimal toxicity [3,4]. The DNA nanoparticle small size (< 20 nm) is thought to facilitate the transport of genetic material through the nuclear pore. A Phase I clinical trial completed in 2004 found most patients had evidence of partial to complete correction of the electrophysiological defect after nasal dosage [5]. Other recent approaches to increase gene transfer mediated by nonviral vectors include the addition of a ligand to mediate cellular uptake, such as serpin enzyme complex receptor (SecR) [6] or glycods [7], the synthesis of polymer conjugates with HIV-1 TAT-derived peptides [8], or the addition of a protein moiety [9].

A variety of nonviral vectors, including cationic lipids, polycationic polymer complexes, and proteolipidic vectors, have been developed for administering DNA by inhalation [10]. Recently, it has been reported that the branched form of polyethylenimine (PEI) stabilised DNA during the nebulisation process and exhibited a higher degree of specificity for the lungs, with virtually no plasmid DNA in evidence in other tissues after this mode of delivery [11-13]. Inhalation of PEI-DNA complexes resulted in higher level of transgene expression in the lungs than cationic lipid-based formulations, without inducing high levels of proinflammatory cytokines [14].

Table 1 presents the most important results achieved in the field of nonviral gene delivery to the lung in the period 2003-2006, including clinical trials, studies in mice and in vitro in airway epithelial cells.

Table 1: Non viral cationic carriers for gene transfer to the airway epithelium.

CLINICAL TRIALS IN CF PATIENTS			
Cationic polymers	Reference	Study	Results
Polyethylene glycol substituted 30-mer lysine peptides	[5]	Double-blind, dose escalation gene transfer Phase I trial of nasal administration	Placebo (saline) or compacted DNA was superfused onto the inferior turbinate of the right or left nostril. Twelve subjects were enrolled: 2 in dose level I (DLI) (0.8 mg DNA), 4 in DLII (2.67 mg), and 6 in DLIII (8.0 mg). No serious adverse events occurred. Day 14 vector PCR analysis showed a mean value in DLIII nasal scraping samples of 0.58 copy per cell. Partial to complete NPD isoproterenol responses were observed in eight subjects: one of two in DLI, three of four in DLII, and four of six in DLIII. Corrections persisted for as long as 6 days (1 subject to day 28) after gene transfer.
EXPRESSION OF CFTR IN MOUSE MODELS			
Cationic polymer		Administration route	Results and Comments
PEIs of different molecular weight (22, 25, 50 kDa)	[15]	Intranasal	Linear 22kDa (L-PEI), branched 25 kDa PEI (B-PEI) and DcChol/DOPE lipoplexes were evaluated for luciferase reporter gene transfer into human airway epithelial cell line 16HBE. The L-PEI was significantly better than all the other reagents in confluent and polarised cells. The L-PEI gave consistently higher gene expression than B-PEI and lipoplexes in the lungs, nose and trachea. Re-dosing mice at 7 days with L-PEI/luciferase resulted in lower levels of reporter gene expression than observed after the first dose. Higher expression was obtained following re-dosing after 25 days than after 7 days. CF null mice (<i>Cftr</i> ^{tm1CAM}) were perfused onto the nasal cavity with L-PEI complexed with a plasmid encoding the human CFTR cDNA (pT10CFTR2) or a control plasmid (pT10). Four out of nine mice showed Cl ⁻ secretion in response to genistein which was higher than that observed in the control group and is not significantly different from the genistein response of normal mice treated with L-PEI alone.
EXPRESSION OF TRANSGENES IN MICE			

Cationic polymer/cationic lipid		Administration route	Results and Comments
Polyethylene glycol-substituted poly-L-lysine	[3]	Intratracheal and intranasal	Mice dosed with compacted DNA nanoparticles had peak activity of luciferase in lung at 2 days postinstillation, which declined in log-linear fashion with a half-life of 1.4 days. Luciferase activity in animals dosed with naked DNA was 200-fold less. Addition of polyethylene glycol to the complex was necessary for efficient gene. Immunohistochemical staining for bacterial beta-galactosidase 2 days after administration revealed expression predominantly in the dependent portions of the right lungs of mice, in alveolar and airway epithelial cells, though macrophages and sometimes endothelial cells also were transfected. Tissue survey for transgene expression shows expression only in lung and trachea following intranasal administration.
Polyethylene glycol-substituted poly-L-lysine	[4]	Intranasal	There was no systemic response to 10 and 100 microg of nanoparticles in serum chemistries, hematologic parameters, serum complement, IL-6, or MIP-2 levels or in the activity, growth, and grooming of the mice. Nanoparticles containing 10 microg DNA induced responses comparable to saline in all measures, including BAL cell counts and differentials and cytokine levels and histology. However, mice dosed with 100 microg DNA in nanoparticles had modest increases in BAL neutrophils 48 and 72 h after dosing, modest increases in BAL IL-6 and KC beginning 24 and 48 h, respectively, after dosing, and, on histology of the lung, a trace to 1+ mononuclear cell infiltrates about the pulmonary veins at 48 h, which were markedly reduced by 10 days and gone by 28 days after dosing. BAL neutrophil and cytokine responses were no greater than those entrained by naked DNA for up to 24 h. However, compared to administration of only 10 microg E. coli genomic DNA, the response to compacted DNA was much less. A low dose of lipofectin-complexed DNA (5 microg DNA) induced the same response as 20-fold higher doses of DNA nanoparticles.
Nonionic amphiphilic block	[16]	Intratracheal and aerosolisation	Lutrol-DNA and, to a lesser extent, PE6400-DNA complexes promoted efficient gene transfection into mouse airways in a dose-dependent manner. Lutrol delivered significantly higher DNA amounts than current nonviral vectors, without the formation of

copymers			colloidally unstable complexes. Lutrol-DNA permitted prolonged gene expression for up to 5 days whereas with PEI polyplexes, expression peaked on days 1-2 postinstillation. Aerosolized delivery of Lutrol-DNA complexes gave 5- to 15-fold higher reporter gene expression compared to PEI-DNA polyplexes. The inflammatory response after intratracheal instillation of Lutrol-DNA complexes was reversible. Lutrol could efficiently deliver genes to the airways of CF mice.
Lipofectin (L), integrin-targeting peptide (I), DNA (D)	[17]	Intratracheal	The LID vector formed in water was 10-fold more efficient than the same vector prepared in PBS. Luciferase expression was still evident 1 week after instillation. The inflammatory effects of instilling the LID vector in water were minimal, even after three administrations of the LID vector, with only mild alterations in cytokine and bronchoalveolar lavage fluid (BALF) cell profiles.
Lipofectin (L), integrin targeting peptide (I), DNA (D)	[18]	Intratracheal	The LID vector was administered 30 min after EGTA (100 mM) in saline or saline alone. Animals pre-treated with EGTA demonstrated a six-fold enhancement of luciferase expression compared with a control group pre-treated with saline. Transfection with LID formulated in saline with EGTA did not enhance transfection over LID in saline alone.
TAT-PEG-PEI	[8]	Intratracheal	A TAT-PEG-25 kDa PEI conjugate mediates high levels of gene transfer in bronchial and alveolar cells. TAT-PEG-PEI is more efficient than PEI alone and endowed with low toxicity.
PEI 25 kDa, chitosan	[19]	Intratracheal	RNA was isolated from the lungs 24 or 72 h after administration. Gene expression was monitored using a micro-array incorporating 149 genes. Chitosan-pLuc upregulated genes that protect the cell from oxidative stress and inflammation, such as heme oxygenase-1 and catalase, whereas PEI-pLuc upregulated genes involved in inflammatory processes, such as the cyclooxygenase 1 and 2. Both polymers activated genes involved in reaction to stress, such as DNA damage repair. Furthermore, in the PEI group, chaperone genes (CCT) and members of the p38 mitogen-activated protein kinase pathway (which are involved in the activation of endocytosis <i>in vivo</i>) were also upregulated.
PEI 25 kDa	[20]	Aerosol and intratracheal	The same group had previously reported that aerosolisation of branched PEI 25 kDa-DNA complexes resulted in higher gene expression in the lung as compared with linear

			PEIs of 22 and 25 kDa [21]. Luciferase gene expression mediated by branched PEI 25 kDa-DNA complexes formulated in distilled water after aerosol application was significantly, 57- and 185-fold, higher when compared with complexes formulated in 5% glucose and HBS, respectively. FITC-labeled PEI complexes were found in detectable in both the alveolar and the bronchiolar epithelium one hour after nebulisation. Aerosolised nanogram quantities of pDNA complexed to PEI (350 ng) yielded transfection levels 15-fold higher than a 140-fold higher dose (50 µg) of the same vector applied directly to the lungs of mice via intratracheal intubation.
PEI/albumin	[9]	Intravenous	750 kDa PEI /albumin polyplexes causes almost null inflammatory response, as evaluated as cytokine levels. A further increase in gene transfer levels is obtained by administering PEI/albumin concurrently with dexamethasone.
PEI 22 kDa	[22]	Intravenous	Polyplexes formed between linear 22 kDa PEI and pDNA transfect the lung at the level of endothelial cells, type I and II pneumocytes and epithelial cells of the respiratory bronchioli. Normal and CF mice show the same transgene expression pattern .
Poly (4-vinylimidazole) (P4V), PEI	[23]	Intravenous	P4V forms DNA condensates of small size (<110 nm) and in a pH-sensitive manner. P4V or PEI complexed to a plasmid co-expressing human osteoprotegerin (hOPG) and YFP was injected into the tail vein of Sprague-Dawley rats. After 3 days, expression of hOPG was observed by RT-PCR performed on spleen and lung tissue. Expression was substantially higher when the DNA was complexed with P4V than with PEI.
Lipofectamine 2000 or cationic lipid 67 (GL67) coupled to superparamagnetic particle TransMAG ^{PEI}	[24]	Perfusion of nasal cavity	The addition of the magnetic particles to the GL67/pDNA formulation resulted in an approximately 50-fold decrease in gene expression. Further, exposure to the magnetic field for a total of approximately 30 min, during and after the perfusion, did not increase transfection efficiency. The formulation precipitated during the 15-min perfusion, with the precipitate retained in the catheter. Optimisation of TransMAG ^{PEI} (i.e. non-precipitated formulation) resulted again in reduction of gene transfer as compared with plain polyplexes.
STUDIES IN VITRO			
Cationic		Cell lines	Results

polymers /cationic lipids			
PEI 25 kDa	[25]	16HBE14o- Calu-3 CFBE41o- CFSMEo-	Low molecular weight heparin (3 kDa) added at the end of the polyplex incubation time (2 hours) significantly increases the viability of cells, without interfering with the transfection efficiency.
PEI 25 kDa/albumin	[26]	A549 9HTEo- CFT1-C2	Addition of human serum albumin (HSA) to preformed PEI/DNA complexes increased gene transfer efficiency of PEI by 500-1000 fold. The ternary complexes determined detectable CFTR gene transfer and expression at the apical membrane in polarized CFT1-C2 cells, as evaluated by confocal microscopy. CF sputum inhibited PEI-mediated gene transfer by 7-186 fold. Although luciferase expression mediated by PEI-HSA was still inhibited by CF sputum, these levels were 18-83.8 fold higher than with PEI.
Cationic phosphonolipids	[27]	Primary airway epithelial cells obtained from non-CF and CF Δ F508 nasal polyps	Transfection of luciferase with the phosphonolipid KLN 30 achieved higher levels than those obtained with DOTAP/DOPE. Correct expression of CFTR was achieved in Δ F508 nasal cells with KLN 30. A significant luciferase expression was detected up to 35 days after transfection with both KLN 30 and DOTAP/DOPE. KLN 30 appears to be less toxic than DOTAP/DOPE for a charge ratio of 4, which is the best for optimal transfection.
Poly-lysine peptides	[28]	HepG2 and CF/T1 cells	Luciferase gene expression mediated by the alkylated-Cys-Trp-Lys ₁₈ (CWK ₁₈) DNA condensing peptide was increased by low molecular weight proteasome inhibitors (MG 115 and MG 132) by 10-30 fold. The gene expression was not influenced by inhibition of the lysosomal enzyme cathepsin B. The luciferase expression mediated by either Lipofectamine or PEI was reduced in the presence of proteasome inhibitors. A series of novel gene transfer CWK ₁₈ peptides containing intrinsic proteasome inhibitor derived from the Epstein-Barr virus nuclear antigen-1 were more potent gene transfer peptides relative to CWK ₁₈ only.
Lactosylated PEI 25 kDa	[29]	CF-KM4 cells, primary bronchial	In 70% confluent and in highly confluent differentiated CF-KM4 cells, Lac-PEI is more efficient than unsubstituted PEI and lactosylated polylysine in the presence of

(Lac-PEI), glycosylated polylysines		epithelial cells	chloroquine. In differentiated CFMK4 cells, a low gene transfer efficiency was observed that could not be attributed to low expression of membrane lectins or low uptake of glycosylated complexes. Bronchial epithelial cells were grown as an explant-outgrowth. Using a plasmid encoding the GFP gene complexed to various glycosylated polylysines or glycosylated PEIs, very few cells were transfected.
Lac-PEI	[30]	Σ CFTE29o-, primary bronchial epithelial cells	After three transfections of 1 h performed daily, 60% of Σ CFTE29o- cells were transfected with lac-PEI, whereas 25% of cells were transfected with unsubstituted PEI. Cell viability was 1.8-fold higher with lac-PEI as compared to unsubstituted PEI. Both forms of PEI showed an endosomal and lysosomal accumulation. In the presence of a proton pump inhibitor, bafilomycin A1, the level of lactosylated and unsubstituted PEI-mediated gene expression was reduced more than 20-fold, whereas the cell viability increased to almost 100%. For both complexes, nuclear localization was observed for less than 5% of intracellular complexes. Transcription of DNA complexed with lac-PEI or unsubstituted PEI was slower than that of free plasmid DNA.
Lac-PEI, glycosylated polylysines	[7]	Σ CFTE29o-, primary bronchial epithelial cells	Electron microscopy studies show that the cellular processing of complexes varied with their size and the polycation derivative used: large complexes (>200 nm) made with all polycationic vectors studied were internalized by macropinocytosis. In contrast, intermediate (100-200 nm) glycosylated polylysine and PEI complexes primarily entered through clathron-coated pits. Complexes were than found in endosomal vesicles, accumulated in lysosomes or vesicles near the nucleus. For the population of small complexes (<100 nm) obtained with PEI derivatives, they were internalized through caveolae and pursued a traffic pattern of potocytosis to the endoplasmic reticulum. Glycosylated-polylysine complexes were never seen caveolae or in the ER. Some complexes exited the cells either by regurgitation when Lac-PEI was used or through an exosome-like pathway for glycosylated-polylysine complexes.
Lipofectin (L), integrin-targeting peptide (I), DNA (D) [18]	[18]	1HAEo-, 2CFSMEo-	The LID vector transfected 8% of non-dividing epithelial cells in culture compared to 45% of subconfluent, dividing cells. Formulation of the vector with EGTA induced a four-fold increase in the transfection efficiency of confluent, non-mitotic cells. The LID vector efficiently transfected cells blocked in mitosis by aphicodilin treatment. The EGTA enhancement of transfection correlated with a four-fold increase in vector binding

			to the cells.
Cationic lipids [31]		16HBE14o ⁻ , CFBE41o ⁻ , IB3-1, CFT-1, MDCK, non-CF and CF human bronchial epithelial cells	LipofectAMINE PLUS and LipofectAMINE 2000 are more efficient with minimal toxicity at promoting gene transfer in 16HBE14o ⁻ and IB3-1 cells than other cationic lipids. Strong expression of CFTR “band C” is achieved upon transfection of IB3-1 (null for CFTR) with LipofectAMINE PLUS. LipofectAMINE PLUS, Effectene, LipofectAMINE 2000 and FuGENE 6.0 efficiently transfected the luciferase gene in polarised 16HBE14o ⁻ , MDCK, IB3-1 and CFT-1 monolayers. However, GFP expression analysis demonstrated that LipofectAMINE 2000 was the most efficient in terms of percentage of positive cells (20-30%). Primary cultures of non-CF human small airway epithelial cells and non-CF human sinus epithelial cells, grown as monolayers in air:fluid interface culture, were transfected best with LipofectAMINE 2000. Monolayers of CF human polyps epithelial cells were transfected best by a combination of LipofectAMINE PLUS and LipofectAMINE 2000. RTE of CFBE41o ⁻ dropped upon transfection of wt-CFTR and not of ΔF508-CFTR, suggesting functional complementation of endogenous ΔF508 CFTR. Transfection of CFTR in polarised CFT-1 monolayers enhanced RANTES expression and secretion across the apical membrane. LipofectAMINE PLUS and LipofectAMINE 2000 transfected IB3-1 and CFT-1 monolayers effectively so that CFTR could be detected by immunoprecipitation.

Abbreviations:

BALF:	Broncho-alveolar lavage fluid
DC-Chol:	3 beta (N(N',N-dimethylaminoethane) carbamoyl) cholesterol
DOTAP:	N-[1-(2,3-Dioleoyloxy)]-N,N,N-trimethylammonium propane methylsulfate
HBS:	Hepes-buffered saline
kDa:	Kilodalton
HSA:	Human serum albumin
NPD:	Nasal potential difference
PCR:	Polymerase chain reaction

PEG: Polyethylenglycol
PEI: Polyethylenimine
R_{TE}: Transepithelial resistance
TAT: Trans-activating sequence from the human immunodeficiency virus type 1

Cell lines:

A549: Type II pneumocytes
Calu-3: Human lung (submucosal gland) epithelial cells
CFBE41o-: CF human bronchial epithelial cells (Δ F508/ Δ F508)
CF-KM4: CF human tracheal gland serous cells (Δ F508/ Δ F508)
CFSMEo-: CF submucosal epithelial cells (Δ F508/unknown)
CF/T1: CF tracheal epithelial cells (F508/ F508).
 Σ CFTE29o-: Human tracheal epithelial cells (Δ F508/ Δ F508)
1HAEo-: Human airway epithelial cells
16HBE14o-: Human bronchial epithelial cells
HepG2: Human hepatocellular carcinoma cells
9HTEo-: Human tracheal epithelial cells
MDCK: Madin-Darby canine kidney cells

Perpectives

The delivery of CFTR gene to the target cells of the airways, e.g. by nebulisation, might be enhanced by a variety of adjunctive physical interventions including electroporation or complexing the vector with magnetic particles and applying a magnetic field [2].

The duration of CFTR gene expression, which usually lasts for 1-4 weeks, can be extended by substituting a human housekeeping promoter for the commonly used viral promoters. By using such a promoter (ubiquitin C) the duration of expression was extended to 6 months or more after a single administration to the murine lung [32].

In the first trial of liposome-mediated CFTR to the lower airways, all patients showed mild flu-like symptoms over a few hours immediately after nebulisation to the lungs [33]. This unfavourable outcome might be related to the unmethylated CpG dinucleotide motifs present in bacterial DNA. The actual strategy is aimed to design plasmids with the minimal number of such motifs [34].

2) VIRAL VECTORS

Background

Recombinant adenoviral vectors (Ad) are easy to produce, transduce non-dividing cells (such as those of the airway epithelium) and possess a large cloning capacity. Moreover, they were demonstrated efficient and able to recover the CFTR function in CF cells. Thus, Ad vectors were chosen as gene transfer system to be tested in CF patients. The use of Ad vectors in Cystic Fibrosis (CF) gene therapy has been decreased in the last years, since the first clinical trials in the 1990s demonstrated that they were unable to achieve and ensure long lasting expression of the therapeutic protein and generated detrimental host immune response [35].

Helper-dependent (HD) adenoviral vectors, which are depleted of all viral genes, are less immunostimulatory and have improved safety profiles compared to first- and second-generation viruses, which have only a subset of viral genes deleted. They would be feasible for repeat administration to the lung.

Adeno-associated viral (AAV) vectors have emerged as interesting vectors for gene therapy of CF lung disease since they were capable of long-term gene transfer and expression in the bronchial epithelium of rabbits and nonhuman primates and were devoided of a strong inflammatory potential [36].

Lentiviral (LV) vectors derived from human immunodeficiency virus type 1 (HIV-1) and feline immunodeficiency virus (FIV) are retroviruses which can be adequately pseudotyped and efficiently transduce the airway epithelium [37,38]. In the nose of CF mice, they can mediate CFTR gene expression and correction of the electrophysiological defect up to 110 days [39]. Lack of receptors for LV vectors on the apical surface of the airway epithelium limit their use so far.

Results recently achieved

AAV vectors with serotype 2 (AAV2) were tested in clinical trials with repeat administrations in CF patients. In the safety study by Flotte *et al.* [40], one vector dose in the nose followed by one administration in the superior lower lobe of the right lung resulted in no adverse events and no increment in cytokine levels in bronchoalveolar lavage and serum of vector receiving patients. Subjects who were treated with the high

vector dose developed anti-AAV2 neutralizing antibodies. Clinical benefit after repeated doses of aerosolized CFTR-AAV2 in the lower respiratory tract was partial and transient, with improvement in induced sputum interleukin-8 and FEV₁ observed at day 14 and 30 respectively in the rAAV-CFTR recipient group [41]. Gene transfer but not CFTR mRNA expression were detected in bronchial brushings.

The major conclusion from these studies is that AAV vector success was limited by transient transgene expression due at least in part to the immune response to the vector. Epithelial cell specific promoter CK18 was reported to mediate transgene expression in CF target cells (airway cells and submucosal glands), with limited transduction of alveolar cells [42]. The epithelial specificity minimized expression in antigen-presenting cells, resulting in less immune stimulation and increased persistence of transgene expression in target cells.

Several AAV serotypes were identified, bearing tropism for different animal species and posing the question about suitability of adequate animal and cellular models for evaluation of gene transfer [43-45].

The usefulness and application of new viruses (Sendai, Canine adenovirus type II, lentivirus) have been evaluated. Griesenbach and colleagues have showed that gene expression mediated by recombinant Sendai-based vectors is transient and repeated administration seems not feasible because of the neutralizing antibodies developed following the first vector administration [46,47]. Preliminary evaluation of vectors derived from Canine adenovirus type II (CAV2) suggests that CAV2 can be considered for efficient gene transfer in the murine airways since they could enable persistent gene expression inducing a reduced pro-inflammatory and immune response [48].

Pseudotyping with heterologous envelopes [49-51] and modification of the paracellular permeability [52] are the strategies currently used to overcome the paucity of lentiviral receptors on the apical surface of the respiratory epithelium and to reach the basolateral membrane receptors. Recent studies have explored the feasibility of lentiviral vector mediated gene expression in the fetal airway epithelium, considering the advantage of a therapeutic intervention before clinical onset and in the absence of a functional immune response [53,54].

Alternative approaches to gene replacing are investigated, for example CFTR trans-complementation [44]. In these last years, different options to achieve enhancement of gene transfer efficiency of viral vector were investigated. Combination of gene

delivery and administration of pharmacological compounds, such as proteasome modulating agents, is actually exploited as a strategy to overcome low efficiency of viral vectors from the apical membrane of respiratory target cells [55]. Also high-throughput screening of synthetic compounds and natural product extracts, is currently used to identify proteasome or telomerase inhibitors able to enhance viral transduction in airway epithelial cells [56].

Table 2 presents the most important results achieved in the field of viral gene delivery to the lung in the period 2003-2006, including clinical trials, studies in mice and in vitro in airway epithelial cells.

Table 2: Viral vectors for gene transfer to the airway and respiratory epithelium.

CLINICAL TRIALS IN CF PATIENTS			
Vector	Reference	Study	Comments
rAAV2	[40]	Phase I study of intranasal and endobronchial administration of rAAV2 –CFTR vector in CF patients	25 CF patients with mild to moderate lung disease received one vector dose (from 3×10^1 to 1×10^9 replication units (RU)) administered to one side of the nose on day 0 and one delivered to a superior lower lobe of the right lung via fiber optic bronchoscopy on day 1. All the parameters for vector safety, immune response and DNA transfer outcomes were studied for 30 days and on day 90. Vector related serious adverse events were observed only in 1 of 25 subjects (pulmonary exacerbation). There was no evidence of increment of cytokine levels in BAL of vector treated patients and alteration in chest CT scans, chest x rays or pulmonary functions. No development of neutralizing antibodies was observed in the patients receiving the lower dose, however the cohort which received the higher dose demonstrated greater than 4 fold increase in anti-AAV2 antibodies titres. DNA vector shedding was present in the sputum of one subject on day 1 and disappeared at day 30. DNA transfer was observed via PCR in the nasal brushing obtained from 1 of 3 subjects receiving 1×10^7 RU in the nose and in 1 subject receiving 1×10^8 RU at day 14. DNA transfer in the lung was reported at day 30 for 2 of 3 subjects receiving a bronchial dose of 1×10^9 RU. There was no significant difference in nasal transepithelial potential difference (TEPD) values, as measured at the baseline and on day 1-7-14-21-30 and 90 after vector administration. CFTR-RNA expression was not evaluated due to low level of DNA expression
rAAV2	[41]	First randomized double-blind placebo-controlled phase II trial of repeated doses of	Three doses of rAAV2-CFTR or placebo were administered at 30-day intervals using Pari LC Plus nebulizer. No adverse events were reported. Improvement in induced sputum interleukin-8 (IL-8) and FEV ₁ were observed at day 14 and 30 respectively in the group receiving rAAV-CFTR. Gene transfer but not CFTR mRNA expression were detected in bronchial brushings of six out of eight subjects receiving rAAV-

		aerosolized rAAV2 containing CFTR cDNA in the lower respiratory tract of CF patients with mild lung disease, defined as FEV ₁ ≥ 60% predicted	CFTR who underwent bronchoscopy. No difference among patients homozygous for the ΔF508 mutation and patients with other genotypes were observed
rAAV2	[57]	CFTR expression and functional correction in primary cultures of bronchial and nasal respiratory cells harvested from rAAV2-CFTR recipients of the gene therapy trial performed by Flotte <i>et al.</i> [40]	This study demonstrates a correlation between the presence of rAAV2-CFTR vector DNA, CFTR mRNA expression and cAMP-activated chloride channel activity in primary cultures of CFTR corrected airway epithelial cells.

EXPRESSION OF CFTR IN ANIMAL MODELS

Vector	Reference	Study	Comments
HD-Ad with a nuclear localization signal, under the control of a human	[58]	Protection of CFTR knockout mice from acute lung infection	CFTR expression mediated by human adenovirus 5-based helper dependent – adenovirus under the control of a human cytokeratin 18 (K18) expression cassette was properly localized at the apical plasma membrane in primary differentiated human CF bronchial cells grown at air liquid interface. In the airway epithelia of mice, CFTR

cytokeratin 18 (K18) expression cassette			mRNA and protein were present in the whole lung and bronchioles for 28 days after gene delivery. In vitro and in vivo gene transfer was performed after pretreatment of the epithelia with EGTA, to disrupt intercellular tight junctions and access the basolateral viral receptors. Acute inflammation in vector treated lungs was minimal to moderate, as assessed by histopathological evaluation. Cftr knockout mice receiving CFTR-HDA Δ 7 days before Bcc challenge showed less severe histopathology, and the number of bacteria recovered from lungs was reduced to the level found in Cftr $^{+/+}$ mice. These data suggest that CFTR gene transfer could reduce susceptibility to opportunistic pathogens in the respiratory tract
HIV-1-derived LV pseudotyped with VSV-G	[39]	Efficacy of CFTR expression after a single dose of a VSV-G-LV in the nasal epithelium of CF mice	Pretreatment of the epithelium with lysophosphatidylcholine (LPC) prior to intranasal instillation of LV vector expressing β galactosidase in mice produced significant transgene expression in the nasal respiratory epithelium for at least 92 days, suggesting that transduction of airway progenitor cells had occurred. Transduction with LV expressing CFTR after conditioning achieved sustained CFTR expression and partial correction of the electrophysiological defect in the nose of CF mice for at least 110 days. This report is the first demonstration of efficacy of CFTR function recovery mediated by a LV vector in combination with preconditioning of the airways

EXPRESSION OF TRANSGENES IN ANIMAL MODELS

Vector	Reference	Study	Comments
AAV2/2, AAV2/5 and AAV2/9 serotypes	[45]	Transduction efficiency in human airway epithelial cells, efficiency and persistence in the mouse lung and nasal epithelium.	AAV2/2 vector application to the apical surface of well differentiated human airway epithelial cells resulted in transduction of very few cells. In contrast, after basolateral application of AAV2/2 vector, 80% of the cells were transduced. For AAV2/5, EGFP-expressing cells were observed after apical as well as basolateral vector application, with basolateral administration being most efficient. AAV2/9 was equally effective in transduction of airway cells after apical or basolateral application. AAV2/9 and AAV2/5 vectors expressing the nuclear Lac Z gene were instilled in

		Feasibility of repeated administration	mice by intratracheal instillation. AAV2/9 transduced mainly alveolar cells and few conducting airway cells, whereas AAV2/5 transduced cells of both the alveoli and conducting airways at levels greater than that observed with AAV2/9. AAV2/9-mediated <i>nLacZ</i> gene transfer in nasal and lung airways was relatively stable for 9 months, suggesting that a progenitor airway cell population was transduced. The human α -1- antitrypsin (hAAT) gene administered by two routes: intratracheally and intranasally. AAV2/9-mediated human α -1-antitrypsin expression in serum was 60-fold better than that of AAV2/5. Intratracheal delivery resulted in a significant 3-fold increase in the level of AAV2/9-mediated hAAT expression compared with intranasal route. AAV2/9 could be readministered in the presence of high levels of serum-circulating neutralizing antibodies as early as 1 month after initial exposure, with minimal effect on overall reporter gene expression, rendering AAV2/9 a promising gene transfer vector candidate for use in humans.
AAV1, AAV2, AAV5 serotypes	[59]	Efficiency of AAV vectors with capsid serotypes with greater tropism for the apical surface of respiratory cells (rAAV1 and rAAV5) and with strong promoter (CMV enhancer / beta actin hybrid promoter) in murine airway epithelia	In this study, rAAV2, rAAV5 or rAAV1 vectors, expressing luciferase reporter gene or the human alpha-1 antitrypsin α cDNA, were intratracheally instilled in C57BL6 mice. The transduction efficiency of rAAV5 and rAAV1 were similar and much higher than rAAV2. This work suggests rAAV1 as a useful capsid serotype for gene transfer in the mouse lung
AAV2 serotype	[43]	Transgene expression with serial doses of	This study addresses whether repeated dosing of AAV2 results in development of neutralizing antibodies and inflammatory response prohibitive to transgene expression. Serial aerosolizations with AAV2 containing the

		aerosolized AAV2 vector in Rhesus Macaques	Green Fluorescence protein (GFP) reporter gene resulted in successful transgene expression in bronchial cells. Mild to moderate peribronchial/bronchiolar lymphocytes or eosinophilic infiltration was observed in localized areas of the airways. Re-exposure to the vector produced anti AAV2 neutralizing antibodies capable of blocking transgene expression in vitro. This study shows that the repeated dosing of AAV2 vector could elicit immune response but did not inhibit gene transfer in the airways of rhesus macaques
AAV5, FIV- and HIV-1- based LV vectors pseudotyped with the baculovirus <i>Autographa californica</i> GP64 envelope	[52]	Efficiency of viscoelastic gel formulations of different viral vectors in human airway epithelial cells and in murine airways	Gene transfer with adenoviral vectors, AAV5 vectors, FIV and HIV-1 based lentiviral vectors was enhanced by formulation in viscoelastic gels (methylcellulose, carboxymethylcellulose sodium, poloxamer 407 polymer) which increased the vector residence time into epithelia by slowing the mucociliary clearance
E1/E3 deleted Ad and AAV2/5	[60]	Gene transfer in the murine airway epithelium with viral vectors complexed with the anti-inflammatory cationic lipid dexamethasone-spermine	After intranasal instillation in mice, formulation of an Ad vector with cationic steroid liposomes containing dexamethasone –spermine (DS)/dioleoylphosphatidylethanolamine (DOPE) and DC-Chol/DOPE resulted in transgene expression limited to the airway epithelial cells, with poor expression in alveolar cells, while Ad vector alone mediates high alveolar transduction. The dexamethasone and DS/DOPE formulation reduced airway inflammation compared to vector alone, while DC-Chol/DOPE did not. Formulation of vectors with DS/DOPE improves targeting to the airway epithelium in vivo and reduces vector-induced inflammation through the anti-inflammatory activity of dexamethasone-spermine
HD-Ad containing an epithelial- cell specific expression cassette cytokeratin 18 (K18)	[42]	Efficiency and persistence of gene expression in murine airway epithelium	Following intranasal instillation in mice, transgene (β -galactosidase) expression was observed in airway epithelia (basal and ciliated cells) and submucosal glands for up to 28 days, with poor expression in alveolar cells. In contrast with first Ad vector inflammation was negligible. This study suggests that epithelial cell specific promoter CK18 drives transgene expression in CF target cells (airway cells and submucosal

			glands), with limited transduction of alveolar cells. The epithelial specificity minimizes expression in antigen-presenting cells, resulting in less immune stimulation and increased persistence of transgene expression in target cells. The vector was also capable to transduce Clara cells which have airway stem cell properties
HD-Ad with a nuclear localization signal, under the control of a human CK18 expression cassette	[61]	Aerosol delivery of an improved version of adenoviral vector expressing β -galactosidase reporter gene and formulated with L α -lysophosphatidylcholine (LPC) to rabbit respiratory tract using intratracheal catheter	Administration of a solution of 0.4M EGTA or 0.01%-0.1% LPC, 30 min prior to vector aerosolization, enhanced gene transfer in the rabbit airways. Large airway transduction was not obtained when the vector was delivered in 0.4M EGTA but it was achieved when HD-AdV was formulated with 0.1%LPC. All type of cells of the surface epithelium were transduced; in the trachea 66% of cells were transduced after 0.1%LPC administration, pretreatment with 0.01% LPC resulted in transduction of 24% of cells. Fever was observed in animals receiving LPC and this adverse affect together with the presence of granulomatous lesions in the rabbit treated with the higher LPC dose, suggest a role for LPC in inflammation and the necessity to determine the minimum effective dose for LPC
HD-Ad and first generation Ad type 5	[62]	Persistence of transgene expression and humoral immune response to the vector after readministration of an helper dependent adenovirus into the	Readiministration of HD-Ad to murine airways in mice resulted in minimal loss of transgene expression. In contrast when first generation Ad vector was administered first, followed by HD-Ad or first generation Ad, transgene expression was greatly reduced. Lower concentrations of neutralizing antibodies were detected in serum or BAL (bronchoalveolar lavage) fluid from mice wich were treated with two dose of HD-Ad in comparison to animals which received first generation Ad followed by HD-Ad vector. The higher dose of both vectors induced the greater antibody response, indicating the relevance to use the minimum vector concentration required to obtain a therapeutic level of transgene expression. This study suggests that readministration of HD-Ad vector is feasible without reduction of gene expression

		mouse lung	
Canine Ad type-2, HD-Ad5 and Ad. All the vectors were deleted of the E1 region	[48]	Efficiency and persistence of gene transfer in vivo to the murine airways and ex vivo in well-differentiated human airway epithelia. Immune response elicited by CAV2	CAV-2 vector expressing marker genes transduced well differentiated cultures of human pulmonary epithelial cells, when the virions were applied from the apical surface of the epithelia. The efficiency of this vector was increased when infection was performed from the basolateral membrane of the epithelia, suggesting that CAR is the primary receptor for CAV-2 attachment. Intranasal administration of FK228 peptide, which has been shown to increase cell surface expression of CAR and integrin V in human lymphocytes and enhance HAd5 efficiency, prior to CAV β -gal delivery in mice, increased β -gal expression by threefold. When efficiency of CAV-2 and HAd5 were compared in vivo both vectors was equally efficient in transducing the murine lung. CAV mediated transduction was poorly inhibited by sera containing HAd5 neutralizing antibodies and could achieve long lasting gene expression. CAV induced TNF α and inflammatory cells migration into mice 24h post instillation compared to mock, however it was less immunogenic than HAd 5. This paper shows that vectors derived from CAV can be considered for efficient gene transfer in the murine airways since they could enable persistent gene expression inducing a reduced pro-inflammatory and immune response
FIV-based LV pseudotyped with the JSRV envelope glycoprotein	[50]	Gene transfer into polarized primary cultures of human respiratory epithelial cells and rabbit respiratory tract	JSRV –FIV transduced polarized primary cultures of respiratory epithelial cells derived from human trachea and bronchi with poor efficiency. Expression of hyaluronidase 2 (HYAL2), the reported receptor for JSRV, on the apical surface of the airway cultures did not overcome the inefficient transduction. Intratracheal administration of JSRV –FIV formulated in 12mM EGTA in rabbits, showed that transgene expression was occasionally found in the lung parenchyma, in cells morphologically similar to alveolar type II pneumocytes. This study indicates that JSRV-FIV vectors have cellular tropism for distal airways in vivo and alveolar type II cells in vitro
FIV-based LV	[49]	Persistence of gene transfer in the mouse nasal	A FIV vector pseudotyped with the GP64 derived from the baculovirus <i>A. californica</i> transduced differentiated human airway epithelia from the apical surface with high efficiency.

		epithelia	Intranasal instillation of the vector formulated in methylcellulose resulted in persistent gene expression in the mouse nose, with β -galactosidase expression detected in olfactory and respiratory epithelial cells 3 months after LV administration and luciferase expression observed one year post-infection. GP64 FIV transduced the mouse respiratory epithelia with much greater efficiency than FIV pseudotyped with VSV-G envelope
HA or EIAV pseudotyped HIV-1-based LV vector	[51]	Transduction efficiency in models of human and mouse airway epithelium	HA-LV vectors could efficiently transduce, via the apical membrane surface, polarized cultures of human airway epithelial cells, well-differentiated primary cultures of mouse tracheal cells and mouse tracheal epithelium in vivo. Both ciliated and non-ciliated cells were transduced, with a preference for ciliated cells
HIV-1-based LV pseudotyped with VSV-G	[54]	In vivo gene transfer in the tracheobronchial tree of fetal rabbits	A VSV-G lentiviral vector expressing the GFP marker gene was delivered to the tracheobronchial tree of fetal rabbits via direct tracheal injection or intramniotic administration. Marker gene transduction was observed four days after LV delivery with both techniques, with GFP expression detected in luminal epithelium within fetal trachea and alveolar lining cells in fetal lungs. Direct tracheal injection was associated with high fetal mortality rate (75%) and elevated degree of transgene expression in the control fetuses, compared with amniotic delivery. This is the first report of in vivo LV mediated reporter gene transfer to fetal respiratory epithelium in intact animals
Self-inactivating (SIN) HIV-1-derived LV pseudotyped with VSV-G	[53]	Safety and efficiency of intrapulmonary fetal gene transfer in rhesus monkeys	Self-inactivating (SIN) HIV-1 derived lentivirus pseudotyped with VSV-G and expressing GFP under the control of CMV or SP-C (human Surfactant Protein-C) promoter was injected into the parenchyma of lungs of fetuses of rhesus monkeys. Tissue analyses were done at term and 3 months post-natal age. Whole tissue fluorescence analysis shows that transgene expression was observed with lenti-CMV but not LV-SP-C. No adverse effects were observed, lung development and function were not altered by gene transfer, no difference in cell counts in BAL fluid was found between transduced and control fetuses. Fluorescence microscopy analysis of whole-tissue sections at 3 months post-natal age (6 months after gene transfer) showed GFP expression in the lung lobes, thoracic cavity, pericardium, heart and diaphragm,

			<p>similar to that found for term fetuses.</p> <p>Analysis of lung sections shows that transgene expression was restricted within the pulmonary vasculature and alveolar cells</p>
First generation E1-E3 deleted serotype 5 Ad vectors	[63]	Intra-amniotic injection of Ad vectors in mice	<p>Intra-amniotic delivery of a first generation E1-E3 deleted serotype 5 adenoviral vector encoding the beta galactosidase reporter gene under the control of RSV promoter in mice at the day 16 of gestation resulted in transduction of fetal lungs</p> <p>At this time of development, the intensity of fetal breathing is sufficient to carry the vector into the fetal airways. The breathing movements can be enhanced by combination of intra-amniotic theophylline administration or exposure of the dam to elevated CO₂ concentrations. The dose of vector is a limiting factor to achieve efficient transduction, as both level of transgene expression and number of transduced lungs increased with increasing doses of vector</p>
Non transmissible, replication competent recombinant SeV developed by deleting the envelope Fusion (F) gene (SeV/ΔF)	[46]	Gene transfer to respiratory epithelial cells, in ex vivo models of differentiated airway epithelium and in vivo in murine airways	<p>SeV/ΔF mediated β-galactosidase reporter gene expression in human nasal epithelial cells. To evaluate the effect of mucus, the vector was applied to mucus-covered or mucus depleted tissues in an ex vivo model of sheep tracheal epithelium. Transduction efficiency was affected by mucus coverage at higher vector dose, while at low dose vector efficiency increased after mucus depletion. Efficient transduction was also observed in an ex vivo model of rhesus monkey tracheal explants. After SeV/ΔF vector application to the nasal cavity of mice, transduction was observed in nasal turbinates only with the higher vector dose, and increased with prolonged exposure to the vector particles, while in the lung transgene expression was dose and time dependent, with the highest level of expression at day 2 post LV delivery, and background levels by day 14. This study demonstrates that a new recombinant non transmissible Sendai virus can mediate gene transfer to respiratory epithelia with the same efficacy of wild-type transmissible SeV</p>
Neuroaminidase-deficient Influenza virus	[64]	Gene transfer to murine airways to study the feasibility of delivery of	<p>Intranasal administration of a Neuroaminidase-deficient Influenza virus expressing GFP achieved transgene expression in the epithelial cells of nasal mucosa and bronchioli at day 1 post infection, and in alveoli at day 2.</p> <p>Transgene expression decreased to background levels by day 4. These data suggest</p>

		foreign protein and production of vaccines against infectious disease	that influenza virus can be an useful vector for expression of foreign genes and vaccine production against infectious disease in the respiratory tract
SeV deleted of matrix (M) and fusion (F) genes, (SeV/ Δ M Δ F)	[65]	Production and propagation of SeV/ Δ M Δ F vector in packaging cell line, cytotoxicity and gene transfer efficiency in murine airways in vivo	Following intranasal instillation of SeV/ Δ M Δ F expressing GFP in mice, strong fluorescence was observed on the surface of the lung recovered from infected animals 2 day after vector administration. Detailed analysis of the lung sections showed transgene expression in alveolar cells. SeV/ Δ M Δ F is an improved RNA vector, which retains efficient gene transfer, gains non transmissible properties with less cytopathic effect

IN VITRO STUDIES

Vector	Reference	Study	Comments
AAV5	[66]	Development of a short adeno-associated virus expression cassette for CFTR gene transfer to differentiated CF airway epithelia	An AAV5-CFTR Δ R vector with truncated CMV immediated early enhancer promoter, addition of the SV40 19S/16s intron to enhance expression, shortened polyadenlation sequences and a CFTR transgene deleted of the R domain (CFTR Δ R) restored expression of functional CFTR Cl ⁻ channel in differentiated primary CF airway epithelia
AAV2 and AAV5	[67]	Characterization of proteasome modulating agents for their ability to	N-acetyl-L-leucyl-L-leucyl- norleucine (LLnL) and doxorubicin increased AAV transduction efficiency from the apical membrane in IB3 and A549 immortalized respiratory cells and in fully differentiated polarized human airway epithelia derived from lung transplants. Combined administration of LLnL and doxorubicin

		enhance AAV transduction	synergistically augmented rAAV transduction from the apical membrane of polarized cells. Doxorubicin and LLnL both enhance nuclear accumulation of rAAV particles in respiratory cells. A lipid encapsulated formulation of doxorubicin (Doxil) and ZLL improved rAAV efficiency in mouse lungs and human bronchial xenografts
AAV2 and AAV2/5	[55]	Investigation of pharmacological strategies to improve efficiency of CFTR gene delivery with AAV vectors while simultaneously inhibiting ENaC activity in CF cells	N-acetyl-L-leucyl-L-leucyl- norleucine (LLnL) and doxorubicin increased AAV transduction and correction of CFTR mediated short circuit currents in CF airway epithelia. At the same time these agents inhibited ENaC activity independent on vector administration, through downregulation of γ -ENaC subunit mRNA expression and increase in γ -ENaC subunit promoter methylation. This is the first report concerning identification of molecules with dual therapeutic action, capable to improve CFTR gene transfer efficiency and ameliorate primary aspects of CF disease pathophysiology
AAV, E-1 deleted Ad vector and LV vector	[56]	High-throughput screening of 23.000 compounds and natural product extracts able to enhance transduction mediated by different vectors encoding the luciferase or green fluorescence protein reporter gene	Seven compounds increased gene transfer efficiency up to several hundred fold above control in polarized HT29 cells. Two of them, NSC143491 and NSC675865, induced gene transfer in polarized CF bronchial cells. The activator NSC143491 (duanomycin 3-oxime hydrochloride) is a derivative of daunorubicin and acts as a topoisomerase II inhibitor. Enhanced transduction by unrelated vectors (AAV, adenovirus, lentivirus and liposomal) was observed
AAV2	[68]	Role of heparan	Enzymatic removal of heparan sulfate from the membrane of human respiratory

		sulfate in AAV2 transduction of human airway epithelial cells	epithelial cells resulted only in moderated decrease in susceptibility to AAV2 transduction, indicating that other glycosaminoglycans (GAGs) may be involved in adeno-associated mediated transduction . This study suggests that targeting alternative membrane GAGs may be a strategy to improve the efficacy of AAV2 for lung gene therapy
AAV2 and AAV5	[69]	Comparison of transduction efficiency of AAV2 and AAV5 in mouse and human Air Liquid Interface (ALI) cultures. Evaluation of the utility of ALI epithelia derived from Δ F508 mice for studies of CFTR complementation	This study indicates that mouse ALI epithelia show a preference of AAV serotypes transduction (AAV5>>rAAV2) different than observed in human cells (rAAV5=rAAV2). Differences in intracellular trafficking for rAAV2 and rAAV5 vectors could explain the altered serotypes efficiencies in transduction from the apical membrane. No difference in Isc profiles were observed in cultures of tracheal cells obtained from CF and non CF mice indicating that CF murine ALI cultures are not a suitable model for the study of CFTR complementation
AAV2	[70]	Evaluation of alternative serotypes and promoters to enhance transduction mediated by AAV vectors	The activity of AAV5 and AAV2 serotypes and cytomegalovirus (CMV) promoter, Rous Sarcoma Virus (RSV) promoter, the CMV enhancer / beta actin (CB) promoter and the CMV enhancer / RSV promoter was assessed in vitro in CF bronchial cells. The CB promoter was the most efficient, the rAAV5 achieved the higher level of transduction. rAAV5-CB-promoter driven CFTR minigene vector was used to correct chloride transport defect in vitro and the hyperinflammatory lung phenotypes in CF mice in vivo. This study provides functional characterization of a new version of rAAV-CFTR vector
rAAV2 and rAAV5	[44]	Rescue of CFTR chloride	AAV vectors were generated to carry a pre-trans-splicing molecules (PTMs) engineered to bind intron 9 of CFTR pre-mRNA and then trans-splice the normal

		conductance in polarized human CF airway epithelial cells, using a spliceosome-mediated RNA trans-splicing (SMaRT) approach	sequence for human CFTR exons 10-24 into the endogenous pre-mRNA. Human CF polarized airway epithelia were infected from the apical membrane with rAAV2 or rAAV5 CFTR-PTM vectors in the presence of proteasome-modulating agents (doxorubicin and N-acetyl-L-leucinyl-L-leucinyl-L-norleucinal) to enhance transduction. Epithelia were then evaluated for cAMP-sensitive short-circuit currents 2 weeks postinfection. Levels of CFTR correction observed with rAAV2 CFTR-PTM and rAAV5 CFTR-PTM constructs were similar, and correspond to conductance equivalent to 14.2 and 13.6% of that present in non-CF human polarized epithelia, respectively. RT-PCR analysis demonstrated the existence of wild-type CFTR transcript in CFTR-PTM-corrected epithelia, whereas only DeltaF508 mRNA was detected in polarized cells infected with control rAAV LacZ-PTM vectors. These results provide evidence that rAAV vectors are capable of using SMaRT to correct CFTR function after apical transduction of human CF airway epithelia
HD-Ad	[71]	Gene transfer in sweat glands in human skin organ culture	CFTR was efficiently delivered and expressed by human sweat glands after transduction with a helper dependent adenoviral vector containing cytokeratin 18 regulatory elements. This study proposes gland cells as alternative model for CF gene therapy in humans
First generation and E1 deleted Ad, HD-Ad-CMV and HD-Ad-K18	[72]	Activity of CMV promoter and CK18 regulatory elements in CFTR gene transfer and expression of functional CFTR Cl ⁻ channels in airway epithelia. Study of the level of CFTR expression required for correction of	CMV promoter induced 50-fold more transgene expression than K18 promoter, but K18 promoter generated more transepithelial Cl ⁻ current at high vector dose. When CFTR was overexpressed, some CFTR channels were present in the basolateral membrane of the epithelia reducing the transepithelial Cl ⁻ transport. These data indicate that a small fraction of CFTR expressing cells is sufficient to correct the CF epithelial Cl ⁻ transport defect and suggests the relevance of the percentage of cells expressing CFTR rather than the level of CFTR expression in single cells

		the epithelial Cl ⁻ transport defect	
HIV-1 derived VSV-G pseudotyped LV vector	[73]	Recostitution of well differentiated human airway surface epithelium with lentiviral transduced cells derived from fetal human tracheas or airway xenografts and cultured in 3-D spheroid structures	Respiratory epithelial cells obtained from mature human fetal tracheas or airway xenografts were transduced in suspension with a HIV-1 derived VSV-G pseudotyped lentiviral vector expressing GFP and allowed to develop 3-D spheroid structures. 12% of spheroid cells were positive for GFP expression and marker gene expression was maintained for the duration of the study (80 days) without altering the chloride channel activity. The 3-D spheroid structures were able to reconstitute a fully differentiated surface airway epithelium. Presence of clusters of GFP positive cells in basal ciliated and secretory cells in the reconstituted epithelium at 10 weeks after engraftment suggests targeting of progenitor cells.
HIV-1-derived lentivirus pseudotyped with the Jaagsiekte Sheep Retrovirus (JSRV) envelope glycoprotein	[74]	Evaluation of transduction efficiency in primary and immortalized human lung epithelial cells	JRSV can pseudotype HIV derived lentiviral vector and were able to transduce BEAS-2B human bronchial cells and CF16 nasal cells obtained from a CF patient. JSRV transduced primary human tracheal epithelial cells with low efficiency
Ad2 e Ad5	[75]	Role of glycocalyx and tethered mucins in restriction of Ad transduction from the apical membrane of respiratory epithelial cells	Expression of a glycosylphosphatidylinositol (GPI)-linked Ad receptor (GPI-CAR) on the apical surface of polarized cultures of human airway tracheobronchial epithelial cells did not enhance AdV gene transfer, because glycocalyx limited access to the GPI-CAR. Proteolytic digestion of the tethered mucins or inhibition of O-linked glycoylation improved gene transfer, indicating that tethered mucins are a component of glycocalyx which inhibits Ad transduction from the apical surface of polarized epithelia. AdV gene transfer to murine airway epithelia was inefficient in GPI-CAR overexpressing transgenic mice, but was increased in the lung of Muc 1 Knockout

			(Muc1 ^{-/-}) mice
E1/E3 deleted Ad	[76]	Expression and activity of functional GFP-tagged CFTR	A549 (type II pneumocyte) cells were transduced with E1/E3 deleted adenoviral vectors containing wt CFTR or ΔF508CFTR with N-terminal GFP tag. Electrophysiology measurement of channel activity showed that functional CFTR channels were observed in CFTR-GFP transduced cells, whereas functional GFP-ΔF508CFTR channels required the presence of sodium butyrate in the culture medium. These data indicate that GFP-tagged CFTR constructs have the same single-channel properties like those of untagged forms
Ad5	[77]	Phage biopanning in CFTR deficient human tracheal glandular cells (CF-KM4) to find specific peptide ligands that confer cell target specificity and enhance gene transfer mediated by Ad5 vector	The peptide GHPRQMSHVY enhanced transduction increasing the number of endocytosed virions and promoting the escape of the viral particles from the early endosomes

ANTIINFLAMMATORY THERAPY			
Vector	Reference	Study	Comments
HD-Ad vector	[78]	Expression of a small hairpin (sh)RNA targeting human IL-8 in cultured airway epithelial cells	A helper-dependent adenoviral vector was used to express a small hairpin (sh)RNA targeting human IL-8 in IB3-1 (Cftr ^{-/-}) and C38 (Cftr-corrected) respiratory epithelial cells stimulated with TNF-alpha, IL-1beta or heat-inactivated Burkholderia cenocepacia. IL-8 expression in IB3-1 and C38 cells was significantly reduced by shRNA expression. The shRNA targeting IL-8 had no effect on the activation of NF-kappaB, or on the protein levels of IkappaB or IL-6, suggesting that this anti-IL-8

			strategy was highly specific, and therefore may offer potential for the treatment of inflammatory diseases
Ad5	[79]	Modulation of NF- κ B activity and inflammatory signaling following adenoviral delivery of RelA or a dominant inhibitor of NF- κ B in murine airways before <i>Pseudomonas aeruginosa</i> lung infection	Intratracheal administration of RelA-Ad vector increased bacterial clearance in murine lungs, due to induction of NF- κ B activity. TNF α level was up-regulated in BAL fluid in mice receiving RelA-Ad, whereas NF- κ B inhibition reduced MIP-2 expression and neutrophil recruitment following <i>P. aeruginosa</i> infection. This study indicate that the NF- κ B regulates the innate immune response to <i>P. aeruginosa</i>
HIV-1-derived LV pseudotyped with the Zaire strain of the Ebola virus glycoprotein (EboZ)	[80]	Functional expression of the mouse Relaxin and mouse Relaxin-3 in the mouse lung	EboZ GP-LV vectors expressing the luciferase marker gene was delivered via endotracheal intubation to the respiratory tract of C57BL6 mice. EboZ pseudotyped vector but not VSV-G LV could mediate transgene expression in the lung as early as 4 days after LV administration. Mice transduced with EboZ GP-LV vectors expressing mouse relaxins exhibited increased activity of MMP-2 and MMP-9 in the lung. This study offers potential for treatment of pulmonary diseases characterized by dysregulation of extracellular matrix protein accumulation

Abbreviations:

AAP:

AAT:

AAV:

alpha-1-antitrypsin

Adeno-associate virus

Ad:	Adenovirus
ALI:	Air liquid interface
BAL	bronchoalveolar lavage
CAR:	Coxsackie adenovirus receptor
CAV:	Canine adenovirus
CK18:	Cytokeratin 18
CMV:	Cytomegalovirus
CT:	Computed tomography
DS:	Dexamethasone–spermine
DOPE:	Diioleoylphosphatidylethanolamine
DC-Chol:	3 beta (N(N',N-dimethylaminoethane) carbamoyl) cholesterol
EboZ:	Zaire strain of the Ebola virus
EGTA:	Ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EIAV:	Equine infectious anemia virus
ENaC:	Epithelial Sodium Channel
FEV:	Forced expiratory volume
FIV:	Feline immunodeficiency virus
GFP:	Green fluorescent protein
GMCSF:	Granulocyte-macrophage colony stimulating factor
GPI:	glycosylphosphatidylinositol
HA:	Hemagglutinin
HD-Ad:	Helper-dependent adenovirus
HIV:	Human immunodeficiency virus type 1
IL-8:	Interleukin-8
ITR:	Inverted terminal repeats
JSRV:	Jaagsiekte sheep retrovirus
LlnL:	N-acetyl-L-leucyl-L-leucyl- norleucine
MMP:	metalloproteinase
NAC:	N-acetylcysteine
NM:	Neurominidase

LPC:	Lysophosphatidylcholine
LV:	Lentivirus
PIV:	Parainfluenza virus
PTM:	Pre-trans-splicing molecule
SeV:	Sendai Virus
SMART:	Splicesome-mediated RNA trans-splicing
SP-C:	Surfactant protein-C
TEPD:	Transepithelial potential difference
TNF:	Tumor necrosis factor
VSV-G:	Glycoprotein G of the Vesicular Stomatitis Virus

Cell lines:

A549:	Type II pneumocytes
BEAS-2B:	Human bronchial cells
IB3-1:	CF bronchial epithelial cells (Δ F508/W1232X)
C38:	IB3-1 cells corrected with a retrovirus expressing wild-type CFTR
CF16:	CF nasal cells
CFBE41o-:	CF human bronchial epithelial cells (Δ F508/ Δ F508)
CFBE41o-:	CF human bronchial epithelial cells (Δ F508/ Δ F508)
CF-KM4:	CFTR deficient human tracheal glandular cells
HT-29:	Human colorectal carcinoma cells
MDCK:	Madin-Darby canine kidney cells

Perspectives

Novel generations of rAAV-CFTR are being developed by a number of groups, comprising those pseudotyped with serotype 5 capsid and carrying a minigene version of CFTR that omits sequences that are not necessary for cAMP-activated chloride channel [70]. Integrating lentiviral vectors seems to be the most promising viral vector for CF gene therapy. However important safety concerns, including generation of replication-competent HIV vectors, recombination during wild-type HIV-1 infection and mutagenesis following integration have to be addressed before considering lentivirus for application in humans [38]. Optimal vector formulation and interventions to overcome the paucity of lentiviral receptors on the apical surface of the respiratory epithelium and to reach the basolateral membrane receptors have to be exploited, considering that the airways conditioning before LV administration may be not compatible with the CF environment since CF airways are infected with opportunistic pathogens which may gain access to subepithelial tissues. Another important issue to be taken in consideration for gene transfer in that respiratory epithelium is the right choice of cellular and animal models, since studies on AAV delivery have demonstrated interspecies differences in transduction efficiency for different viral serotypes.

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