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Gene and Cell Therapy for the Treatment of Cystic Fibrosis

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ABSTRACT

Phase I/II gene therapy trials for the treatment of cystic fibrosis (CF) lung disease have demonstrated that CFTR cDNA transfer into respiratory epithelial cells is feasible, but a clinical effect is still far from reality. In addition, the duration of gene expression has been shown to be limited, lasting 1-4 weeks only. Nonviral cationic lipids and polymers, used as carriers of the CFTR gene, and recombinant viruses encounter anatomical, cellular and immunological barriers in the process of delivering genes to the relevant target cells, i.e. the epithelium lining the conducting airways. New nonviral vectors (among which polycations and chitosans) have been studied, which give higher levels of transfection in airway epithelial cells. Alternative and safer delivery methods of these nonviral vectors (magnetofection, electroporation, ultrasound) are being developed. Among recombinant viral vectors, adeno-associated viruses and lentiviruses are considered good candidates for achieving prolonged transgene expression in the airways. New model systems that are more representative of the barriers to gene transfer in the human airways are clearly needed to develop protocols and vectors for gene therapy of CF. The pig, sheep and ferret models are discussed. The final goal of CF gene therapy is to correct target cellular compartments in a lasting way. This could be only accomplished by introducing the CFTR gene in a staminal/progenitor niche in the respiratory epithelium. It has been demonstrated in mice and humans that engraftment of bone marrow-derived hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) in epithelia-lined organs can occur, provided that damage to the epithelium is done. The possibility of using HSCs and MSCs (and embryonic stem cells) in cell therapy of CF is discussed.

Keywords: airway epithelium, bone marrow-derived stem cells, cystic fibrosis, cystic fibrosis transmembrane conductance regulator, gene transfer, gene therapy, hematopoietic stem cells, mesenchymal stem cells, nonviral vectors, viral vectors
Abbreviations: AAV, adeno-associated virus; **Ad**, adenoviral vector; **BM**, bone marrow; **CF**, cystic fibrosis; **CFTR**, CF transmembrane conductance regulator; **CK**, cytokeratin; **EGFP**, enhanced green fluorescent protein; **ESC**, embryonic stem cell; **HD**, helper-dependent; **FIV**, feline immunodeficiency virus; **HIV-1**, human immunodeficiency virus type 1; **HSCs**, hematopoietic stem cells; **IL**, interleukin; **LV**, lentiviral vectors; **MSCs**, mesenchymal stem cells; **PEI**, polyethylenimine; **SeV**, Sendai virus; **SNCT**, somatic cell nuclear transfer

CONTENTS

INTRODUCTION

Cystic Fibrosis (CF) is the most common life-shortening autosomal recessive disorder in Caucasian populations and its clinical symptoms are the consequence of mutations in the CF transmembrane conductance regulator (CFTR) gene on chromosome 7.

CF affects the epithelial cells of several organs, including the respiratory tract, exocrine pancreas, intestine, vas deferens, hepatobiliary system and the exocrine sweat gland. Lung disease is the chief cause of morbidity and mortality in CF patients and current therapies are aimed at controlling the respiratory symptoms by antibiotic and anti-inflammatory treatments (Ratjen and Doring 2003). In patients with end-stage lung disease, lung transplantation is the ultimate therapeutic choice. Although the median age of survival rose from 14 years in 1969 to 35.1 years in 2004 (Cystic Fibrosis Foundation 2005), it is mandatory to find a curative treatment for CF patients.

In this review, we will focus on the barriers that oppose

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Invited Review

to an efficient gene transfer to the CF respiratory epithelium and attempts to overcome them. In the second part, we will discuss the strategies to achieve a long-term expression of the CFTR protein in the airways and the search for therapeutic end-points alternative to electrophysiologic measurements. Recent work has indicated that adult bone marrow-derived hematopietic and mesenchymal stem cells have the ability to repopulate a damaged lung epithelia. We will consider the perspectives of achieving permanent correction of the CF genetic defect by means of bone marrow-derived stem cells as well as of embryonic stem cells.

GENETICS AND PATHOPHYSIOLOGY OF CYSTIC FIBROSIS

CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily and serves as a protein kinase A (PKA)-regulated epithelial ion transporter of chloride. CFTR also regulates the airway surface liquid depth through regulation of other proteins, most prominently the epithelial sodium channel (ENaC). The missing downregulation of ENaC results in increased absorption of sodium ions and fluid across airway epithelia leading to the depletion of the perciliary liquid layer and to the depression of mucus clearance (Stutts *et al*. 1995; Matsui *et al*. 1998). CFTR also regulates other transport proteins, including K^+ channels, ATP-release mechanisms, anion exchangers, sodium-bicarbonate transporters, and aquaporin water channels (Mehta 2005). Considering all these functional interactions, it is not surprising to find that CFTR is expressed in several functionally diverse tissues, including kidney, pancreas, intestine, heart, vas deferens, sweat duct and lung (Crawford *et al*. 1991; Trezise and Buchwald 1991; Engelhardt *et al*. 1992; Trezise *et al*. 1992). In the airways, CFTR expression depends on the cell type: high levels have been found in serous cells of submucosal glands (Engelhardt *et al*. 1994), at the apical surface of ciliated cells in submucosal gland ducts and in the apical plasma membrane of all ciliated epithelial cells in the superficial epithelium (Kreda *et al*. 2005). In very recent reports it was demonstrated that CFTR is expressed in human lung alveolar epithelial type II cells (Fang *et al*. 2006; Leroy *et al*. 2006). CFTR is also expressed by cells of the immune system, like human lymphocytes (Mc Donald *et al*. 1992), human and murine alveolar macrophages (Di *et al*. 2006), and human neutrophils (Painter *et al*. 2006). Although it has been recongnized that each of these cell types display a biochemical or a functional defect linked to CFTR (Conese *et al*. 2003), the role of CFTR in the immune system is still an open question.

Over 1,400 mutations have been identified in the CFTR gene (Cystic Fibrosis Mutation Database: http://www.genet.sickkids.on.ca/cftr/), and a single mutation, deletion of phenylalanine at position 508 (ΔF508) is present in at least one chromosome in 50-90% of CF patients (Riordan *et al*. 1989). Mutations in the CFTR gene disrupt CFTR function by six different mechanisms (Gibson *et al*. 2003; Mishra *et al*. 2005; Rowe *et al*. 2005) identifying six classes: 1) premature transcription termination (class I); 2) missense mutations, including ΔF508-CFTR, causing protein misfolding and premature degradation (class II); 3) abnormal channel activation by ATP (class III); 4) reduced capacity to conduct chloride ions across membranes (class IV); 5) abnormal or alternative splicing with reduction of the amount of functional protein (class V); 6) alteration in CFTR stability at the plasma membrane (class VI). CFTR mutations are classified as severe or mild, depending on the effect on the functional protein and clinical effect (Zielenski 2000). Generally, severe mutations result in no synthesis or blocked processing (Class I, II, and III), whereas mild mutations show altered conductance or reduced synthesis (Class IV, V and VI).

CFTR is synthesized in the endoplasmic reticulum (ER) and transported to the Golgi complex where, after N-glycosylation, it becomes a mature protein that via the secretory pathway reaches the plasma membrane. The folding of CFTR occurs by complex interactions between newly synthesized CFTR and chaperones such as heat shock cognate (Hsc) 70 and, later, the ER chaperones calnexin and heat shock protein 70 (Hsp70) (Yang *et al*. 1993; Pind *et al*. 1994; Meacham *et al*. 1999). The ER quality-control mechanisms allow to export the wild-type CFTR but retain ΔF508-CFTR, and target it for degradation by the 26S proteasome (Cheng *et al*. 1990; Ward *et al*. 1995). While the interactions of these chaperones with wild-type CFTR are transient, interactions with ΔF508-CFTR are more stable. The ΔF508 mutation has other consequences, i.e. the ΔF508-CFTR has reduced capacity to transport chloride ions (Denning *et al*. 1992) and its half-life is decreased in polarized human airway epithelial cells (Swiatecka-Urban *et al*. 2005). The search for a drug to treat cystic fibrosis is focused on identifying substances that allow ΔF508-CFTR to escape out of the ER ("correctors") (Pedemonte *et al*. 2005; Servetnyk *et al*. 2006) and to activate ΔF508-CFTR channels that reach the plasma membrane ("potentiators") (van Goor *et al*. 2006; Verkman *et al*. 2006).

CFTR chloride channel activity is regulated at the plasma membrane through several signalling mechanisms, including those involving phosphorylation and dephosphorylation. CFTR assembles into large, dynamic macromolecular complexes that contain signaling molecules, kinases, transporters, ion channels, myosin molecular motors, Ras, GTPases, and PDZ-domain-containing proteins (Guggino and Stanton 2006). PDZ domains are modular protein interaction domains of ~90 amino acids known by the acronym of the first three PDZ-containing proteins identified: the postsynaptic protein PSD-95/SAP90, the *Drosophila* septate junction proteins Discs-large, and the tight junction protein ZO-1 (Hung and Sheng 2002). PDZ-containing proteins are typically involved in the assembly of supramolecular complexes that are involved in localized signaling. The last four amino acids of CFTR (Asp-Thr-Arg-Leu) constitute a consensus sequence known to bind to PDZ domain proteins. The Na^+/H^+ exchange regulatory factor isoform 1 (NHERF1) is able to bind to the C-terminus of CFTR through its PDZ1 domain (Short *et al*. 1998; Wang *et al*. 1998b). In addition, NHERF1 binds to several members of the ezrin/radixin/moesin (ERM) family of cytoskeletal adaptors. In this way, NHERF1 can cross-link multiple transmembrane proteins to the cytoskeleton to form a preapical membrane platform scaffold that can serve as a docking site for polarized membrane traffic. Because NHERF1 associates with ezrin, which binds to the regulatory subunit of PKA (Dransfield *et al*. 1997), it has been hypothesized that NHERF1 targets PKA near CFTR (Short *et al*. 1998). Indeed, NHERF1 plays a key role in the polarization of CFTR to the apical plasma membrane in epithelial cells (Moyer *et al*. 1999) and NHERF1 binding to CFTR also increases the open probability of CFTR channel (Raghuram *et al*. 2001). Importantly, over-expression of NHERF1 in human airway epithelial cells endogenously expressing ΔF508-CFTR increased both apical CFTR expression and apical PKA-dependent CFTR-mediated chloride efflux (Guerra *et al*. 2005).

Overall, these observations highlight that CFTR biogenesis and stability are complex processes, which depend on multiple pathways and proteins. An essay of this complexity has been presented recently by a proteomic approach to identify those proteins which interact with CFTR (called the CFTR interactome) (Wang *et al*. 2006). Therefore, most of the strategies aimed to correct the basic CF defect are unlikely to form treatments for all CF patients.The ultimate goal of research in this area is to provide a basis for the discovery of target-specific drugs that can cure or treat CF patients.

Lung disease in CF patients reflects chronic infection of the conducting airways with a surprisingly low number of bacterial species. *Staphylococcus aureus* and *Haemophilus influenzae* are early colonizers, whereas *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex (Bcc) often occurs later, resulting in progressive loss of lung function

and premature death. Several hypotheses link mutations in CFTR to development of lung disease in CF, whose hallmarks are bacterial infection with opportunistic pathogens and a vicious neutrophil-dominated chronic inflammatory response (Chmiel *et al*. 2002; Ratjen and Doring 2003; Boucher 2004; Rowe *et al*. 2005). Available data support the "low volume" hypothesis which postulates that due to absent chloride transport and increased sodium absorption the height of the ASL is reduced, leading to impaired mucociliary clearance (Matsui *et al*. 1998). Reduced mucociliary clearance leads to formation of thickened dehydrated mucus, which provides an ideal environment for bacterial growth, leading to chronic inflammation and ultimately organ failure in the CF lung. Recently, it has been proposed that bacterial colonization and infection might be facilitated by this pathologic environment. Bacteria invading the CF lung are trapped in the viscous mucus layer on top of respiratory epithelial cells, in which they encounter microaerophilic or anaerobic growth conditions attributable to abnormally high oxygen consumption of the CF cell (Worlitzsch *et al*. 2002). These growth conditions trigger a switch of *S. aureus* and *P. aeruginosa* from non-mucoid to mucoid cell-types, the latter representing the main phenotype in cystic fibrosis lungs (Hoiby 1995). In an *in vitro* model mimicking CF (8% solids) vs. normal (2.5% solids) airway mucus, bacteria grew in both mucus concentrations, but macrocolony formation was detected only in the CF-like mucus (Matsui *et al*. 2006). Biophysical and functional measurements revealed that concentrated mucus exhibits properties that restrict bacterial motility and small molecule diffusion, resulting in high local bacterial densities with high autoinducer concentrations. Concentrated ("thick") mucus inhibited also neutrophil migration and killing (Matsui *et al*. 2005), demonstrating that this is a component in the failure of defence against chronic airways infection in CF.

Whether inflammatory pathways are dysregulated in CF airways independent of infection, or whether the inflammatory response following bacterial infection is exaggerated and disproportional, has been widely debated, but a conclusive consensus has not been reached (Chmiel *et al*. 2002).

GENE THERAPY OF CYSTIC FIBROSIS

Gene therapy might be the definitive cure for the CF lung disease. Shortly after the CF gene was first identified, it was shown that CFTR gene transfer *in vitro* could correct the biochemical defect demonstrating that gene therapy for CF was feasible (Drumm *et al*. 1990). Gene augmentation studies in CF transgenic mice demonstrating the correction *in vivo* of the chloride transport defect (Whitsett *et al*. 1992; Alton *et al*. 1993; Hyde *et al*. 1993) paved the way to human clinical trials. The first clinical trials in CF patients were carried out in 1993 and to date about 30 trial protocols, most of which completed, have been published (see www.wiley.co.uk). In these phase I/II clinical trials the CFTR gene transfer to the airways has been achieved mostly with nonviral (cationic liposomes) or viral (adenoviruses) vectors. Both kinds of gene therapy agents have failed to give a therapeutic correction of the basic defect and persistent expression in human CF nose and lungs (Bragonzi and Conese 2002; Griesenbach *et al*. 2002b, 2004a, 2004b). However, these clinical trials provided proof-of-principle that the CFTR gene could be transferred to the airway epithelium, detection being accomplished either by means of molecular (DNA or mRNA detection) or electrophysiological (mainly by nasal potential difference) techniques. With both cationic lipids and adenoviruses, the expression was shown to last 1-4 weeks. In the following section we review the most recent clinical trials performed in CF patients with viral and nonviral vectors.

Preclinical studies demonstrated that recombinant adeno-associated virus (rAAV) was capable of achieving long-term gene transfer and expression in the bronchial epithelium of rabbits and nonhuman primates, despite the fact that vector genomes appeared to persist predominantly in the episomal state (Flotte *et al*. 1993, 1994; Afione *et al*. 1996; Conrad *et al*. 1996). AAV vectors with serotype 2 (AAV2) were tested in phase I and phase II clinical trials, which demonstrated dose-related DNA transfer and showed some indication of gene expression (Flotte 2005). In the last trial published, clinical benefit after repeated doses of aerosolized CFTR-AAV2 in the lower respiratory tract was partial and transient, with decrease in induced sputum interleukin (IL)–8 and improvement in FEV_1 observed at day 14 and 30 respectively in the rAAV-CFTR recipient group (Moss *et al*. 2004). Gene transfer but not CFTR mRNA expression was detected in bronchial brushings. In an attempt to understand the relationship between AAV molecular action and the observed clinical improvement, CFTR expression and functional correction was studied in primary cultures of bronchial and nasal respiratory cells harvested from rAAV2-CFTR recipients of the gene therapy trial previously performed (Flotte *et al*. 2003). A correlation between the presence of rAAV2-CFTR vector DNA, CFTR mRNA expression and cAMP–activated chloride channel activity was demonstrated (Flotte *et al*. 2005). These results suggest that the vector DNA is capable of providing a sizeable correction of the CF defect with low levels of mRNA expression. The physiological level of endogenous CFTR mRNA in normal individuals is only about one copy per cell (Trapnell *et al*. 1991). Thus, although the copy number of vector-derived CFTR mRNA was not calculated, it could be that the assay used in the original study (Flotte *et al*. 2003) was not sensitive enough to detect very low levels of exogenous CFTR mRNA. Alternatively, the very low level of CFTR mRNA implies the primary role of CFTR as a regulator of other chloride channels rather than a mass-action chloride channel.

A nonviral 'DNA nanoparticle' has been recently developed: it consists of polyethyleneglycol-substituted 30-mer lysine peptides. In preclinical studies, gene transfer to as many as 60-75% of murine bronchial epithelial cells has been observed after a single intrapulmonary dose (Ziady *et al*. 2003a). No side effects or histological abnormalities were evident (Ziady *et al*. 2003b). The DNA nanoparticle small size (<20 nm) is thought to facilitate the transport of genetic material through the nuclear pore. Compacted DNA nanoparticles infused onto the nasal epithelium of CFTR knockout mice generated significant NPD correction, which was correlated with the transfection efficiency (Ziady *et al*. 2002). A Phase I clinical trial completed in 2004 demonstrated in most patients evidence of partial to complete correction of the electrophysiological defect after nasal dosage (Konstan *et al*. 2004). 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 copies per cell. Partial to complete nasal potential difference isoproterenol responses were observed in eight subjects. Corrections persisted for as long as 6 days (1 subject to day 28) after gene transfer. Aerosols of compacted DNA can be formulated, and these complexes retain structural integrity and full biological activity (Oette *et al*. 2004). A follow-on clinical trial looking at single-dose aerosol delivery of DNA nanoparticles is currently planned.

Barriers to efficient gene delivery to the CF airways

It is clear from the clinical trial results that gene transfer vectors have to overcome anatomical and cellular barriers prior to delivery of the DNA to the relevant site, i.e. the nucleus of target cells. Many investigators have therefore pursued the aim to understand which are the most relevant hurdles to efficient gene delivery to the airway epithelial cells in CF. Gene transfer vectors are blocked by the blanket-like mucus, by apical membrane glycocalyx, by the lack

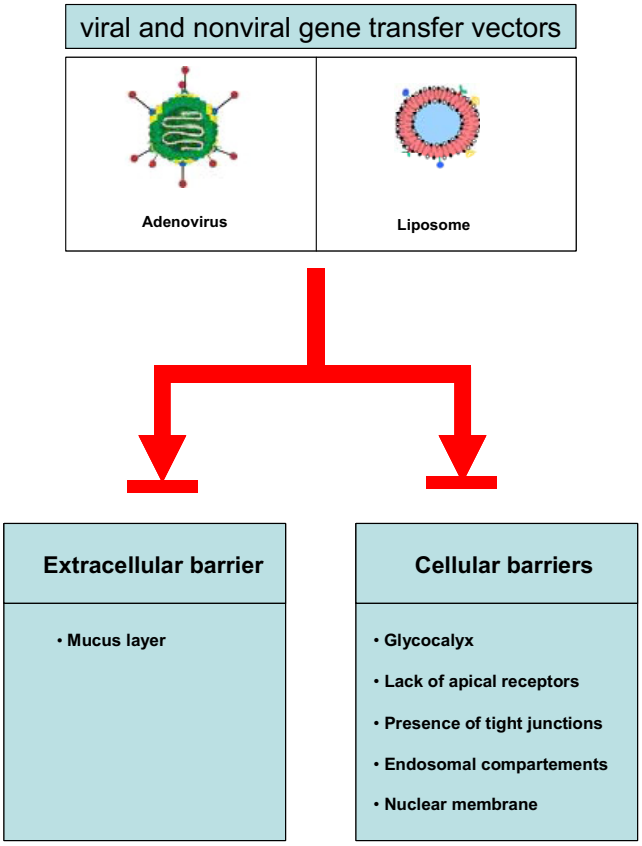

Fig. 1 Viral (adenovirus) and nonviral (cationic liposomes) gene transfer vectors do not succeed in entering the airway epithelial cells because of the presence of both extra-cellular (a thick mucus layer) and cellular barriers (the apical membrane glycocalyx, the lack of appropriate receptors in the same location, the tight junctions between the cells, the intracellular endosomo-lysosomal compartments where vectors degradation eventually occurs, and the nuclear membrane).

of appropriate receptors in the same location, by the tight junctions between the cells, by the intracellular endosomo-lysosomal compartments (Zabner *et al*. 1995; Coonrod *et al*. 1997), where their degradation eventually occurs, and finally by the nuclear membrane (Mortimer *et al*. 1999; Escriou *et al*. 2001) (**Fig. 1**).

The airway surface liquid (ASL) consists of at least two layers, a mucus layer and a periciliary liquid layer (PCL). The mucus layer consists of high-molecular weight, heavily glycosylated macromolecules, products of at least two distinct genes (*MUC5AC* and *MUC5B*), that behave as a tangled network of polymers. It appears that mucin macromolecules are well adapted to binding and trapping inhaled particles for clearance from the lung, at least in part because of the extraordinary diversity of their carbohydrate side chains (Lamblin *et al*. 2001). Most delivered vectors alighting on airway surfaces is promptly lost via airway mucociliary clearance mechanisms by epithelial cell cilial beating (Boucher 2002; Knowles and Boucher 2002). Mucociliary clearance, assisted by cough clearance, is remarkably effective in preventing airway particles, including gene vector particles, from reaching airway epithelial cells. In CF airways inspissated mucus and mucus plaques will enhance the barrier to airway gene transfer even further. The CF mucus is extremely thick and viscoelastic due to the presence of high amounts of DNA, proteins (albumin, actin and mucins), phospholipids, and inflammatory products, which originate from lysed neutrophils, bacteria and exfoliated epithelial cells (Lethem *et al*. 1990; Sheils *et al*. 1996; Sanders *et al*. 2001). Hydrogen bonds, disulphide bonds, van der Waals' forces, ionic bonds and physical entanglements between the polymeric components maintain the viscoelasticity of CF sputum and form a network that sterically hinders the diffusion of macromolecular drugs and colloidal drug carriers (Sanders *et al*. 2000a). Liposomal and adenoviral vectors are not efficient in the presence of CF mucus in a model of native sheep respiratory epithelium (Kitson *et al*. 1999). Sanders *et al.* have shown that CF sputum blocks the transport of anionic nanoparticles and cationic lipoplexes in a size-dependent fashion (Sanders *et al*. 2000b, 2003). CF sputum with high concentrations of linear DNA caused dissociation of plasmid DNA from lipoplexes (Sanders *et al*. 2001), likely causing inhibition of cationic liposome-mediated gene transfer. Both CF sputum and a mixture of DNA, mucin and actin, the three main component of CF sputum, have been shown to retard the diffusion of carboxylated 200 nm nanospheres, which were used to model the size of liposomal gene therapy vectors (Broughton-Head *et al*. 2006). Interestingly, this study also showed that the synthetic mucus sample hampered nanosphere diffusion less effectively than it was observed for CF sputa. These results underline the role of additional components in CF sputum such as alginate, phospholipids, plasma proteins and other debris of the inflammatory response that might increase the microviscosity of sputum samples. Although actin did not contribute to the barrier function of mixtures of DNA and mucin, it inhibited the ability of the mucolytics DNase and *N*-acetylcystein to enhance nanosphere diffusion through these mixtures (Broughton-Head *et al*. 2006). Overall this study indicates that none of the mucolytics tested, either alone or in combination, are likely to improve delivery of liposomal gene therapy vectors through CF sputum, thus indicating that alternative approaches are needed.

The efficiency of viscoelastic gel formulations of different viral vectors in human airway epithelial cells and in murine airways was evaluated by Sinn and colleagues (Sinn *et al*. 2005c). Gene transfer with Ad, AAV5, FIV and HIV-1-derived lentiviral vectors was enhanced by formulating them in viscoelastic gels (methylcellulose, carboxymethylcellulose sodium, poloxamer 407 polymer) which increased the vector residence time into epithelia by slowing the mucociliary clearance.

Recently, we have shown that addition of human serum albumin (HSA) to preformed polyethylenimine (PEI)/DNA complexes increased gene transfer efficiency of PEI by 500-1000 fold in immortalised airway epithelial cells (Carrabino *et al*. 2005). 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 inihibits PEI-mediated gene transfer, whereas in the presence of albumin PEI showed increased levels of gene transfer. The presence of HSA in the complexes may have impeded interaction between positively charged PEI polyplexes and negatively charged albumin exuded in the CF sputum.

Beside its function as a physical barrier, CF mucus can inhibit adenovirus (Ad)- and AAV-mediated gene transfer by the presence of preexisting antibodies (Perricone *et al*. 2000) and elevated levels of neutrophil defensins (Virella-Lowell *et al*. 2000), respectively.

Particles that do successfully evade the mucus barrier may still be captured by the glycocalyx on the airway epithelium lumenal surface. The glycocalyx is composed of several families of carbohydrate-rich molecules, including glycoproteins (most notably the mucins) (Pickles *et al*. 2000; Wang *et al*. 2002). A major component of the airway glycocalyx are the "tethered" mucins, particularly the large (>1 megadalton), heavily glycosylated MUC1 and MUC4 glycoproteins. With respect to airway gene transfer, sialoglyconjugaytes (including MUC1) expressed on the apical surface of polarized epithelial cells inhibit Ad- (Arcasoy *et al*. 1997; Pickles *et al*. 2000) and retroviral-mediated gene transfer (Wang *et al*. 2002).

Tight junctions (TJs) and uneven expression of viral receptors are formidable barriers for most viral vectors (Pilewski 2002). The TJ protein complex separates the apical from the basolateral domain and regulates permeability in epithelial and endothelial layers, acting as a selec-

tive barrier to passage of ions and other non-charged molecules (Anderson 2001). It has been established that the expression of viral receptors is more abundant on the basolateral than on the apical surface of the airway epithelium (Wang *et al*. 1998, 1999). *In vitro* and *in vivo* studies have indeed demonstrated that both adenovirus- and retrovirus-mediated gene transfer was enhanced by ethylene glycol-bis(β-aminoethylether)-tetraacetic acid (EGTA) pretreatment (Wang *et al*. 1998, 2000; Chu *et al*. 2001). The airway lumen-facing columnar cell is relatively resistant to nonviral transfer agents. This has been attributed to a decrease in binding of the cationic lipid and/or to a decline in the rate of internalization of bound complexes (Matsui *et al*. 1997; Jiang *et al*. 1998; Chu *et al*. 1999).

Uptake, intracellular trafficking and transport to the nucleus represent the major cellular impediments to successful gene delivery. There are no specific reviews dedicated to these barriers in the airway epithelium, however recent reviews cover these subjects for both nonviral (Dean *et al*. 2005; Lechardeur *et al*. 2005; Khalil *et al*. 2006; Rejman *et al*. 2006; van der Aa *et al*. 2006) and viral vectors (Anderson and Hope 2005; Campbell and Hope 2005; Ding *et al*. 2005).

Inflammation and immunity limit the efficiency and duration of gene transfer by viral and nonviral vectors

Immunological defence mechanisms can limit gene delivery and expression mediated by viral vectors (Thomas *et al*. 2003). Results from studies in experimental animals and clinical trials have shown that inflammation, antibody and T cell responses can limit the duration of transgene expression and as well as the therapeutic value of repeated administration of the gene transfer vector. Reported strategies aimed at overcoming these immunological hurdles to lung gene therapy include pharmacological treatments (immunosuppressant drugs, corticosteroids), induction of tolerance, and modification of the vector backbone, especially in the case of adenovirus (reviewed in Ferrari *et al*. 2003b).

Gene transfer into the murine airway epithelium with viral vectors (E1/E3 deleted Ad and AAV2/5) complexed with the anti-inflammatory cationic lipid dexamethasone-spermine (DS) was studied by Price and collegaues (Price *et al*. 2005). After intranasal instillation in mice, formulation of an Ad vector with cationic steroid liposomes containing DS/dioleoylphosphatidylethanolamine (DOPE) and DC-Chol/DOPE resulted in transgene expression limited to the airway epithelial cells, with poor expression in alveolar cells, whereas 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 dexametahsone-spermine.

In the search for formulations of AAV vectors alternative to serotype 2 (see below), it has been established that AAV vectors bearing capsid proteins from AAV type 5 or 6 show high transduction rates in rodent lungs and in cultured human epithelia, with transduction rates achieved by AAV6 in the range estimated to be sufficient for treating CF (Zabner *et al*. 2000; Halbert *et al*. 2001). In order to understand whether preexisting immunity may limit AAV5 and AAV6-mediated gene therapy for CF, Halbert and colleagues have measured neutralizing antibodies against AAV type 2, 5, and 6 vectors in serum from children and adults with CF (Halbert *et al*. 2006). More than 70% of CF adults and more than 85% of CF children lack serum neutralizing antibodies against AAV type 2, 5, or 6. Furthermore, 95% of CF children aged 0–10 years lacked serum neutralizing antibodies against AAV type 5 or 6. The prevalence and strength of the immune responses indicate that vectors made with AAV5 and AAV6 capsids will be most useful for avoiding preexisting immunity during lung gene therapy.

Although nonviral vectors generally do not elicit a specific humoral immune response, an inflammatory response is frequently observed with increased levels of TNF-α, IL-6 and IL-12 cytokines (Tousignant *et al*. 2000). These nonspecific responses increase the lethality of such systems, severely limiting their therapeutic potential as gene delivery vectors. Indeed, proinflammatory cytokines down-regulate the commonly used viral promoters (Paillard 1997). To avoid the use of viral promoters and to increase the duration of CFTR gene expression, which usually lasts for 1-4 weeks, Gill and coworkers substituted a human housekeeping promoter for the commonly used viral promoters (Gill *et al*. 2001). 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.

In the first trial of lipsosome-mediated CFTR transfer to the lower airways, all patients showed mild flu-like symptoms over a few hours immediately after nebulisation to the lungs (Alton *et al*. 1999). This unfavourable outcome might be related to the unmethylated CpG dinucleotide motifs present in bacterial DNA. Recognition of these motifs by the host leads to a pleiotropic inflammatory response that includes the activation of B cells, monocytes, macrophages, dendritic cells, and natural killer cells. The immunostimulatory CpG motifs within the plasmid DNA vector contribute substantially to the induction of proinflammatory cytokines by cationic lipid-DNA complexes instilled into the lungs (Freimark *et al*. 1998; Li *et al*. 1999; Yew *et al*. 1999). The current strategy is aimed at the design of plasmids with a minimal such motifs (Yew *et al*. 2000). However, it has been shown that, unlike adenovirus, lipoplexes can be readministered successfully in CF patients without apparent loss of efficacy (Hyde *et al*. 2000), even if inflammatory effects due to CpG motifs in plasmid DNA are observed (Ruiz *et al*. 2001).

We refer the readers to recent state-of-the-art articles that have reviewed the field of cystic fibrosis gene- and cell-based therapeutic approaches (Anson *et al*. 2006; Davies 2006; Griesenbach *et al*. 2006b). Here we give an overview of the most recent advances in viral and nonviral vectors as well as in delivery systems to the lung. In the last part, we will review recently acquired knowledge about stem cell capacity to differentiate into a fully differentiated airway epithelium and its application in cystic fibrosis.

New viral vectors

In the last three years an ample variety of new viral vectors have been developed and tested in animal models. They are summarized in **Table 1**. Here we focus on brief discussion concerning each type of viral vectors currently used.

The availability of vector pseudotypes in which a common AAV2-based genome is packaged in capsids from different AAV isolates has brought to the evaluation of these serotypes in the respiratory system. The most efficient in transducing cells of airway epithelium were shown to be AAV5 (Zabner *et al*. 2000), AAV6 (Halbert *et al*. 2001), and AAV1 (Wilson 2004; Virella-Lowell *et al*. 2005). Recombinant AAV2 requires access to the basolateral surface of airway epithelial cells where its main receptor, heparan sulfate proteoglycan, is most abudant (Duan *et al*. 1998b). The receptor for rAAV 5/5, 2,3-*N*-linked sialic acid, is expressed on the apical surface of airway epithelial cells (Zabner *et al*. 2000; Walters *et al*. 2001). Recenltly, it has been shown that rAAV5/5 is more efficient than rAAV2/2 and gives prolonged transgene expression (up to 32 and 52 weeks in the nose and the lung, respectively) (Sumner-Jones *et al*. 2006). Sustentacular cells of the olfactory epithelium in the nose and alveolar type 2 cells in the lung were found almost exclusively to express the transgene.

Limberis and colleagues have recently shown that AAV2/9 is equally effective in transduction of polarized airway cells after apical or basolateral application (Limberis and Wilson 2006). AAV2/9-mediated nuclear β-galactosidase gene transfer in nasal and lung airways was relatively

Table 1 Recent research on viral vectors and airway epithelium in animal models and *in vitro*.

Vector	Study	Reference
1) *In vivo* studies		
AAV2/2, AAV2/5 and AAV2/9 serotypes	Transduction efficiency in human airway epithelial cells, efficiency and persistence in the mouse lung and nasal epithelium. Feasibility of repeated administration.	Limberis and Wilson 2006
AAV1, AAV2, AAV5 serotypes	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 / βactin hybrid promoter) in murine airway epithelia.	Virella-Lowell *et al*. 2005
AAV2 serotype	Transgene expression with serial doses of aerosolized AAV2 vector in Rhesus Macaques.	Fischer *et al*. 2003
AAV5, FIV- and HIV-1-derived LV vectors pseudotyped with the baculovirus *Autographa californica* GP64 envelope	Efficiency of viscoelastic gel formulations of different viral vectors in human airway epithelial cells and in murine airways.	Sinn *et al*. 2005c
E1/E3 deleted Ad and AAV2/5	Gene transfer into the murine airway epithelium with viral vectors complexed with the anti-inflammatory cationic lipid dexamethasone-spermine.	Price *et al*. 2005
HD-Ad containing an epithelial-cell specific expression cassette cytokeratin 18 (CK 18)	Efficiency and persistence of gene expression in murine airway epithelium.	Toietta *et al*. 2003
HD-Ad with a nuclear localization signal, under the control of a human CK 18 expression cassette	Aerosol delivery of an improved version of a HD-Ad vector expressing β-galactosidase reporter gene and formulated with LPC into rabbit respiratory tract using intratracheal catheter.	Koehler *et al*. 2005
HD-Ad and first generation Ad type 5	Persistence of transgene expression and humoral immune response to the vector after readministration of an helper dependent adenovirus into the mouse lung.	Koehler *et al*. 2006
Canine Ad type-2, HD-Ad5 and Ad. All the vectors were deleted of the E1 region	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.	Keriel *et al*. 2006
FIV-derived LV pseudotyped with the JSRV envelope glycoprotein	Gene transfer into polarized primary cultures of human respiratory epithelial cells and rabbit respiratory tract.	Sinn *et al*. 2005b
FIV-derived LV	Persistence of gene transfer into the mouse nasal epithelia.	Sinn *et al*. 2005a
HA or EIAV pseudotyped HIV-1-derived LV vector	Transduction efficiency in models of human and mouse airway epithelium.	McKay *et al*. 2006
HIV-1-derived LV pseudotyped with VSV-G	*In vivo* gene transfer into the tracheobronchial tree of fetal rabbits.	Skarsgard *et al*. 2005
SIN HIV-1-derived LV pseudotyped with VSV-G	Safety and efficiency of intrapulmonary fetal gene transfer into rhesus monkeys.	Tarantal *et al*. 2005
First generation E1-E3 deleted serotype 5 Ad vectors	Intra-amniotic injection of Ad vectors into mice.	Buckley *et al*. 2005
Non transmissibile, replication competent recombinant SeV developed by deleting the envelope Fusion (F) gene (SeV/ΔF)	Gene transfer into respiratory epithelial cells, in *ex vivo* models of differentiated airway epithelium and *in vivo* in murine airways.	Ferrari *et al*. 2004
Neuroaminidase-deficient influenza virus	Gene transfer into murine airways to study the feasibility of delivery of foreign protein and production of vaccines against infectious disease.	Shinya *et al*. 2004
SeV deleted of matrix (M) and fusion (F) genes (SeV/ΔMΔF)	Production and propagation of SeV/ΔMΔF vector in packaging cell line, cytotoxicity and gene transfer efficiency in murine airways *in vivo*.	Inoue *et al*. 2004
2) *In vitro* studies		
AAV5	Development of a short adeno-associated virus expression cassette for CFTR gene transfer to differentiated CF airway epithelia.	Ostedgaard *et al*. 2005
AAV2 and AAV5	Characterization of proteasome modulating agents for their ability to enhance AAV transduction.	Yan *et al*. 2004
AAV2 and AAV2/5	Investigation of pharmacological strategies to improve efficiency of CFTR gene delivery with AAV vectors while simultaneously inhibiting ENaC activity into CF cells.	Zhang *et al*. 2004
AAV, E1 deleted Ad vector and LV vector	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.	Sorscher *et al*. 2006
AAV2	Role of heparan sulfate in AAV2 transduction of human airway epithelial cells.	Boyle *et al*. 2006
AAV2 and AAV5	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 ΔF508 mice for studies of CFTR complementation.	Liu *et al*. 2006b
AAV2	Evaluation of alternative serotypes and promoters to enhance transduction mediated by AAV vectors.	Sirninger *et al*. 2004
rAAV2 and rAAV5	Rescue of CFTR chloride conductance in polarized human CF airway epithelial cells, using a spliceosome-mediated RNA *trans*-splicing (SMaRT) approach.	Liu *et al*. 2005
HD-Ad	Gene transfer into sweat glands in human skin organ culture.	Lee *et al*. 2005
First generation and E1 deleted Ad, HD-Ad-CMV and HD-Ad-CK18	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 the epithelial Cl^- transport defect.	Farmen *et al*. 2005
HIV-1–derived VSV-G pseudoytyped LV vector	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.	Castillon *et al*. 2004

Table 1 (Cont.)

Vector	Study	Reference
HIV-1-derived lentivirus pseudotyped with JSRV envelope glycoprotein	Evaluation of transduction efficiency in primary and immortalized human lung epithelial cells	Liu *et al*. 2004
Ad2 and Ad5	Role of glycocalix and tethered mucins in restriction of Ad transduction from the apical membrane of respiratory epithelial cells	Stonebraker *et al*. 2004
E1/E3 deleted Ad	Expression and activity of functional GFP-tagged CFTR	Vais *et al*. 2004
Ad5	Phage biopanning in CFTR deficient human tracheal glandular cells (CF-KM4) to find specific peptide ligands that confer cell target specificy and enhance gene transfer mediated by Ad5 vector	Gaden *et al*. 2004

Ad = adenovirus; AAV = adeno-associated virus; HD-Ad = helper-dependent adenovirus; CK 18 = cyokeratin 18; EIAV = Equine infectious anemia virus; FIV = feline immunodeficiency virus; HA = hemoagglutinin; HIV-1 = human immunodeficiency virus type 1; JSRV = Jaagsiekte sheep retrovirus; LPC = Lα-Lysophosphatidylcholine; LV = lentivirus; SeV = Sendai virus; SIN = self inactivating.

stable for 9 months. 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 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.

Helper-dependent (HD)-Ad 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. Following intranasal instillation in mice of a HD-Ad containing an epithelial cell-specific expression cassette cytokeratin 18 (CK 18), 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 (Toietta *et al*. 2003). In contrast with first-generation Ad vectors inflammation was negligible. This study suggests that epithelial cell specific promoter CK 18 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. Aerosol delivery of an improved version of a HD-Ad expressing nuclear β-galactosidase reporter gene to rabbit respiratory tract was accomplished when formulated with Lα-lysophosphatidylcholine (LPC) (Koehler *et al*. 2005). All type of cells of the surface epithelium were transduced; in the trachea 66% of cells were transduced after 0.1% LPC administration, while pretreatment with 0.01% LPC resulted in transduction of 24% of cells. Re-adiministration of HD-Ad to murine airways in mice resulted in minimal loss of transgene expression, as compared to readministration of a first generation Ad vector (Koehler *et al*. 2006), indicating that these vectors would be suitable for repeated administration to the lung.

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 tranduce the airway epithelium (Copreni *et al*. 2004; Wilson 2004). In the nose of CF mice, they can mediate CFTR gene expression and correction of the electrophysiological defect up to 110 days (Limberis *et al*. 2002). Lack of receptors for LV vectors on the apical surface of the airway epithelium limit their use so far. Pseudotyping with heterologous envelopes (Sinn *et al*. 2005a, 2005b; McKay *et al*. 2006) and modification of the paracellular permeability (Sinn *et al*. 2005c) 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 (Skarsgard *et al*. 2005; Tarantal *et al*. 2005).

Recombinant Sendai virus (SeV) is a murine paramixovirus which requires short contact time with the target cells for internalization and replicates in the cytoplasm of transduced cells, circumventing the nuclear membrane barrier. SeV transduced airway epithelial cells in a variety of animal models efficiently *in vivo* (Yonemitsu *et al*. 2000; Griesenbach *et al*. 2002a). Preliminary data show that SeV carrying the CFTR cDNA is able to partially correct the characteristic CFTR-dependent chloride transport defect in the nasal epithelium of CF knockout mice (Ferrari *et al*. 2003a). However, 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 (Ferrari *et al*. 2004; Griesenbach *et al*. 2006a).

New nonviral vectors

There is a constant search for new nonviral vectors with optimal efficiency/toxicity profile that are able to deliver therapeutic genes to the lung. **Table 2** summarizes the most recent studies in this field. The use of polymers instead of lipids confers several advantages, due to their ease of preparation, purification and chemical modification as well as their enorrmous stability (Lungwitz *et al*. 2005). Among the variety of cationic polymers, polylysine, PEI, and chitosans seem to be the most promising gene transfer agents to the lung.

A modified polylysine-based vectors has been shown to be efficient and safe when administered to the airways in pre-clinical animal models for CF and in CF patients and it has been discussed in a previous section (Ziady *et al*. 2003a, 2003b; Konstan *et al*. 2004).

The polymer PEI has been shown to be one of the more effective agents for DNA delivery to the lung of animal models (Lemkine and Demeneix 2001). Various forms of PEI have been tested in the airways, with a more favourable therapeutic index (i.e. less toxicity with increased efficiency) than cationic lipids (Gautam *et al*. 2001; Smolarczyk *et al*. 2005). Recently, Dif and colleagues have shown that within 18-20 hours after injection through the tail vein, DNA/PEI complexes have already crossed the capillary barrier resulting in high levels of expression of reporter genes in the lungs (Dif *et al*. 2006). Transgene expression is observed in endothelial cells, in type I and type II pneumocytes, and in septal cells. Coexpression of the transgene and of the endogenous *Cftr* gene was observed in some of the targeted epithelial cells. Levels and sites of expression were similar in normal and in CFTR-mutant mice.

To overcome the limited transgene expression in the lung obtained with nonviral vectors, a new gene delivery vector based on the *Sleeping Beauty* (SB) transposase was used. The SB transposase is an enzyme that recognizes the ends of a transposon (indirect repeats, IRs), excises the transposon from its location in the genome, and reinserts it elsewhere into chromosomal DNA. By introducing a therapeutic gene between transposon IRs and supplying the transposase function, it is possible to use this transposon as a vector for gene therapy. Belur and colleagues demons-

Table 2 Recent research on nonviral vectors and airway epithelium in animal models and *in vitro*.

Cationic lipid/cationic polymer/molecular conjugate	Study	Reference
1) Studies with CF mice		
Linear PEI 22 kDa, branched PEI 25 kDa, branched PEI 50 kDa	Efficiency of gene expression in the airways after intranasal instillation. Efficacy study in CF-null mice.	Wiseman *et al*. 2003
PEI 22 kDa	Localization of transfected cells at the level of the lung after ntravenous in wild-type and CF mice.	Dif *et al*. 2006
2) *In vivo* studies		
PEG-substituted poly-L-lysine	Efficiency of gene expression in the mouse lung after intratracheal and intranasal instillation.	Ziady *et al*. 2003a
PEG-substituted poly-L-lysine	Safety (lung and systemic inflammation) after intranasal instillation in mice.	Ziady *et al*. 2003b
Nonionic amphiphilic block copymers	Efficiency and safety after intratracheal injection and aerosolization in mice.	Desigaux *et al*. 2005
Lipofectin (L), integrin-targeting peptide (I), DNA (D)	Efficiency and safety after intratracheal instillation in mice. Repeated administrations.	Jenkins *et al*. 2003
Lipofectin (L), integrin targeting peptide (I), DNA (D)	Effect of EGTA pretreatment on the efficiency of the vector administered *via* intratracheal injection in mice.	Meng *et al*. 2004
Cationic lipid GL67	Efficiency and toxicity following bronchoscopic instillation in lung segments of sheep.	Emerson *et al*. 2003
Cationic lipid GL67, PEI 25 kDa	Efficiency, dose-response effect and toxicity following aerosol delivery into the ovine lung.	McLachlan *et al*. 2007
Cationic lipid GL67	Efficiency and efficacy study (on ENaC) in mice following intranasal instillation of antisense oligonucleotides and synthetic siRNAs.	Griesenbach *et al*. 2006c
DOTAP/cholesterol protamine, naked DNA	Efficiency and distribution of oligonucleotides following intravenous delivery in mice. Effect of inflammatory mediators.	Holder *et al*. 2006
TAT peptide-PEG-PEI	Efficiency and toxicity of the vector administered via intratracheal injection in mice.	Kleemann *et al*. 2005
PEI 25 kDa, chitosan	Study of gene expression by microarray analysis after intratracheal administration to mice.	Regnstrom *et al*. 2006
PEI 25 kDa	Efficiency and biodistribution of DNA complexes after aerosol and intratracheal in mice.	Rudolph *et al*. 2005
PEI 25 kDa	Biodistribution and clearance from the lungs after nebulization in different mouse strains.	Dames *et al*. 2006
PEI/albumin	Cytokine levels in blood after intravenous injection in mice. Effect of dexamethasone.	Smolarczyk *et al*. 2005
Chitosan	Structure-function relationship. Comparison of chitosan and high-molecular-weight chitosan *in vitro* and after intratracheal administration to the mouse lungs.	Koping-Hoggard *et al*. 2003
Chitosan	Comparison of chitosan and high-molecular-weight chitosan after intratracheal administration to the mouse lungs.	Koping-Hoggard *et al*. 2004
Chitosan, PEI	Efficiency study with a new nebulization catheter device. Comparison with intratracheal instillation.	Koping-Hoggard *et al*. 2005
Thiolated chitosan	Biophysical characterization of nanoparticles. Efficiency of transfection after intranasal administration in BAL cells.	Lee *et al*. 2007
Trisaccharide-substituted chitosan oligomers	Efficiency *in vitro* and after intratracheal administration to the mouse lungs.	Issa *et al*. 2006
Poly (4-vinylimidazole) (P4V), PEI	Expression of human osteoprotegerin (hOPG) in the spleen and the lung after intravenous injection in mice.	Ihm *et al*. 2005
Lipofectamine 2000 or cationic lipid 67 (GL67) coupled to superparamagnetic particle TransMAG^{PEI}	Perfusion of nasal cavity with TransMAG^{PEI} in mice.	Xenariou *et al*. 2006
PEI 22 kDa/*Sleeping beauty* transposase	Duration of gene expression in the lung after a single intravenous injection in the mouse.	Belur *et al*. 2003
PEI 22 kDa/*Sleeping beauty* transposase	Delivery of a transposon encoding the human gene indoleamine-2,3-dioxygenase to the rat lung via intratracheal injection. Therapeutic efficacy in a model of lung allograft.	Liu *et al*. 2006a
3) *In vitro* studies		
PEI 25 kDa	Transfection efficiency and viability of immortalised CF airway epithelial cells in the presence of low molecular weight heparin (3 kDa).	Dragomir *et al*. 2004
PEI 25 kDa/albumin	Efficiency of PEI/DNA complexes added with HSA in human airway epithelial cells in the presence of CF mucus.	Carrabino *et al*. 2005
Cationic phosphonolipids	Efficiency and toxicity of KLN 30 in primary airway epithelial cells obtained from non-CF and CF ΔF508 nasal polyps. Expression of wild-type CFTR.	Montier *et al*. 2004
Poly-lysine peptides	Efficiency of the alkylated-Cys-Trp-Lys$_{18}$ (CWK$_{18}$) DNA condensing peptide in HepG2 and CF/T1 cells. Effect of the proteasome inhibitors.	Kim *et al*. 2005b
Lactosylated PEI 25 kDa (Lac-PEI), glycosylated polylysines	Efficiency in CF-KM4 cells and primary bronchial epithelial cells.	Fajac *et al*. 2003
Lac-PEI	Efficiency in ΣCFTE29o- and primary bronchial epithelial cells. Analysis of transcription rates.	Grosse *et al*. 2004
Lac-PEI, glycosylated polylysines	Electron microscopy study in ΣCFTE29o- and primary bronchial epithelial cells.	Grosse *et al*. 2005
Uronic acid-PEI and uronic acid-PEI-PEG copolymers	Efficiency and receptor-mediated endocytosis study in 16HBE14o$^-$, HeLa and HepG2 cells.	Weiss *et al*. 2006
Lipofectin (L), integrin-targeting peptide (I), DNA (D)	Efficiency in 1HAEo- and 2CFSMEo- cells. Effect of EGTA.	Meng *et al*. 2004
Cationic lipids	Effect on CFTR and RANTES expression in polarized CF human bronchial epithelial cells.	Tucker *et al*. 2003

BAL=bronchoalveolar lavage; DOTAP= 1,2-dioleoyl-3-trimethylammonium propane; PEG= polyethyleneglycol; PEI=polyethylenimine; siRNA=small interference RNA; TAT=*trans*-activating transcriptional activator from HIV-1

trated transgene expression after 24 hours in the lungs of all animals intravenously injected with the luciferase transposon complexed to PEI (linear 22 kDa), but expression up to 3 months required co-delivery of a plasmid encoding the SB transposase (Belur *et al*. 2003). Transgene expression was localized to the alveolar region of the lung, with transfection of mainly type II pneumocytes.

SB-mediated gene delivery was recently shown to be therapeutically useful in rat model of lung allograft fibrosis (Liu *et al*. 2006). An improved SB transposon encoding the human gene indoleamine-2,3-dioxygenase (IDO), an enzyme that possess both T cell-suppressive and antioxidant properties, was complexed with linear PEI and delivered together with a tranposase plasmid to the donor lung via intratracheal injection 24 hours prior to transplantation. IDO activity produced in lung allografts showed a remarkable therapeutic response, as evident by near pulmonary function, histological appearance, and reduced collagen content in lung allografts.

Chitosans, a family of linear binary polysaccharides comprised of (1-4) linked 2-amino-2-deoxy-β-D-glucose (GlcN) and the N-acetylated analogue (GlcNAc) are biocompatible cationic polymers, suitable for plasmid DNA gene delivery to the lung, with low cytotoxicty (Koping-Hoggard *et al*. 2001). Recently, the most effective gene delivery after lung administration *in vivo* was seen with chitosan oligomers with a number-average degree of polymerisation (DPn) of around 18 monomer units (DPn18) (Koping-Hoggard *et al*. 2003, 2004). The oligomer-based polyplexes had reduced viscosity and were less prone to aggregation in the more concentrated solutions used for *in vivo* application. Importantly, these polyplexes released plasmid DNA more easily than conventional high molecular weight chitosans, resulting in a faster onset and higher levels of *in vivo* gene expression.

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) (Ziady *et al*. 2002) or glycids (Grosse *et al*. 2005; Issa *et al*. 2006), the synthesis of polymer conjugates with HIV-1 TAT-derived peptides (Kleemann *et al*. 2005), or the addition of a protein moiety (Smolarczyk *et al*. 2005).

New delivery systems for nonviral vectors

A variety of nonviral vectors, including cationic lipids, polycationic polymer complexes, and proteolipidic vectors, have been developed for administering DNA by inhalation (Densmore 2006). Recently, it has been reported that the branched form of PEI stabilised DNA during the nebulization process and exhibited a higher degree of specificity for the lungs, with virtually no evidence of plasmid DNA in other tissues after this mode of delivery (Densmore *et al*. 2000; Gautam *et al*. 2000; Koshkina *et al*. 2003). 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 (Gautam *et al*. 2001).

The delivery of the CFTR gene to the target cells of the airways, e.g. by nebulization, might be enhanced by a variety of adjunctive physical interventions including electroporation or magnetofection (Griesenbach *et al*. 2004a). Magnetic targeting of gene delivery is achieved by application of a magnetic field to superparamagnetic iron oxide particles associated with the gene vectors (Scherer *et. al*. 2002; Plank *et al*. 2003). In this technique, cationic polymer PEI coated superparamagnetic nanoparticles are complexed to plasmid DNA under the addition of free PEI ($TransMAG^{PEI}$). Magnetofection was compared with conventional nonviral gene transfer methods such as lipofection and polyfection in permanent and primary airway epithelial cells (Gersting *et al*. 2004). Magnetofection was shown to deliver DNA to the cells more rapidly than PEI-polyfection and led to increased vector accumulation in the target cells. Magnetofection was the most efficient when applied to primary human airway epithelial cells. It also led to significant gene expression at very short incubation times in an *ex vivo* porcine airway epithelium organ model. However, a recent study has failed to demonstrate a positive effect of magnetoparticles and magnetic field on the *in vivo* efficiency of the cationic lipid GL67 (Xenariou *et al*. 2006). In experiments performed with perfusion of the mouse nasal cavity, the authors showed a decrease in gene expression for GL67 coupled to $TransMAG^{PEI}$ compared to non-magnetic particles. Better formulations and probably other animal models will be required to optimize the magnetofection technique *in vivo*.

In vivo gene transfer to the lung has been attempted by electroporation. After intratracheal instillation of naked plasmid DNA, mice were given a series of eight wave electric pulses each at an optimal field strength of 200 V/cm through electrodes placed on the chest (Dean *et al*. 2003). Gene expression was detected already one day after electroporation and further increased reaching its maximum between 2 and 5 days. By the day 7, expression was back to baseline. In contrast, essentially no gene expression was detected in the absence of electric pulses. The cell types expressing gene product include alveolar type I and type II epithelial cells. No inflammation or injury was observed in the lung neither after 1 nor 24 hours after electroporation, as detected by histology and cytokine measurements.

A further improvement of this method has been applied to the rat lung (Machado-Aranda *et al*. 2005). Purified plasmid was delivered to the lungs of anesthetized rats through an endotracheal tube, and a series of square-wave pulses were delivered via electrodes placed on the chest. Relatively uniform gene expression was observed in multiple cell types and layers throughout the lung, including airway and alveolar epithelial cells, airway smooth muscle cells, and vascular endothelial cells. Gene expression was dose- and pulse length-dependent. Most importantly, no inflammatory response was detected. To demonstrate efficacy of this approach, the β1 subunit of the Na^+, K^+-ATPase was transferred to the lungs of rats with or without electroporation, and 3 days later, alveolar fluid clearance was measured. Animals electroporated with the β1 subunit plasmid showed a two-fold increase in alveolar fluid clearance and Na^+,K^+-ATPase activity as compared with animals receiving no electroporation.

A recent study combined *in vivo* electroporation and a long-acting promoter system for gene transfer to the lung (Gazdhar *et al*. 2006). Plasmids expressing luciferase under the control of the cytomegalovirus immediate-early promoter/enhancer (CMV-IEPE) or human polyubiquitin c (Ubc) promoter suspended in water were instilled into the left lung of anesthetized rats, followed by left thoracotomy and electroporation of the exposed left lung. Gene expression with the CMV-IEPE promoter was highest 24 hours after gene transfer and returned to baseline by day 3; at day 5 no expression was detected, whereas gene expression under the Ubc promoter was detected up to day 40. Arterial blood gas (PaO_2), histological assessment and cytokine measurements showed no significant toxicity neither at day 1 nor at day 40. Overall, these studies provide evidence that *in vivo* electroporation is a safe and effective tool for nonviral gene delivery to the lungs. If this method is used in combination with a long-acting promoter system, sustained transgene expression can be achieved.

Recently, it has been assessed if high-frequency ultrasound (US) can enhance nonviral gene transfer to the mouse lung (Xenariou *et al*. 2007). Cationic lipid GL67/pDNA, PEI/pDNA and naked plasmid DNA were delivered via intranasal instillation, mixed with Optison microbubbles. Subsequently, the animals were exposed to 1 MHz US. Addition of Optison alone significantly reduced the transfection efficiency of all three gene transfer agents. US exposure did not increase GL67/pDNA or PEI/pDNA gene transfer compared to Optison-treated animals. However, it increased naked pDNA transfection efficiency by approximately 15-fold compared to Optison-treated animals, suggesting that

despite ultrasound being attenuated by air in the lung, sufficient energy penetrates the tissue to increase gene transfer. However, it should be noticed that the application of US induced lung haemorrhage, assessed histologically. The left lung was more affected than the right and this was mirrored by a lesser increase in naked pDNA gene transfer. The positive effect of US was dependent on Optison, as in its absence US did not increase naked pDNA transfection efficiency. Further refinement of US-mediated gene transfer by cationic vectors in the murine lung is needed.

New animal models

Designing and implementing new gene transfer protocols of relevance to human clinical trials demands assessment using appropriate *in vivo* model systems. For initial screening, the mouse is the preferred animal model, due to its small size and usefulness to obtaining large experimental numbers. However, one should be aware that barriers, which might impede gene transfer in the murine lung, differ from those found in the human lung. Human lungs possess abundant submucosal glands in bronchi which mice do not, which leads to the production of fewer secretions in the murine lung than in human. Furthermore, CFTR mRNA levels are lower throughout murine pulmonary epithelium than in the human lung. Experimental data indicate that Cl^- secretion in murine lung is performed predominantly by an alternative Cl^- channel (Rochelle *et al*. 2000). Therefore, CF mice do not develop the severe inflammatory response and thickened mucus associated with CF lung disease, which limits their value for the assessment of efficacy of gene therapy for CF lung disease (Grubb and Boucher 1999).

Recently, a new murine animal model was created. To study the mechanistic links between the altered ion transport processes and pathogenesis of CF lung disease mice with airway-specific overexpression of β subunit of ENaC were generated (Mall *et al*. 2004). The increased airway Na^+ absorption *in vivo* caused ASL volume depletion, increased mucus concentration, delayed mucus transport and mucus adhesion to airway surfaces. Defective mucus transport caused a severe spontaneous lung disease sharing features with CF, including mucus obstruction, goblet cell metaplasia, neutrophilic inflammation and poor bacterial clearance. This study reveals that increasing airway Na^+ absorption initiates CF-like lung disease and produces a model for studying novel therapeutic approaches in the presence of mucus. It is important to note that CFTR expression and function is normal in this animal model. Double transgenic mice (knock out for CFTR and over-expressing βENAC) should give an answer about the relevance of these mice for the comprehension of CF lung disease and for application of therapeutic interventions.

Pigs and sheep seem to be better animal models to develop protocols and vectors for gene therapy of CF. Pig lungs share many anatomical and physiologycal similaraties with those of human lungs. Their bronchi show similar patterns of branching and histology, possess a similar abundance of submucosal glands and have similar patterns of glycoprotein synthesis and secretion (Hartmann *et al*. 1984; Mills *et al*. 1986). Similarities have also been reported in the immune system in the lungs of the pigs and humans that may be relevant to the evaluation of inflammatory and immune responses to vector treatment (Pabst and Binns 1994; Pabst 1996). Moreover, pigs express CFTR in their bronchial epithelium and submucosal glands, which mediates secretion of chloride and bicarbonate anions and liquid, similar to humans (Ballard *et al*. 1999). Bronchoscopic administration of a nonviral vector to the right lower lobe of piglets resulted in high efficiency of gene transfer with 46% of large bronchi staining positively (Cunningham *et al*. 2002). There was no evidence for vector-specific inflammation assessed by leukocytosis and TNF-α production. The successful production of cloned piglets by somatic cell nuclear transfer (SCNT) (Polejaeva *et al*. 2000) has opened up the possibility of generating genetic knockouts (Rogers *et al*. 2006), which surely will play a role in designing efficient and safe gene therapy vectors for CF.

Both anatomical and physiological features make the sheep lung an attractive model for gene therapy Primary and secondary bronchioles consist of basal, intermediate, ciliated, and nonciliated (Clara) cells and in general the fine structure, localization, and composition of cellular populations of the bronchiolar and alveolar epithelium are similar to those of other mammalian species (Boulijhard and Lieipold 1994). Moreover, there is a very high degree of evolutionary and functional similarity between the human and the sheep CFTR gene (90% identity at the protein level; (Tebbutt *et al*. 1995). The ovine model was used to test the relative efficacy of different gene transfer agents by direct instillation into the lung (Emerson *et al*. 2003). A recent study demonstrated distribution and reporter gene expression throughout the lung after aerosol administration of plasmid DNA complexed to the cationic lipid GL67 or PEI (McLachlan *et al*. 2007). GL67 and PEI achieved significant levels of gene expression compared to controls in the more distal region of the lung. Importantly, a major difference between aerosol and instillation delivery of DNA/GL67 relates to toxicity. Instillation caused a severe airway-centric acute inflammatory response, which often extended to the peribronchiolar interstitial tissue leading to consolidation, bronchiolar destruction, extensive alveolitis, and alveolar neutrophil exudates (Emerson *et al*. 2003). Aerosol delivery resulted in consistently less severe pathological changes than instillation, as previously observed in mice (Eastman *et al*. 1997). This is probably due to the improved distribution and absence of pooling effects.

Importantly, it has been recently demonstrated that specific anti-human CFTR antibodies can detect vector-derived human CFTR in sheep trachea after instillation of plasmid DNA/PEI complexes or in cryosections of sheep airways after whole lung aerosol delivery of DNA/PEI complexes (Davidson *et al*. 2006).

The domestic ferret, *Mustela putorius furos*, has proven to be an excellent animal model for studying CFTR lung biology In contrast to mice, the ferret lung has marked similarities to the human lung in terms of physiology, airway morphology, and cell types (Plopper *et al*. 1980; Leigh *et al*. 1986; Oldham *et al*. 1990; Duan *et al*. 1998a; Kishioka *et al*. 2001; Wang *et al*. 2001). Moreover, the expression of CFTR in the ferret airway epithelium and submucosal glands is identical to that in humans (Engelhardt *et al*. 1992; Sehgal *et al*. 1996). In addition, amino acid identity between ferret and human nucleotide binding domain 1 (NBD1) of CFTR is a striking 97% (Sehgal *et al*. 1996), which is just as high as for non-human primates (96%, *Macaca nemestrina*) and is significantly higher than for rodents (80%, rat and mouse). Fourth, the ferret had been a useful model for viral and bacterial lung infections seen in humans (Leigh *et al*. 1995a; Kishioka *et al*. 1999). Furthermore, the ferret, with a gestation period of 42 days and 6 months to sexual maturity, has obvious advantages over larger animal models. These features make the ferret an ideal choice for modeling genetic lung disease such as CF (Li and Engelhardt 2003). Recently, Engelhardt's group has reported the successful production of live cloned, reproductively competent, ferrets using species-specific SNCT methodologies (Li *et al*. 2006).

The search for new therapeutic end-points

In clinical trials, the primary endpoints to evaluate efficiency include quantification of vector DNA, mRNA, and protein (mostly as chloride channel activity). Although the "gold standard" for gene therapy of CF lung disease is the measure of nasal and bronchial potential difference (Griesenbach and Boyd 2005), some concerns have been raised. The question is whether the change of few millivolts obtained upon application of CFTR-bearing vectors may be considered to represent successful gene therapy (Zeitlin 2000). Thus, alternative functional secondary assays have been searched for, in particular ones directed towards eva-

luation of the inflammatory response and bacterial infection.

None of the several mouse models produced thus far was found to develop the lung disease characteristic of CF spontaneously (Davidson and Rolfe 2001), although it was soon discovered that lung disease could be induced by exposure to high levels of bacteria (Davidson *et al*. 1995).

Koehler *et al.* have developed a HD-Ad vector which harbors control elements from CK 18 to express CFTR (Koehler *et al*. 2001). Wild-type mice repeatedly instilled with *Burkholderia cepacia* complex (Bcc) cleared the bacteria within 9 days, whereas CF knock out mice retained Bcc and succumbed to severe bronchopneumonia, exhibiting many of the histological signs of human CF lung disease including neutrophilia (Saijan *et al* 2001). $CFTR^{-/-}$ mice receiving HD-Ad-CFTR 7 days before Bcc challenge had less severe histopathology, and the number of lung bacteria was reduced to the level seen in $CFTR^{+/+}$ littermates (Koehler *et al*. 2003).

van Heeckeren *et al.* observed that pretreatment of gut-corrected CF knockout mice with an adenoviral vector expressing CFTR (Ad2/CFTR-16) improved survival following challenge with *P. aeruginosa*-laden agarose beads (van Heeckeren *et al*. 2004). However, no statistically significant difference in survival was observed between mice pretreated with Ad2/CFTR-16 and those treated with the empty vector (Ad2/EV). The other hallmark of the response of CF mice to *P. aeruginosa*-laden agarose beads, the increased inflammatory response, was not diminished in the mice pretreated with Ad2/CFTR-16 compared to mock-treated animals. Moreover, there was no effect on the bacterial clearance from the lungs in Ad2/CFTR-16-treated mice as compared to those treated with the empty vector. A possible explanation of the different outcome of the two studies is that the Ad vector used by van Heeckeren *et al.* is highly inflammatory, whereas the HD-Ad vector used by Koehler *et al.* does not express adenovirus genes and therefore causes little inflammation. These results highlight the notion that new vectors with a better efficiency/safety profile are needed for correcting the hypersusceptibility of CF airways to bacterial challenge.

Analysis of inflammatory markers in sputum and broncho-alveolar lavage fluid (BALF) may be an important secondary endpoint. Previous studies of the bronchopulmonary secretions of CF subjects showed increased concentrations of inflammatory markers, such as IL-8, IL-6, IL-1β, TNF-α, leukotriene B_4, and free neutrophil elastase, and decreased IL-10 (Dean *et al*. 1993; Konstan *et al*. 1993; Lawrence and Sorrell 1993; Bonfield *et al*. 1995; Khan *et al*. 1995; Salva *et al*. 1996; Noah *et al*. 1997; Muhlebach *et al*. 1999). Interestingly, a small change in cytokine expression has been reported in sputum in at least three gene therapy trials. Alton *et al.* reported a decrease in sputum IL-8 after administration of liposome/CFTR gene transfer (Alton *et al*. 1999). Wagner *et al.* described an increase in the anti-inflammatory cytokine IL-10 after administration of adeno-associated virus-CFTR (AAV-CFTR) to the maxillary sinus of CF patients (Wagner *et al*. 2002). In the first repeated-administration lung trial of AAV2 to the CF lung a reduction in IL-8 in induced sputum after the first, but not after subsequent administrations, was observed. This reduction in efficacy on re-administration of the AAV vector may in part be caused by the development of an immune response after the first administration (Moss *et al*. 2004).

Other more updated techniques may play a role in clinical trials in the near future, such as custom-made chips for microarray analysis of gene expression after CFTR transfer and mass spectrometry to detect subtle changes in CFTR and other proteins in CF biological fluids (Griesenbach and Boyd 2005).

CELL THERAPY OF CYSTIC FIBROSIS

The final goal of gene therapy of cystic fibrosis (CF) is to permanently correct the genetic defect in the target cellular compartment. This could be conceivably be achieved by gene transfer into the ‘‘stem’’ cell compartment of the respiratory epithelium. Although the identification of a resident pulmonary multipotent stem cell still remains to be accomplished, it is clear that local stem or precursor cells contribute to the repopulation of the injured epithelium in different anatomical regions of the airways (Otto 2002; Neuringer and Randell 2006; Randell 2006). Different approaches have led to the identification of local repopulating cells in trachea and bronchi (basal, mucous, secretory), bronchioles (Clara), and alveoli (type II pneumocytes). Most recently another epithelial niche in the bronchiolar epithelium has been identified in the zone where airways terminate and form alveoli (Kim *et al*. 2005).

In search of a potential use of autologous human airway epithelial cells, Castillon and colleagues have used polarized and well-differentiated 3-D spheroid cultures produced from isolated airway epithelial cells (Castillon *et al*. 2002). These 3-D spheroid structures can be maintained in culture for several months without any alteration of their polarized and differentiated state. Moreover, they have beating cilia at the surface and functional choride channels (Castillon *et al*. 2004). The group has shown that the 3-D spheroids are suitable to study a gene and cell therapy approach to CF. Respiratory epithelial cells obtained from human fetal tracheas or fetal airway xenografts were transduced in suspension with a HIV-1-derived VSV-G pseudoytyped lentiviral vector expressing GFP and allowed to develop 3-D spheroid structures (Castillon *et al*. 2004). Transgene expression was maintained for the duration of the study (80 days) without altering the epithelium reconstitution or the chloride channel activity. Transduced spheroids were then assayed in an *ex vivo* and an *in vivo* model of airway-epithelial denuded trachea, to investigate their capacity to regenerate a differentiated airway epithelium. Presence of clusters of GFP positive basal, ciliated and secretory cells in the reconstituted epithelium at 10 weeks after engraftment suggests targeting of progenitor cells. This study shows that lentiviral vectors can achieve efficient and long-lasting gene expression in polarized and differentiated 3-D spheroid structures of human airway epithelial cells. Transduced spheroids can be then used as a pool of autologous corrected cells and as a potential source of human airway progenitor cells for reimplantation on a denuded basement membrane, often present under CF pathological conditions.

Alternatively to”hitting” a local stem cells compartment in the lung, it may be conceivable to induce circulating adult hematopoietic stem cells (HSCs) to home to the damaged respiratory epithelium during regeneration (Spencer and Jaffe 2004; Conese and Rejman 2006). This concept has been exploited for muscle and nerve regeneration, in view of a cell therapy approach to muscular dystrophy (Ferrari *et al*. 1998) and neurodegenerative diseases (Biffi *et al*. 2004).

The potential of adult-derived bone marrow cells to home to the airways is an attractive, novel therapeutic approach for pulmonary repair. Much scientific debate has focused on the ability of bone marrow (BM)-derived cells to be engrafted into non-hematopoietic tissues and adopt an epithelial phenotype (Herzog *et al*. 2003; Wagers and Weissman 2004). BM-derived cells in the liver and kidney have been shown to arise as a consequence of fusion (Alvarez-Dolado *et al*. 2003; Camargo *et al*. 2004). However, other reports showed no evidence of fusion in lung, skin, liver, and epidermis (Harris *et al*. 2004; Brittan *et al*. 2005).

Studies with lung injury models in mouse

It was demonstrated that transplanted BM cells could repopulate the airways after irradiation of the recipient. Harris *et al.* made use of the Cre/lox recombinase system to examine whether fusion occurs between BM-derived stem cells and

host cells after BM transplantation (Harris *et al*. 2004). Tissues from the recipient were analyzed 8 to 12 weeks after lethal irradiation and transplantation for the presence of BM-derived (Y chromosome-positive) epithelial cells and EGFP expression. Only 0.6% of total pneumocytes were EGFP-positive.

After transplantation of total BM or enriched HSCs into irradiated recipient animals, engraftment of BM-derived cells in liver, lung, gut and skin epithelial have been detected (Krause *et al*. 2001). The level of pneumocyte engraftment was significantly higher (up to 18.7%) than that in other epithelial cell compartments in which bone marrow engraftment could be demonstrated (0.19-3.39% in gastrointestinal lining cells, bile ducts, skin, and hair follicles).

Theise *et al.* studied BM transplantation from male B6D2F1 mice into irradiated female mice and assessed the kinetics of engraftment by measuring the percentage of surfactant B protein-producing cells derived from the donor using co-fluorescent *in situ* hybridation (FISH) for surfactant B mRNA and the Y-chromosome (Theise *et al*. 2002). The percentage of marrow-derived type II pneumocytes increased over time from an average of 0.9% at day 5 to 11-14% at month 6, demonstrating either progressive expansion of the engrafted population or continuous engraftment of circulating marrow cells.

Herzog *at al.* studied the engraftment of marrow-derived pneumocytes into mice that had received varying doses of total body irradiation and quantifying the degree of lung damage associated with irradiation (Herzog *et al*. 2006). They found that only at doses that induced lung injury could marrow-derived lung epithelium be identified following BM transplantation. With irradiation doses less than 1,000 centigray (cGy), there was little to no apparent injury to the lung, and there were no marrow-derived pneumocytes despite high levels of hematopoietic chimerism. In contrast, 4 days after either split or single-dose 1,000 cGy irradiation, nearly 15% of lung epithelia were apoptotic, and with this dose, marrow-derived type II pneumocytes (0.2%) were present at 28 days. These data indicate a critical relationship between lung injury and the phenotypic change from BM-derived stem cells to lung epithelial cells.

Kotton *et al.* used whole bone marrow or side population (SP) cells to engraft irradiated mice and use a bleomycin damage protocol (Kotton *et al*. 2005). The SP population is isolated by virue of its ability to exclude the DNA-binding dye Hoechst 33342 and it is highly enriched in HSC activity. In contrast to their earlier reported work with mesenchymal bone marrow-derived cells (Kotton *et al*. 2001), there was no evidence of engraftment in type II pneumocytes. However the bleomycin used in this study as the damaging agent is known to induce lung fibrosis (Xu *et al*. 2006). This may mean that the type of donor bone marrow-derived cells engrafted into the lung would not be pneumocytes but fibroblasts (Hashimoto *et al*. 2004).

Other studies have evaluated whether lung injury obtained by local treatments increases recruitment of the bone marrow-derived cells (**Table 3**). MacPherson and colleagues injected the bone marrow-derived SP cells from ROSA26 mice (constitutively expressing β-galactosidase) into irradiated hosts before polidocanol treatment (MacPherson *et al*. 2005). They demonstrated that mice engrafted with SP cells have donor-derived cells present in the epithelial lining of the trachea following damage and repair. Donor-derived cells (Y-chromosome$^+$) were found at a frequency of 0.83%. Confocal microscopy analysis revealed that 55% of the cells expressing cytokeratins were donor – derived cells. Analysis of X-gal staining and allele-specific ROSA26 PCR indicated that these cells did not have the ability to contribute to the developing blastocyst, nor were they able to contribute to primary epithelial cultures grown at an air liquid interface, or denuded tracheal xenografts. Clearly, the necessary signals/factors are not present in these *in-vitro* and *ex-vivo* systems to allow marrow-derived SP cells to contribute to the formation of the epithelia. Very recent work by the same authors extended and confirmed these observations (MacPherson *et al*. 2006). Indeed, they show that whole BM donor cells also contribute to the tracheal epithelium following damage but without damage the numbers of donor cells is 10 fold less. In the animals transplanted with SP cells, Y chromosome FISH was used to identify donor-derived cells and deconvolved imaging to confirm localization of these cells with the epithelial marker pan cytokeratin (CK). The majority (60.2%) of donor-derived cells express CK, and some of these also express the CD45 hematopoietic marker.

Beckett *et al.* studied the lung engraftment following different treatments producing lung damage (Beckett *et al*. 2004). Adult female C57Bl/6 mice were irradiated and engrafted with marrow from adult male transgenic GFP mice, followed by NO_2 or endotoxin administration. Under all experimental conditions evaluated, small numbers of CD45$^-$ donor-derived cells in alveolar septae stained positive for pro-surfactant protein C. Rare donor-derived cells located in the airway epithelium stained positive for cytokeratin. The treatment with endotoxin or NO_2 or both did not increase the number of donor-derived cells acquiring type 2 alveolar epithelial cell phenotype. These results suggest that lung injury by NO_2 or endotoxin lung injury does not improve engraftment of marrow-derived cells in lung. The fact that these results are in contrast with those reported by MacPherson and colleagues, implies that that the type of lung injury is important.

Abe *et al.* used parabiotic pairs of transgenic enhanced green fluorescent protein (EGFP) expressing and wild-type (wt) littermate mice in which the wt mouse was either injured, or lethally irradiated or received intratracheal elastase or both irradiation and intratracheal elastase (Abe *et al*. 2004). Radiation greatly increased engraftment of circulation-derived cells into the lung of wt mice. Elastase infusion alone had little effect, but elastase in combination with radiation showed slightly more engraftment than radiation alone. Approximately 5 to 20% of lung fibroblast and rare type I pneumocytes cells from injured wt mice were EGFP$^+$, indicating their blood-born origin.

In the study published by Ortiz *et al.* mesenchymal stem cells (MSCs) from male bleomycin-resistant BALB/c mice were transplantated into female bleomycin-sensitive C57Bl/6 mice, after bleomycin lung injury (Ortiz *et al*. 2003). FISH analysis revealed that engrafted male cells were localized in the areas of bleomycin-induced injury and exhibited an epithelium-like morphology (type II pneumocytes, ~1%). Moreover, purification of type II epithelial cells from the lungs of transplant recipients resulted in a 3-fold enrichment of male, donor-derived cells as compared with whole lung tissue. MSC administration immediately after exposure to bleomycin also significantly reduced the degree of bleomycin-induced inflammation and collagen deposition within lung tissue.

In the study by Rojas and colleagues MSCs from C57Bl/6 were transplanted into busulfan-treated mice after bleomycin lung injury (Rojas *et al*. 2005). Myelosuppression increased mice susceptibility to bleomycin injury but MSC transfer protected the animals from lung damage. The protection was associated with the differentiation of engrafted MSCs into specific and distinct lung cell phenotype, with an increase in circulating levels of G-CSF and GM-CSF and with a decrease in inflammatory cytokines. Transplanted stem cells were localized in the injured lung and assumed type I alveolar (vimentin$^+$) and type II alveolar (aquaporine$^+$) epithelial phenotype.

In two studies (Ishizawa *et al*. 2004; Yamada *et al*. 2004), BM was reconstituted by injecting GFP$^+$ fetal liver cells. In one of these studies (Yamada *et al*. 2004), GFP$^+$ mice with elastase-induced emphysema were treated with all-*trans* retinoic acid (ATRA) and granulocyte colony-stimulating factor (G-CSF) or both, while in the other one (Ishizawa *et al*. 2004) recipient mice were treated with LPS. In both reports alveolar epithelial phenotype (GFP$^+$, CK$^+$, CD45$^-$) and pulmonary capillary endothelial cells (GFP$^+$, CD34$^+$, CD45$^+$) were found. Overall, these data suggest that

Table 3 Ability of bone marrow-derived stem cells to give rise to airway epithelial cells in mouse models, cell cultures, and in the human lung.

Source and phenotype of stem cells	Study	Phenotype and % of BM-derived epithelial cells	Reference
1) Studies with lung injury models in mouse			
Lin^-, $CD34^+$, $Sca\text{-}1^+$ HSCs	Male HSCs were injected into lethally irradiated female recipients	Anti-CK $Cam5.2^+$ type II pneumocytes (20%). Anti-CK $AE1/AE3^+$ bronchial epithelial cells (4%).	Krause *et al*. 2001
(Sca^+, $Gr\text{-}1^+$) SP cells	Marrow-derived SP cells from ROSA26 mice were transplanted into irradiated hosts before polidocanol treatment.	At 3 months post-transplantation: Y^+ tracheal epithelium (0.83%). CK^+ cells: 55% of donor cells.	Macpherson *et al*. 2005
(Sca^+, $Gr\text{-}1^+$) SP cells	Whole marrow or marrow-derived SP cells from ROSA26 mice were transplanted into irradiated hosts before polidocanol treatment.	At 3 months post-transplantation: 1.0-1.6% (with total BM) and 0.6-1.5% (with SP cells) Y^+ tracheal epithelium. CK^+ cells: 60.2% of donor cells.	MacPherson *et al*. 2006
Sex-mismatched tracheal transplantation	1) Tracheal transplants from C57BL/6 females into C57BL/6 males; 2) Tracheal transplants from wild-type C57BL/6 female tracheas into C57BL/6 male GFP^+ mice; 3) Tracheal transplants from wild-type female tracheas into male CK5-GFP mice.	1) At day 21 posttranplantation, CISH Y^+: 18.4%; 2) At day 21 posttranplantation, GFP^+: 48.7%. 3) At day 21 posttransplantation, $CK5^+$, GFP^+: 18.6%.	Gomperts *et al*. 2006
2) Studies with CF mice			
1. Plastic-adherent marrow stromal cells from adult C57Bl/6 mice.	1) Transplantation of male MSCs into female *Cftr* KO mice following naphthalene treatment;	1) Y^+, $CD45^-$, $CCSP^+$ (0.025%), some of which (0.01%) were $CFTR^+$. 2) Y^+, GFP^+, $CD45^-$, pro-SPC^+ (0.1%).	Loi *et al*. 2006
2. CD3- total bone marrow from adult male transgenic GFP-expressing mice	2) Transplantation of total BM from male wt mice into irradiated female *Cftr* KO mice and before naphthalene treatment.		
BM cells from adult mice	Transplantation of GFP-expressing total BM into irradiated CF mice. Functional studies (rectal and nasal potential difference) show modest level of CFTR-dependent chloride secretion.	Y^+, CK^+, $CD45^-$ (0.01-0.1%) in GI tract.	Bruscia *et al*. 2006
3) Studies with cell cultures			
hMSCs	GFP-expressing hMSCs were co-cultured with heat-shocked small airway epithelial cells. A subset of the hMSCs rapidly differentiated into epithelium-like cell and restored the epithelial monolayer.	GFP^+, CK 17^+, CK 18^+, CK 19^+, $CC26^+$.	Spees *et al*. 2003
GFP^+ human mesenchymal stem cells (MSCs)	CF MSCs co-cultured with CF airway epithelial cells. Partial recovery of the chloride channel activity.	Co-culture: 80% epithelial cells 20% wild-type MSCs from normal individuals are GFP^+, CK 18^+ (some cells) GFP^+, occludin$^+$ (10%)	Wang *et al*. 2005
Rat MSCs	MSCs were cultured on compartmentalized permeable support and allowed to differentiate.	CK 5^+ e 8^+. mRNA for ENaC, CFTR e ZO-1.	Shu *et al*. 2006
4) Studies with chimerism of the human lung			
Human lung transplant Human BMT	Study to investigate whether 1) in human lung allografts or 2) in lungs of BM-transplanted patients, recipient-derived cells are of bone-marrow origin.	1) Bronchial epithelium (6-26%), type II pneumocytes (9-20%), submucosal glands (9-24%) of recipient origin. 2) No lung cell types of donor origin.	Kleeberger *et al*. 2003
Human BMT	Group of sex-mismatched allogenic BMT patients to investigate whether the tissues of the lung might be derived from extrapulmonary sources.	Lung epithelium (2.5 to 8.0%), rare distal bronchial cells. Lung endothelium (35.7 to 42.3%).	Suratt *et al*. 2003
Human BMT	Study to examine the nasal epithelium of female patients up to 15 years after gender-mismatched bone marrow transplantation.	No nasal epithelium of donor origin.	Davies *et al*. 2002
Human HSCT	Lung-tissue specimens were obtained at autopsy from four female patients, two with male donors, after nonmyeloablative HSCT.	Lung epithelial cells: XY^+, CK^+, $CD68^-$ type II pneumocytes (2%).	Mattsson *et al*. 2004
Human lung transplant	Longitudinal study to investigate the stem cell engraftment in the lung after sex mismatched lung transplantation in two CF patients.	Y^+, CK^+ in alveolar region (2.3-5-5%) and in bronchial tissue (0-6.6%).	Spencer *et al*. 2005
Human lung transplant	Re-cut sections were obtained from lung biopsy specimens from seven male recipients of transplanted lungs from female donors.	Y^+ type II pneumocytes were found in 9 of 25 biopsy specimens (0-0.553%).	Zander *et al*. 2005
BM transplantation	Re-cut sections were obtained from five lung biopsy specimens and autopsy lung tissues from four female recipients of transplanted mobilized peripheral blood stem cells or bone marrow from male donors.	Y^+ type II pneumocyte was found in one lung biopsy from one HSC transplant recipient (1.75%).	Zander *et al*. 2006
Human lung transplant Human BMT	Cross-gender transplantation of lung (female in male) or bone marrow (male in female).	Y^+ type II pneumocytes.	Albera *et al*. 2005

BM = bone marrow; BMT = bone marrow transplantation; CC26 = marker of Clara, serous and goblet cells; CCSP = Clara cell Secretory Protein; CD45 = antigen expressed by cells of haematpoietic origin; CD68 = antigen express on the membrane of monocytes, macrophages, neutrophils, basophils and great lymphocytes; Cftr = Cystic fibrosis transmembrane conductance regulator; CISH = Chromogenic *in situ* hybridization; CK = cytokeratin; GFP = green fluorescent protein; GI = gastro-intestinal; HSCT = hematopoietic stem cells transplantation; Gr-1 = a 25-30 kDa cell surface antigen expressed on myeloid cells but not on lymphoid or erythroid cells; Lin^- = lineage minus cells; Sca = a marker of HSC belonging to *Ly-6* gene family; SP = side population; SPC = surfactant protein C; wt = wild type.

BM-derived stem cells play an important role in the regeneration of lung parenchyma.

Gomperts and colleagues have used a mouse model of sex-mismatched tracheal transplantation (Gomperts *et al*. 2006). This model is associated with tracheal ischemia, followed by reperfusion from neovascularization posttransplantation. The airway injury is associated with complete sloughing of the epithelium from the basement membrane with gradual re-epithelization starting by day 3 post-transplantation. Full regeneration of the pseudostratified columnar epithelium occurs by day 21 post-transplantation. The authors demonstrated that a population of oriented progenitor cells expressing the epithelial marker CK5 and the chemokine receptor CXCR4 is harvested in the bone marrow; these cells passing into the circulation provide a cellular pool able to repair damaged tracheal epithelium. Depletion of CXCL12 prevents precursor recruitment and appropriate epithelial repair and favors squamous metaplasia. These findings demonstrate that $CK5^{+}CXCR4^{+}$ cells have a crucial role in the re-epithelialization of tracheal transplants and that the CXCL12/CXCR4 axis is involved in epithelial precursor mobilization and recruitment at sites of injury.

Studies with CF mice

Recently two groups have reported bone marrow transplantation of CF mice with wild type cells (**Table 3**). Loi *et al*. determined whether transplantation of adult marrow cells containing the gene for wild type Cftr might result in functional Cftr expression in the lung epithelium (Loi *et al*. 2006). The authors transplanted two populations of bone marrow-derived cells, cultured stromal marrow and total bone marrow cells containing the wild type Cftr gene, into transgenic Cftr knock-out (KO) mice. Administration of plastic adherent stromal cells to naïve non-irradiated mice resulted in the engraftment of donor-derived airway epithelial cells, although in small number (approximately 0.025%). In contrast, no donor-derived airway epithelial cells were detected in irradiated mice treated with total marrow cells. Cftr mRNA and protein could only be detected in the lungs of Cftr KO recipients treated with isolated adherent bone marrow stromal cells. However the total number of chimeric lung epithelial cells exhibiting Cftr expression was small (0.01%) and unlikely to affect overall Cftr-dependent chloride transport and other functions in airway epithelium.

Bruscia *et al*. transplanted CFTR+/+ GFP^{+} BM cells into CFTR-/- mice after receiving different doses of irradiation (Bruscia *et al*. 2006). Very low levels of engraftment (0.01-0.1%) were observed in the gut, correlating with very low CFTR mRNA expression. Surprisingly the bioelectric profile of CF mice transplanted with wild type bone marrow was significantly improved in both gut and nose compared to those transplanted with bone marrow from CF mice. This implies that a very low level of cell therapy produced an amplified electrophysiological effect. A study using mouse models suggested that 5% of normal levels of CFTR is sufficient to rescue the intestinal phenotype apparent in these animals (Dorin *et al*. 1996).

Studies with cell cultures

Spees *et al.* studied the differentiation, the cell fusion and nuclear fusion during *ex-vivo* repair of epithelium by human adult stem cells (Spees *et al*. 2003). GFP-expressing hMSCs were co-cultured with heat-shocked small airway epithelial cells. A subset of the hMSCs rapidly differentiated into epithelium-like cells, and they restored the epithelial monolayer. Immunocytochemistry and microarray analyses demonstrated that the cells expressed many proteins of epithelial cells such as keratins (CK 17, 18, and 19), structural proteins of intermediate filaments, and CC26 (a lung epithelial marker of Clara, serous, and goblet cells). The results demonstrated that adult stem cells of mesenchymal origin could be a source of cells for the repair of damaged epithelium *ex-vivo*.

Very promising results have been obtained recently by Wang and colleagues using human MSCs (Wang *et al*. 2005). In their experiments MSCs obtained from bone marrow of healthy volunteers were mixed with airway epithelial cells (AECs) and grow in air-liquid interface cultures on semi-impermeable filters. Almost 10% of the MSCs acquired an epithelial phenotype, as judged by the expression of CK 18 and occludin. Moreover, MSCs obtained from CF patient corrected *ex-vivo* with a CFTR-encoding retrovirus and mixed with CF AECs effectuated partial resumption of CFTR-mediated chloride current.

Shu *et al.* induced rat MSCs into epithelial cells by culturing them onto compartmentalized permeable supports (Shu *et al*. 2006). Hematoxylin staining showed that after growing for 4 days on permeable supports, MSCs formed an epithelial-like monolayer. Immunofluorescence of the MSC cultured on permeably-supported monolayers, but not those grown in culture flasks, showed positive signals for epithelial markers, CK 5 and CK 8. RT-PCR results showed the presence of ENaC and CFTR mRNA, as well as of the tight junction protein ZO-1 in the MSC-derived monolayers grown on permeable supports. However, the western blot analysis revealed only the presence of ZO-1 protein but not ENaC and CFTR.

The compartmentalized culture conditions provide a suitable environment for rMSCs to differentiate into epithelial progenitor cells with tight junction formation; however, this condition is not sufficient for functional expression of epithelial ion channels associated with well-differentiated epithelia.

Johnson and colleagues have shown in CF cell-mixing experiments that 6 to 10% of CFTR-expressing cells were required to restore normal levels of chloride secretory function to an epithelium *in vitro* (Johnson *et al*. 1992). These findings were confirmed by Farmen and colleagues, which made use of mixed freshly isolated wild-type and CF (homozygous ΔF508) airway epithelial cells in varying proportion and then used them to generate well-differentiated cultures of airway epithelia (Farmen *et al*. 2005). Epithelia with ~20% wild-type cells generated ~70% of the transepithelial Cl^{-} current of epithelia containing 100% wild-type cells.

However, *in vitro* and *in vivo* studies suggest that nearly every cell in the sample must be corrected with CFTR to reverse the excess activity of ENaC (Johnson *et al*. 1992; Goldman *et al*. 1995; Johnson *et al*. 1995). These findings would imply that BM cell-based treatment of CF lung disease should achieve the correction of approximately every cell in the airway epithelium.

Studies with chimerism of the human lung

Human studies following HSC or lung transplantation have been performed (**Table 3**). Suratt *et al.* examined a group of sex-mismatched allogeneic HSC transplanted patients to determine whether the tissues of the lung might be derived from extrapulmonary sources in the humans (Suratt *et al*. 2003). They found significant epithelial (2.5-8.0%) and endothelial (37.5-42.3%) chimerism. This study provided the first evidence of chimerism in the human lung after HSC transplantation.

Mattsson *et al.* showed evidence of donor derived cells presence (chimerism) in the necroscopic and surgical lung biopsy tissue (Mattsson *et al*. 2004). Lung-tissue specimens were obtained at autopsy from four female patients, two with male donors, after nonmyeloablative HSC transplantation. Immunohistochemical staining for cytokeratin was used to identify lung epithelial cells. The tissue sections were analyzed for the presence of donor-derived lung epithelial cells with the use of FISH analysis. XY–cytokeratin-positive, CD68-negative, and surfactant-positive cells were detected, indicating engraftment of type II pneumocytes. The authors concluded that circulating donor stem cells might differentiate into lung epithelial cells after allogeneic

HSC transplantation.

Kleeberger and colleagues investigated whether recipient-derived cells of bone marrow origin could be found in human lung allografts or in the lung of BM-transplanted patients. They found chimerism as early as 4 days and up to 7 years in seven archived human lung allografts explanted because of organ failure (Kleeberger *et al*. 2003). Bronchial epithelial cells (6-26%), type II pneumocytes (9-20%), submucosal glands (9-24%) of recipient origin were found. Interestingly, the epithelial structures displaying signs of chronic injury, such as squamous metaplasia, showed a markedly higher degree of chimerism (24% versus 9.5%). No lung cell types of donor origin were found in the in lungs of three BM-transplanted patients. Limited size and poor quality of the lung tissues available from recipients of BM transplantation might provide an explanation of these negative results.

Most notably, analysis of nasal epithelium of patients after gender-mismatched BM transplantation did not show integration of donor cells at the level of respiratory epithelium in healthy lungs (Davies *et al*. 2002).

Spencer and colleagues investigated the stem cell engraftment in the lung after sex mismatched lung transplantation in two CF patients through transbronchial biopsies (Spencer *et al*. 2005). They have shown, for the first time, the evidence of host derived epithelial cells in alveolar region (2.3-5-5%) and in bronchial tissue (0-6.6%) without apparent evidence of cell fusion. One limitation of this study was that it failed to show evidence of epithelial cell function. Future studies should include multiple transbronchial biopsy samples. Transbronchial biopsy specimens are potentially a viable tissue source for investigating the kinetics of stem cell engraftment in the lung and might be useful in future stem cell therapeutic trials.

In two related works, Zander *et al.* evaluated the extent of lung repopulation by type II pneumocyte descendents of adult bone marrow-derived stem cells in allogeneic HSC gender-mismatched transplant recipients (Zander *et al*. 2005, 2006). In one study, one Y chromosome-positive, CK-positive alveolar epithelial cell, estimated to be 0.5% of the alveolar epithelium, was found in 9 of 25 biopsy specimens from 5 of 7 gender-mismatched male lung transplant recipients (Zander *et al*. 2005). Interestingly, the number of type II pneumocytes of male karyotype showed a statistically significant relationship to the cumulative number of episodes of acute cellular rejection. In the other study, recut sections were obtained from five lung biopsy specimens and autopsy lung tissue from 4 female recipients of transplanted mobilized peripheral blood stem cells or BM from male donors (Zander *et al*. 2006). A single Y-chromosome-containing type II pneumocyte was found in one lung biopsy from one hematopoietic cell transplant recipient. After adjustment for the effects of incomplete nuclear sampling, this pneumocyte type represented 1.75% of all type II pneumocytes in the biopsy sample. In both studies, no evidence of cell-to-cell fusion (lack of polyploidy) was obtained (Zander *et al*. 2005, 2006).

Albera *et al.* studied 8 lungs from female donors transplanted into male recipients and also 3 lungs at autopsy from females receiving male BM transplants (Albera *et al*. 2005). Although their results were only qualitative, they suggested the possibility of exogenous cells contributing to lung epithelium.

These findings lead to speculation about the use of stem cells in the repair and regeneration of damaged lung tissue in diseases such as CF. Unfortunately most of the cited reports do not reveal high engraftment rates of BMDCs into bronchial/bronchiolar epithelium, also because of the difficulty in identifying these cells on paraffin section using chromosomal analysis. **Table 3** summarizes all the studies which demonstrate that bone marrow-derived stem cells can give rise to airway epithelial cells of the conducting airways.

Overall, these results strongly suggest that hematopietic stem cells have a great potential for replacement of diseased or degenerating cell populations, tissues and organs, and show promise for a stem cell gene therapy approach (Asahara *et al*. 2000). It has been proposed that further studies, besides those relying on imaging, will help to establish transdifferentiation of BM-derived stem cells into airway and lung parenchymal cell types (Neuringer and Randell 2006). CF is characterized by chronic respiratory infections by opportunistic pathogens that cause remodeling (Baltimore *et al*. 1989) and proliferation (Leigh *et al*. 1995b) of the airway epithelium. Thus, the challenge will be to unveil and characterize those cellular compartments that are involved in these processes and target them with BM-derived stem cells bearing a viable CFTR gene.

Studies with embryonic stem cells

Pluripotent embryonic stem cells (ESCs) offer promise as a potential source of lung endoderm, mesoderm and ectoderm cell types. Murine ESCs can differentiate into Clara cells, as indicated by the expression of Clara cell-secreted protein CC10 mRNA and protein (Coraux *et al*. 2005). Differentiation occurs as early as day 8 of culture when cells are grown on type I collagen, at day 15 when cells are cultured on plastic or in presence of other substrates (gelatin, type IV collagen or type VI collagen). Clara cells obtained from ESCs has been showed to develop into pseudostratified airway epithelial tissue that included basal, intermediated and ciliated cells, similar to the native airway epithelium, when were seeded on type I collagen coated porous membranes and allowed to form air liquid interface cultures. Ciliary beating, and functional cellular junctions such as tigh junctions, desmosomes and hemidesmosomes were observed in the ESC-derived airway epithelium.

Denham and colleagues have demonstrated that murine ESCs can be directed towards a respiratory cell-like phenotype with high efficiency (greater than 24% of all mESC derivatives) *in vitro* by co-culture with dissociated E11.5 mouse lung explants (Denham *et al*. 2006). Murine ESCs derivatives displayed immunoreactivity to the pneumocyte II specific marker surfactant-associated protein C (SFTPC). The same group has recently published that the E11.5 mouse lung inductive niche is supportive of human ESC (hESC) differentiation into epithelial tubules at high frequency (>30% of all hESC derivatives), yet SFTPC immunoreactivity associates with these tubules only at very low frequency (<0.1% of all hESC derivatives) (Denham *et al*. 2007). These data demonstrate that ESCs display species-specific differences in reponse to the E11.5 mouse lung rudiments.

The *in vitro* developmental potential and the success of ESCs in animal models demonstrate the principle of using human embryonic stem cells as a regenerative source for transplantation therapies of human diseases (Wobus and Boheler 2005). However, before therapeutically applicable, any ESC-based treatment must show limited potential for toxicity, immunological reaction, or tumor formation.

CONCLUDING REMARKS

Several hurdles must be overcome before successful gene therapy can become a reality for CF patients. The major challenge is posed by inefficient gene delivery to the differentiated airway epithelium. The biophysical and biological characteristics of DNA nanoparticles bring this gene transfer carrier into consideration as a low-toxicity nonviral gene transfer vector for efficient targeting to the respiratory epithelium. However, even in the most favorable scenario, DNA nanoparticles should be administered several times during the time span of one year. The integrative nature and the low inflammatory profile of lentiviral vectors make them a promising choice for the transfer of CFTR gene to the airway epithelium, although the immunological response has not been fully studied yet. Eventually, an approach considering HSC-based therapy of CF injured lungs through BMT should avoid repeated dosing and achieve a perma-

nent correction of the CF genetic defect. However, developing rational strategies based on cell therapy for CF lung disease will require intensive and scientifically rigorous efforts. Engraftment of bone marrow-derived stem cells into the airway epithelium is a very inefficient process. Further studies on the molecular network governing the homing of circulating stem cells to the airways will be needed to increase this efficiency. Alternatively, much more effort has to be put into the discovery and characterization of stem cell compartment(s) in the airways. Gene therapy agents will be then explored for their targeting (receptor- or transcriptionally-based) to these compartments.

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