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Gene delivery systems—gene therapy vectors for cystic fibrosis

Daniel Klink[a], Dirk Schindelhauer[b], Andreas Laner[c], Torry Tucker[d], Zsuzsanna Bebok[d], Erik M. Schwiebert[d], A. Christopher Boyd[e], Bob J. Scholte[a,*]

[a] *Department of Cell Biology, Erasmus MC, Erasmus University, P.O. Box 1738, Rotterdam DR 3000, The Netherlands*
[b] *Livestock Biotechnology, Life Science Center Weihenstephan, TUM, Freising 85354, Germany*
[c] *Department of Medical Genetics, Ludwig Maximilians University, Munich 80336, Germany*
[d] *Department of Physiology and Biophysics, University of Alabama, Birmingham 35294, USA*
[e] *Medical Sciences (Medical Genetics), Edinburgh University, Edinburgh EH4 2XU, UK*

Abstract

Gene delivery systems (GDS) play a central role in the development of gene therapy strategies for Cystic Fibrosis (CF). Further, these systems are important tools in studies with cultured cells and in animal models. In this review, we describe the properties of several viral and synthetic gene delivery systems, and evaluate their possible application in gene therapy of CF. While many gene delivery systems give satisfactory results in cultured or animal studies, none of these systems has been shown to fulfil all the requirements of safety and efficacy for use in CF patients. The intact airway epithelium, the most important target in CF gene therapy, proves to be well protected against invading vector systems.

Keywords: Gene therapy/ethics/methods; Gene transfer techniques; Genetic vectors; Lung; Cystic fibrosis transmembrane conductance regulator

1. Introduction

Since the discovery of the *CFTR* gene in 1989 [1], many attempts were made to develop a gene transfer system for somatic gene therapy of CF. Cystic Fibrosis (CF) is a recessive disorder, which implies that a single copy of the normal CF gene is sufficient for normal function. Hence, the concept of somatic CF gene therapy is deceptively simple. All we have to do is to supply the affected cells with a gene that expresses CFTR protein. The airway epithelium is the most important target, as lung disease contributes mainly to morbidity and mortality in CF patients. For this purpose, many different gene delivery systems (GDS), viral and synthetic, have been developed and investigated for efficacy in vitro and in vivo. So far, what we have learned is that a good gene transfer system in vitro is not necessarily a useful gene therapy vector. Systemic application of vectors in a clinical setting is much more challenging than transfection of cells in a dish. Initial optimism about the use of adenoviral vectors was weakened by their lack of transduction efficiency in intact human lung, and by safety concerns caused by the inflammation response that was observed. Trials with cationic liposomes showed low toxicity, but also low efficacy. In addition, long-term stability and regulated expression in a tissue-specific manner requires not only the coding portions of the *CFTR* gene but also regulatory and functional chromosomal elements. This is a requirement that available vectors do not address.

A decade of vector development since the initial trials has not yet yielded a vector that can be used successfully to treat CF in the clinic [2–4]. However, many of the available systems can be very useful for CF researchers in animal models or in cultured cells in vitro. The European Working Group on CFTR Expression website provides a number of protocols and contacts that may help researchers along [5]. Further, developments in the field of vector development have raised new hope for future clinical applications. This is the main thrust of this review.

Abbreviations: GDS, gene delivery system; AAV, Adeno-associated virus; HIV, human immunodeficiency virus; ORF, open-reading frame; NLS, nuclear localization signal; NPC, nuclear pore complex; PAC, P1 phage-based artificial chromosome; YAC, yeast artificial chromosome.

* Corresponding author. Tel.: +31-10-4087205; fax: 31-10-408-9468.
E-mail address: b.scholte@erasmusmc.nl (B.J. Scholte).

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2. The target: airway epithelium in a patient with chronic lung disease

Airway epithelium, the most important target of CF gene therapy and related research models is a highly complex, multifunctional tissue. It lines the tubular structure of the airways from the nasal cavity, via the trachea into the intricately branched structure of the bronchial tree. It consists of several epithelial cell types, which show marked proximal to distal gradients. These include mucus secreting (goblet) cells that produce a protective coating, ciliated cells that are involved in clearance of bacterial pathogens and other microscopic particles. Further distally, *bronchi* and *broncheoli* are lined by CLARA cells, which secrete a mixture of proteins and peptides that is presumably involved in regulating inflammatory responses. Further, submucosal glands produce a mixture of mucous and serous secretions that is extremely important in lung homeostasis. In between these prominent and well-known cell types, we find basal cells and neuroendocrine cells. Further, there are cells that do not belong to the epithelial lineage, such as macrophages and dendritic cells, but which do contribute to the responses of the tissue. Finally, it should be noted that this epithelial layer grows on a complex matrix, which is produced by mesenchymal cells. The dynamic cross-talk between epithelium and mesenchyme, which can lead to irreversible and pathological tissue remodelling (fibrosis), plays a key role in CF lung pathology. While massive efforts are made to elucidate the molecular basis of this process, and the role that CFTR dysfunction plays, we are a long way from a comprehensive model that could be used as a guide towards therapeutic approaches.

Thus, the gene therapist is faced with an incomplete picture of CFTR function and expression patterns in airway epithelium. We can at present not be sure which cells, and how many of these we have to transduce to obtain clinical benefit. However, several requirements of the gene therapy vector system can be formulated [3,4]. The therapeutic gene should at least be expressed in the epithelial cells that are known to express CFTR, preferably at levels comparable to the endogenous gene. Studies in animal models suggest that transduction of 5–10% of the target cells would be sufficient for clinical benefit [6]. Moreover, expression should be stable, preferably life-long. The vector should be efficient and safe, not only for the patient but also for the environment. Importantly, the vector and its clinical application should be affordable, which may turn out to be the greatest challenge.

3. Barriers for gene delivery and ways around them

Living organisms are generally well protected by intra- and extracellular barriers against invasion of foreign genetic material. This is required to ensure the genetic stability of the species. Therefore, GDS has to be designed to overcome these barriers. A GDS generally consists of a polynucleotide, encoding the therapeutic gene, and a carrier. The carrier has several important properties. First, it condenses the polynucleotide, protecting it from mechanical stress and enzymatic attack. Second, the carrier should facilitate transport of the therapeutic gene from the extracellular space into the nuclear compartment, where transcription can take place. In nature, specialists in gene delivery have evolved, namely viruses. In many different shapes and molecular designs, viruses consist of a polynucleotide genome packaged in a protein complex called a capsid. The capsid proteins are well adapted to allow efficient binding to target cells, penetrate the plasma- or endosomal membrane, and facilitate intracellular transport and subsequent import of the genome into the nucleus. While viruses have found different solutions to this trafficking problem, they all make use of innate cellular transport systems through interactions of their capsid proteins with the host. By replacing part of the viral genome with a therapeutic gene, we can create a recombinant virus, which can be used as a gene delivery system. Alternatively, we can make synthetic carriers that mimic the properties of viral capsids, to package and deliver therapeutic genes.

Depending on the method of administration, the vector needs to penetrate several host-defense mechanisms. When injected into the bloodstream, circulating antibodies and/or complement factors may inactivate the vector [7–9]. One lesson learned in a more than a decade of painstaking preclinical and clinical research is that intact differentiated airway epithelium in situ is a difficult target for every GDS developed so far [4]. Vectors delivered to the lumen of the airways face a spectrum of host defense mechanisms, including a mucus layer that is continuously cleared by cilliae, and scavenging macrophages. Further, the receptors that pathogen derived GDS such as adenoviral vectors rely on are usually inaccessible, located on the basolateral membrane and shielded from the external milieu by tight junctions. Synthetic vectors that are internalised with high efficiency by epithelial cells in vitro prove much less effective in vivo [4].

4. Viral vectors

Gene delivery systems have been derived from various viruses. Adenoviral [10], adeno-associated viral [11], lentiviral vectors [12,13], poxvirus [14,15], Sendai [16] and herpes virus [17] are investigated for gene transfer. In general, the main advantage of viral vectors is the high transduction efficiency in vivo, compared to current synthetic systems. Further, the use of integrating viral vectors such as Adeno-associated virus (AAV) and lentiviral vectors would allow stable expression in the targeted cell pool [18]. This is an important property that none of the available synthetic systems can offer. On the down side, viral-based gene therapy poses serious safety concerns. Administration

of a viral vector can evoke an acute host defense response, either mediated by circulating antibodies or by activation of complement and macrophages [19–21]. This response can range from a relatively mild acute inflammation to an escalated and fatal response, as was observed after intravenous administration of a high dose of adenoviral vector to a patient [22]. Further, a cytotoxic T-lymphocyte response can develop against cells that produce viral antigens encoded by the vector [10]. Apart from the fact that this presents a health risk to the patient, it greatly reduces the stability of expression of the vector, and precludes the possibility of repeatedly delivering viral vectors to compensate for any fall-off in expression. Another concern is that the vector inevitably shares genetic information with the wild type viral genome, which may give rise to recombination events or activation of the vector in the presence of a wild type virus. When integrating vectors are used, there is a risk of germ cell transduction and of insertion mutagenesis. Indeed, recently two patients treated with bone marrow cells transduced with a retroviral vector were diagnosed with leukemia caused by an insertional modification of a protooncogene [23,24]. With these concerns in mind, novel and improved viral systems are being developed.

5. Adenoviral vectors

The first viral GDS for the treatment of CF that was tested in primates and clinical trial was the adenoviral vector. It was a logical, if not inescapable choice. The parent virus is a relatively benign pathogen with a taste for airway infections, able to efficiently transduce nondividing cells. Furthermore, the genome of the adenovirus and its functions had been thoroughly studied; it could be manipulated easily and there were no limitations to the amount of vector that could be produced. Though this vector has recently fallen out of favour for reasons explained below, it is worthwhile to study its history. Many of the drawbacks of the adenoviral vector system are also encountered to some extent with other GDS. In the first versions of the adenoviral vector, the therapeutic gene replaces part of the viral genome. Replication of the vector is achieved in a production cell line that provides the missing viral genes in transit. This approach leaves the viral genome intact but partially transcriptionally inactive. One of the problems with this vector system is that low level residual expression of viral genes can result in a cytotoxic T-cell response, which targets the cells containing the vector [10]. Further, the fact that adenovirus is a common airway pathogen is a mixed blessing. Many potential recipients have circulating antibodies that may reduce vector efficacy and add to the T-cell response. Most importantly however, systemic application of an adenoviral vector causes an acute, and potentially life threatening inflammation response, mainly caused by activation of professional antigen presenting cells [19,25–27].

Adenovirus and adenoviral vectors bind to the cell following two sequential receptor interactions: first the capsid fiber binds to a glycoprotein of the immunoglobulin family called Coxsackie Adenovirus Receptor (CAR) [28] and second, the fibronectin-binding integrin (integrin v 5) binds to the penton base capsid protein [29]. These receptors are located at the basolateral membrane of intact polarised airway epithelial cells [30]. This limits the infection efficiency of adenoviral vectors when applied to the airways. This can potentially be cured by replacing the receptor binding elements of the capsid [31–33]; however, this has not yet resulted in a vector that targets airway epithelium.

6. Minimal adenoviral vectors

The first generation of adenoviral vectors has been extensively modified to reduce their immunogenicity. This was achieved by removing parts of the viral genome, and providing these genes in transit in a suitable production cell line. In the most extreme case, only the viral terminal repeats are retained in the vector backbone (‘minimal’, ‘high capacity’ or ‘gutless’ adenoviral vectors) [34]. This completely abolishes the expression of viral proteins by the vector. In animal models, this strongly reduces the cytotoxic T-cell response against infected cells, and increases the duration of therapeutic gene expression dramatically [34,35]. While this is a very promising result, all available efficient production systems yield vector particles contaminated with helper virus. Moreover, for effective use of gene therapy of CF the vector should be re-targeted to the apical surface of epithelial cells. Further, circulating antibodies and acute inflammation responses are still of concern with minimal adenovectors.

7. Lentiviral vectors

Lentiviral vectors are derived from the human immunodeficiency virus (HIV)-1 retrovirus, the etiologic agent of acquired immunodeficiency (AIDS), or from counterparts of other species. These vectors have advantages compared to many other gene delivery systems [12,13]. It is an integrating retrovirus with a considerable cloning capacity (8–9 kb). Current results suggest that stable and cell specific expression can be obtained using appropriate regulatory sequences, in particular locus control regions (LCRs) [36]. Third generation or ‘self-inactivating’ vectors were generated that contain less than 5% of the original viral genome and cannot be rescued by wild type virus. The limitations of this approach are that production of large batches of clinical grade vector is difficult. Lentiviral vectors pseudotyped with the commonly used vesicular stomatitis virus (VSV)-G envelope protein are not able to transduce intact polarised airway epithelia in situ efficiently when administrated to the

apical surface [37,38]. To overcome this problem, retroviral vectors were pseudotyped with apical membrane-binding envelope proteins, leading to the development of a filovirus-pseudotyped feline lentiviral vector that efficiently transfect airway epithelia in vivo [39]. A further challenge will be to provide the vector with a suitable promoter/enhancer structure. Finally, risk of insertional mutagenesis and protooncogene activation inherent to all randomly integrating vectors has to be addressed.

8. AAV

Adeno-associated virus has been used to create a GDS with interesting properties [11,40]. AAV vectors are small DNA viruses able to transduce nondividing cells. They can integrate into the genome of the target cell, though with lower efficiency and specificity than the parent virus. The vector has a relatively small cloning capacity (4–5 kb), which makes it difficult to create a vector that produces CFTR (the open-reading frame alone spans 4443 bp) under a cell specific promoter/enhancer structure. However, recent data suggest that it might be possible to overcome this problem by making use of homologous recombination between two partial vectors [41].

A receptor (heparan sulfate) for AAV has been located on the basolateral membrane of airway epithelial cells [42]. The apical membrane exposes an abundant high affinity receptor which contains sialic acid in an α-2,3 linkage. The different vector capsid serotypes vary in sialic acid linkage specificity [43,44]. So far AAV6 type vectors appear to be most effective in airway epithelia [45].

Clinical trials (Phases I and II) with different AAV serotype vectors are being performed [46,47]. Initial results, including data submitted or in press, confirm the low toxicity and immunogenicity ascribed to the system. Effective titres in lung tend to be disappointing, however, presumably due to intracellular inactivation and limited access to apical receptors. It is not clear yet whether level and stability of transcription obtained with available vectors will be sufficient for clinical benefit.

9. Sendai virus

A most recent addition to the viral vector repertoire is recombinant Sendai virus. Recombinant Sendai virus was shown to be very effective in transduction of airway epithelial cells in situ both in mice and ferrets [48,49]. The ferret lung is considered a better model than mice since the architecture of distal airways and the frequency of submucosal glands more closely mimics the human situation [50]. Despite this encouraging result, it is clear that many of the concerns raised against other viral GDS still have to be addressed, particularly how Sendai can be administered repeatedly without provoking immune ablation.

10. Synthetic vector systems

Synthetic GDS consist generally of DNA encoding the therapeutic gene, combined with a carrier. This type of vector is theoretically the method of choice, since many of the safety issues involved with the use of viral vectors could be avoided, and it can potentially package any size of DNA. The DNA is usually in the form of a plasmid encoding the therapeutic gene, which can be isolated from bacteria in large amounts and in clinical grade. The carrier is generally a synthetic compound, which mimics the functions of a viral capsid, but should lack its immunogenicity. It condenses the DNA, binds to cells, and helps the vector to escape from the endosomal compartment. Like we have seen with viral vectors, systemic delivery of synthetic GDS has to overcome many barriers, from the complement system to the nuclear membrane. While this development is of major interest, much work needs to be done to accomplish its full therapeutic potential. We will describe a number of these systems that may be of interest to CF researchers. The CFTR Working Group online repository presents a number of basic protocols and contacts that may offer both the novice and experienced GDS researcher an entry into this complex field [5].

Carriers generally contain either cationic lipids or cationic polymers, which bind with high affinity and thus condense the negatively charged DNA. Although many carriers have been used successfully in vitro, their in vivo use is less straightforward. Initial studies and clinical trials that targeted airway epithelia were performed with cationic liposomes. Proof of principle was established; marker genes and *CFTR* could indeed be expressed in this way [51]. However, these initial studies also showed that these carriers lacked efficiency in vivo, further the available plasmid vectors could not establish stable expression in the correct cell specific pattern.

11. Uptake and intracellular transport of synthetic GDS

Non-viral gene delivery systems rely on cellular uptake mechanisms. Polycations complexed to DNA result in positively charged polyplexes that interact electrostatically with negatively charged proteoglycans of the cell membrane, followed by endocytosis [52]. This nonspecific mode of cell entry can be altered by the addition of a targeting ligand to the DNA delivery vehicle. The first targeting ligand used was asialo-orosomucoid for hepatocytes [53]. Numerous ligands are under investigation [54], including transferrin [8], folate [55,56], monoclonal antibodies [57], invasin [58] and carbohydrates [59]. To enhance specificity of uptake, attempts are made to shield the cationic aspect of the complexes with polyethylene glycol (PEG) moieties [8,56,60]. However, the apical membrane of intact epithelial airway cells still proves a formidable barrier to viral and non-viral vectors alike, which should not surprise us since

this is the most well-guarded frontier that separates us from our hostile environment.

One practical solution is to deliberately break down this barrier, by damaging the target tissue with irritants like SO_2 [37], EGTA or fatty acids [61,62]. This results in a transient dissociation of tight junctions, which is associated with exposure of basolateral epitopes and increased endocytotic activity. Indeed, early success with cationic liposomes in animal studies was probably caused by this effect. Whether this approach is acceptable in a clinical context remains to be established.

Non-viral vectors have mechanisms to circumvent lysosomal degradation, though much less efficient than virus derived GDS. Polycationic polymers, such as polyethylenimine [63] and pDMAEMA [64], are able to escape the endosome due to intrinsic properties of the polymer. First, the high buffer capacity of the compounds would result in swelling of the endosomes as the lysosomal proton pump attempts to reduce the luminal pH. Further, cationic polymers tend to destabilize membrane in high concentrations. However, the bulk of the DNA delivered this way does not escape hydrolysis and this remains one of the bottlenecks in delivery by synthetic GDS. In contrast, the gene transfer activity of the cationic polymer polylysine based compounds is low, unless endosomolytic agents are present [65]. Pharmacological agents such as chloroquine can be used to disrupt the internal routing of the GDS from the early endosomes to the lysosomal compartment [66]. Indeed, the presence of chloroquine enhanced transfection efficiency of lactosylated polylysine considerably [67]. Chloroquine is a weak base (pKA 8.1 and 10.2) that accumulates in acidic cellular compartments. Common consensus is that due to the raised luminal pH, osmotic swelling occurs followed by the destabilization of the endosomal membrane and release of the contents into the cytosol [68]. Another strategy to promote endosomal release is the addition of endosome-disrupting peptides such as N-terminal amphilic anionic peptides derived from hemagglutinin [69,70] and glutamatic acid–alanine–leucine–alanine (GALA) [71,72]. Glycerol is another agent that promotes endosomal escape [73]. KALA (lysine–alanine–leucine–alanine) is a cationic amphipathic peptide and was designed to both condense DNA and destabilize the endosomal membrane [74].

Once released from endosomes, the DNA has to reach the nucleus. Little is known about how current synthetic gene delivery vehicles move within the host cell. They may diffuse freely through the cell or move actively (“piggybacking”) after association with own intracellular carriers the host cell. The network of actin or tubulin filaments provides an intracellular transport system that is exploited in various ways by viral and bacterial pathogens for transport from the periphery to the nucleus. It seems attractive to apply the same strategy to future synthetic systems. This is one of the reasons why cationic polymers that can be covalently linked to synthetic peptides, carbohydrates and proteins are of major interest to synthetic GDS development.

12. Nuclear import, a major barrier in non-viral gene transfer

Once in the perinuclear region, the foreign DNA must enter the nucleus to undergo transcription. The nucleoplasm is separated from the cytosol by the nuclear envelope, which consist of an outer and inner nuclear membrane. Very large and complex protein structures called nuclear pore complexes (NPC) form aqueous channels through the double nuclear membrane and thus create passageways for proteins and genetic material [75–77]. Small molecules, up to 9 nm in diameter or proteins up to 50 kDa, can passively diffuse through the NPC, but larger cargo (up to 28 nm in diameter, or $\sim$ 1000 kDa) is transported in an active signal-mediated manner. Nuclear localization signals (NLS) on the cargo bind to cytoplasmatic proteins such as α-importins and transportin, which are responsible for docking the cargo onto the NPC. The vectorial translocation into and across the NPC is still under intense study. A key regulatory molecule, the GTPase Ran, has been reported to be essential for the creation of a concentration gradient [78].

In principle, there are two ways for viral and non-viral vectors to deliver their genetic material into the nucleus. First, the vector resides in the cytosol until the nuclear envelope is disassembled during mitosis. The vector genome can then enter the newly assembling nuclei of the daughter cells. Alternatively, the genomic material can be delivered by active transport through the envelope of the interphase nucleus [79]. Many viruses have developed clever devices to use the nuclear transport systems of the host to transport their genome through the nucleopore into the nuclear compartment [80–83], which allows them to transduce nondividing cells efficiently. It is especially in this aspect that non-viral transfer systems are deficient, they are much more effective in mitotic cells than in non-mitotic cells. As the DNA double helix has a diameter of 2–3 nm (nonhydrated) and turns of the double helix well below 28 nm are possible, transport seems feasible in principle. However, there is no known active transport system for DNA in the cytoplasm. Evidence was presented supporting the hypothesis that nuclear uptake of intact vector DNA can occur exclusively in cells entering mitosis, following breakdown of the nuclear membrane [69,84]. However, it was also reported that transferrin/polylysine complexes used for gene transfer in the presence of glycerol gave high transgene expression levels in growth-arrested fibroblasts [85]. Pollard et al. [86] reported that some polycations facilitate the nuclear uptake of DNA complexes. In vitro, plasmid DNA is thought to form a complex with proteins prior to transit to the NPC and translocation [87]. Likewise, it has been shown that single-stranded DNA/protein complexes were efficiently imported

in mammalian nuclei following the classical importin-dependent nuclear import pathway [88]. Moreover, several groups have reported a significant increase in transfection efficiency after inclusion of an NLS in the carrier system [89–91]. Unfortunately, none of these studies appear completely conclusive. Firstly, in transport studies with fluorescently-labelled DNA, fragmentation of the DNA prior to nuclear transport cannot be excluded. Small DNA fragments (<500 bp) may readily accumulate in the nucleus, in contrast with larger molecules that can accommodate a therapeutic gene. Second, in experiments with supposedly 'non-mitotic cells' actual controls of mitotic activity after addition of the vector are often missing. It should be noted that non-viral GDS are generally cytotoxic, application of which can easily result in a burst of transient mitotic activity. Therefore, much of the confusion could be resolved by using a simple but rigorous device to monitor transfection: continuous time-lapse fluorescence microscopy. This way we can establish without question whether cells that express the vector have, or have not been in mitosis after application of the vector (D. Klink et al., unpublished results). If there is any consensus in this field, it is that none of these developments have yet resulted in a synthetic GDS that demonstrably works sufficiently well in intact airways in vivo, the prime target of CF gene therapy [4].

13. Transfection of epithelial cells in culture

As outlined above, a variety of synthetic GDS ranging from cationic liposomes to modified cationic polymers is available to the researcher. Many of these are commercially available and work well in immortalised cell lines. The online repository presents a number of basic protocols that can be used as a starting point for further exploration [5]. These include the use of liposome carriers [92] and various cationic polymers [93]. It follows from the discussion of uptake and intracellular transport of synthetic GDS that their efficacy is largely determined by the properties of the target cells. In general, mitotically active nonpolarised cells are much easier to transfect than polarised quiescent cells. Therefore, in addition to a careful titration of DNA-carrier ratios and carrier-cell number ratios, optimal conditions for growth and carrier uptake should also be defined. For this, the reader is referred to other papers this issue [94–97] and the repository that describe the properties of epithelial cell lines [5] and primary culture systems [98].

14. The therapeutic gene, the longer the better?

So far, we have concentrated on the carrier rather than the therapeutic gene. Gregory et al. [99] showed proof of principle for CFTR expression by gene delivery. Initial experiments with human *CFTR* cDNA constructs were hampered by the presence of a cryptic prokaryotic promoter at position 930 of the hCFTR mRNA, which severely reduced the viability of the bacterial host. This problem was solved by mutating a single nucleotide in an open-reading frame (ORF)-neutral way. In later versions of hCFTR expression vectors, improving the cloning stability of the constructs and leaving the protein sequence intact [100,101]. A set of such hCFTR cDNAs, including several mutant versions has been made by Dalemans et al. (Transgene, Strasbourg, France). Many early studies were performed with a simple construct comprising a full-length CFTR cDNA and a ubiquitously expressed promoter. The advantage of this approach is that such constructs are relatively small, and can therefore be produced in large quantities and in clinical grade. However, a CF gene therapy vector has to be expressed in a stable and cell-specific manner, which requires more sophisticated expression constructs, in particular transcription control units. The expression pattern of the CFTR in airway cells is complex. Not only do different cell types express CFTR to different levels [102], also this expression pattern is subject to modulation during inflammation and remodelling [103]. Progress has been made using a promoter/enhancer structure derived from the cytokeratin (CK) 18 gene, which has an expression pattern similar to CFTR [104]. However, this kind of core promoter/enhancer constructs does not exactly mimic the responses of the endogenous CFTR gene. Further, they are subject to transcriptional silencing by methylation and chromatin remodelling (positional silencing). Another issue is that these full-length cDNA constructs do not have an intron structure, which results in inefficient mRNA processing and missing regulatory sequences. Most importantly, plasmid derived vectors are not chromosomes: they do not have telomers and centromers, and lack efficient eukaryotic replication sites. As a result, these vectors are intrinsically instable in a population of mitotic cells.

The obvious solution to all of these problems is to create a true mini-chromosome complete with centromere and telomeres, encoding the complete human CFTR gene, including distant regulatory sequences and boundary elements. This proves to be a daunting task, however. The CFTR gene is very big, some 200,000 base pairs excluding flanking control elements. In addition, it seems possible that small chromosomes (<1 Mb) might not be stable. A vector this size is very difficult to handle in bacterial plasmid vectors and with standard molecular biological techniques. Yeast artificial chromosomes (YAC) containing the human CFTR locus (Chr 7q31) were isolated shortly after the identification of the CFTR gene [105]. However, yeast elements are not stable in higher eukaryotes. Several groups created large replicating CFTR mini-chromosomes, using different approaches. In the 'top down' approach, existing mammalian chromosomes are used or further fragmented, and retro-fitted with a gene of interest by recombination in cultured mammalian cells. One such pre-existing mini-chromosome derived from human chromosome 1 was

recombined with a CFTR gene, and stable expression of CFTR could be shown in cultured cells [106]. A circular chromosomal vector into which large fragments can be fitted by Cre/Lox recombination was described and reported to be stable in the mouse germ line [107]. In the 'bottom up' approach, bacterial or yeast vectors are used to provide mammalian centromeric and telomeric sequences [108]. Huxley et al. created a hybrid vector based on a YAC encompassing the CFTR gene and a viral element, Ori-P/EBNA-1, that ensures replication and mitotic segregation in mammalian cells [109]. Another approach avoiding the use of viral replication elements and antigens is to add human centromeric sequences, i.e. long arrays of alpha satellite tandem repeats (>100 kb), which can be stably cloned as a unit copy bacterial plasmid (P1 phage-based artificial chromosome, PAC), and human telomere sequences [110]. These developments may lead to a vector that stably segregates during cell division as a low copy episomal element, carrying a large chromatin context for cell specific expression. Such a PAC has been engineered to contain a large region of the CFTR gene (140 kb) including its natural promoter, fused to a synthetic exon encoding eGFP. Expression from the CFTR promoter by RT-PCR, splicing of all 10 exons, and correct translation of the expected CFTR–GFP fusion protein as well as reliable detection of a stable copy has been demonstrated in mammalian cells (Schindelhauer et al., submitted for publication). However, efficient transfer of such large genetic elements to relevant target cells in a patient requires the development of a novel transfer technology. In a complementary study, PACs containing up to 180 kb of genomic CFTR fused to CFTR cDNA have been constructed which encode the whole CFTR gene and have been shown to express CFTR mRNA after in vitro transfection into epithelial cells [111].

15. Future developments

For monogenetic hereditary diseases, gene therapy offers the potential of correcting the underlying cause for which the responsible gene is known. Since delivery of a CFTR gene in the relevant cells, at the right time, and at the proper expression level seems difficult to achieve with available vector systems, alternatives should be considered. One such alternative is to correct the CF mutation by homologous recombination with a small DNA fragment that encodes the correct sequence [112,113]. This would circumvent many problems associated with stable and functional gene delivery and result in a pool of normal progenitor cells in the targeted organ. So far, the problem with this approach in CF therapy is the low conversion frequency plus the fact that the few corrected cells have no significant proliferation advantage. Exaggerated inflammation, associated with tissue damage and fibrosis, is a hallmark of CF lung pathology [114]. Delivery of a GDS encoding an extracellular modulator of these processes, e.g. an interleukin, could perhaps be effective and more easily achieved [49,115,116]. Recent developments indicate that the delivery of dsDNA is not the only possible application of delivery systems. RNAi and RNA decoys that bind regulatory proteins can be used to modulate gene expression of relevant genes [117]. This could lead to a novel therapeutic approach that aims at modulating the inflammation response and the subsequent fibrotic process in CF and related chronic lung disease (asthma, chronic obstructive pulmonary disease, COPD, bronchiectasis).

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