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Genetic Medicines for CF: Hype versus Reality

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Abstract
Since identification of the *CFTR* gene over 25 years ago, gene therapy for cystic fibrosis (CF) has been actively developed. More recently gene therapy has been joined by other forms of "genetic medicines" including mRNA delivery, as well as genome editing and mRNA repair-based strategies. Proof-of-concept that gene therapy can stabilise the progression of CF lung disease has recently been established in a Phase IIb trial. An early phase study to assess the safety and explore efficacy of CFTR mRNA repair is ongoing, whilst mRNA delivery and genome editing-based strategies are currently at the pre-clinical phase of development. This review has been written jointly by some of those involved in the various CF "genetic medicine" fields and will summarize the current state-of-the-art, as well as discuss future developments. Where applicable, it highlights common problems faced by each of the strategies, and also tries to highlight where a specific strategy may have an advantage on the pathway to clinical translation. We hope that this review will contribute to the ongoing discussion about the hype versus reality of genetic medicine-based treatment approaches in CF.

Introduction

The improved understanding of CF pharmacogenetics has led to licensing of drugs that begin to address the molecular defect caused by certain cystic fibrosis transmembrane conductance regulator (*CFTR*) mutations [1]; these advances provide the first proof-of-concept that the molecular defect in CF can be targeted and functionally corrected. In parallel 'genetic medicines', defined as the delivery of DNA or RNA nucleic acids encoding the CFTR protein and the repair of the *CFTR* gene (genome editing) or the CFTR mRNA (mRNA editing), (**Figure 1**) have been developed over the last two decades and will be reviewed in this article. The strategies currently focus on restoring CFTR function in the lung, given the role of this organ in morbidity and mortality in CF patients.

Several hypotheses have been postulated to explain how mutations in the *CFTR* gene might cause cystic fibrosis (CF). The 'low-volume' hypothesis postulates that, in addition to a reduction in chloride transport, the lack of functional CFTR also leads to sodium hyperabsorption through disinhibition of ENaC, and subsequent increased water absorption into the tissue, leading to reduced airway surface liquid and impaired mucociliary clearance [2]. More recently it has been suggested that reduced bicarbonate secretion through CFTR alters the pH on the airway surface which may affect airway defense mechanisms [3] and alter mucus properties [4]. The development of genetic medicines does not require the cause of disease pathophysiology to be conclusively understood.

Approximately 2000 mutations/genetic variants have been described in the *CFTR* gene (http://www.genet.sickkids.on.ca/app), but a link to

disease causation has only been proven for a small proportion (~10%) [5]. The valuable "Clinical and Functional Translation of CFTR (CFTR2)" initiative is focused on understanding and grouping mutations based on their effects on the protein, although to date this information is only available for a small proportion of putative disease-causing genetic variants (http://www.cftr2.org). Again, gene and mRNA-based therapies should be agnostic regarding the patient's genotype or which of the six mutation classes these might belong to, and should be suitable for the treatment of patients with any mutation. Although both mRNA- and genome *editing* approaches do require more detailed information about the genetic alteration, and not all mutations may be amenable to these repair strategies, these techniques are also not mutation class dependent.

Gene Therapy

Historical overview

Gene therapy is currently the most advanced form of CF genetic medicine. Since cloning of the *CFTR* gene in 1989 extensive pre-clinical research led to approximately 27 clinical trials involving about 600 patients being completed (**see Table 1** for key publications). The older literature related to CF gene therapy has been reviewed in many publications (see for example [6]), and so this review is restricted to a brief description and discussion of the key findings, rather focusing on more recent progress in the field. Over the last 20 years we have learnt that:

1. Gene transfer into the lung is a difficult task

Potent intra- and extracellular barriers that have evolved to protect us from viruses, bacteria and other inhaled particles also "protect" against inhalation and uptake of inhaled gene transfer agents (GTAs) and the accompanying DNA or RNA. Amongst the intracellular barriers, the

nuclear membrane presents a particularly significant hurdle for non-viral gene transfer agents. Strategies based on mRNA delivery, or the repair of CFTR mRNA, which both act in the cytoplasm, have the potential advantage of bypassing the nuclear membrane. Further, there are extracellular barriers including airway mucus, mucociliary clearance, CF mucopurulent sputum [7-9], as well as humoral and cellular immune responses (see [10] for more detailed discussion). Crucially, many of these barriers will also affect the other types of CF genetic medicines, including many editing approaches.

2. Identification of gene transfer agents suitable for clinical translation is challenging

Vectors that carry nucleic acids into cells fall broadly into two categories; viral and non-viral vectors. In general viral vectors are more efficient, because they have evolved to infect cells and, therefore, carry suitable proteins to overcome at least some of the barriers described above. Adenoviruses and adenoassociated viruses (AAV) have a natural tropism for the lungs and seemed obvious choices for early CF gene therapy trials (reviewed in [6]). However, pre-existing and induced immune responses to the viral vector which effect efficacy and duration of expression, limit their usefulness for the treatment of a life-long disease such as CF. To date, we have not seen convincing evidence in either pre-clinical models or clinical trials to demonstrate that *repeated* administration (three or more times) of adenoviral or AAV vectors to immune-competent lungs is feasible without loss of efficacy (reviewed in [6]).

In contrast to viral vectors, the simpler structure of non-viral formulations, which generally do not contain proteins, make them less likely to induce immune responses. Between 1999 and 2004 nine CF gene therapy trials used non-viral gene transfer agents (GTAs) (reviewed in [6].

Combined, these studies presented a mixed picture with some studies detecting vector-specific mRNA and some partial correction of the chloride transport defect, whereas others did not. Proof-of-concept for efficacy (based on detection of mRNA and partial correction of chloride transport) of repeated administration (three doses delivered to the nasal epithelium) of a non-viral vector was only assessed in one study [11].

More recent developments

The UK CF Gene Therapy Consortium (GTC) was founded in 2001, consisting of the three groups in Edinburgh, London and Oxford who had previously conducted CF gene therapy trials. The explicit aim was to share expertise and knowledge in a translational programme to assess whether gene therapy can change the progression of CF lung disease. The GTC is currently the only group conducting CF gene therapy trials and recently completed a Phase IIb multi-dose trial; key data are briefly summarised below:

a. Following an extensive screening programme, determined that the cationic lipid formulation GL67A, first used in the 1990s, remained the most potent GTA for airway gene transfer some two decades later [12].

b. First generation plasmids used in previous trials contained a large number of immune-stimulatory CpG dinucleotides, which may have contributed to the mild flu-like symptoms noted in previous single dose lung trials [13;14]. The first generation plasmid (termed pGM169) was improved by removing the CpG islands, codon-optimising the *CFTR* cDNA and incorporation of the novel regulatory element, hCEFI, consisting of the elongation factor 1α promoter coupled to the human CMV enhancer [15].

c. Regulatory-compliant multi-dose toxicology studies in mice [16] and sheep [17] supporting progression into a multi-dose clinical trial were undertaken. Interestingly, repeated aerosolisation of pGM169/GL67A to mice led to *cumulative* dose-related expression on repeat dosing,

reaching 94±19% of endogenous murine Cftr levels after 12 deliveries. These data further supported progression into a multi-dose clinical trial.

d. A single administration, dose-escalation (5, 10 and 20 ml of pGM169/GL67A) Phase I/IIa safety trial showed that despite CpG-depletion of the plasmid, patients receiving the 10 and 20 ml dose still developed mild flu-like symptoms including a fever [18]. The likely explanation is that both the volume administered to the lung, and the lipid, also contribute to the inflammatory response (in addition to CpG sequences). The 5 ml dose (containing ~12.5 mg plasmid DNA) was chosen for the multi-dose trial.

e. A double-blinded, placebo-controlled Multi-dose Trial was undertaken. Patients (12 years or older with moderate or mild lung disease) received 5 ml of nebulised pGM169/GL67A or 5 ml 0.9% saline every month for 12 months. The primary endpoint was a change in lung function measured as a relative change of % predicted forced expiratory volume in one second (FEV_1). Data from 116 patients (who received nine or more doses) were analysed [19]. The treatment was well tolerated and the trial met its primary endpoint showing a significant, albeit modest, treatment effect in the pGM169/GL67A group versus placebo at 12 months' follow-up (3·7%, 95% CI 0·1–7·3; p=0·046) (**Figure 2a**). Pre-specified subgroup analysis showed that patients with more severe lung disease at the start of treatment responded better than patients with milder lung disease at the start of treatment (**Figure 2b+c**). The reasons for this are currently unknown and various hypotheses have to be tested. One simple explanation may relate to the amount of material deposited in the proximal airways, which is likely higher in patients with more severe lung diseases due to mucopurulent mucus globule restricted deposition distally into the smaller airways.

The significant effect on lung function shown for the first time in this trial was paralleled by only minimal changes in the ion transport assays and no detectable vector-specific mRNA. This discordance may relate to the timing and sensitivity of the assays, the site of measurement and/or the relatively small area of airways assessed when using molecular assays and further questions the use of these assays as go/no-go decision

points in the development of CF gene therapy. It also raises the possibility of non-specific effects of the gene transfer complex on airway function, although this is difficult to rationalise with current knowledge of airway biology.

The outcome of the trial raises a number of questions.

a. Could the dose be increased?

The 5 ml dose was well tolerated when administered repeatedly and a follow-on trial might include a higher dose, supported by preliminary data from our single administration Pilot Study.

b. Was the right dosing-interval chosen?

Although animal studies have shown that gene expression persists for more than a month, it is conceivable that more frequent administration may further increase efficacy. However, moving from monthly to fortnightly or weekly dosing will clearly increase the treatment burden.

c. Was the appropriate placebo used?

The GTC, and others, have extensively deliberated the use of saline as placebo. It is important to first consider the alternatives. The use of lipid alone as a placebo is a poor choice because charge, pH, tonicity and chemical composition are very different compared to lipid/DNA complexes. The alternative could have been to use an empty plasmid or a plasmid carrying a mutant CFTR sequence. However, both these strategies are risky as it would not have be able to rule out expression of an immunologically active peptide or novel non-coding RNA molecules with deleterious biological functions. Thus, 0.9% saline, which has not been shown to negatively affect lung function, is likely the optimal placebo from a range of non-ideal options.

d. What is the best primary endpoint?

Spirometry is a variable and effort-dependent measurement and, therefore, is less than ideal. However, we spent approximately 2 years studying the longitudinal progression of numerous validated and more novel markers of disease severity in about 200 patients ("Run-in" Study, manuscript in preparation) and were unable to identify a more appropriate, regulatory-compliant endpoint.

Alternative gene transfer agents suitable for CF gene therapy

Lentiviral vectors, which integrate into the genome, are able to transduce dividing and non-dividing cells and might, therefore, be suitable for targeting differentiated cells in the lung. Several groups have investigated lentiviral vectors for airway gene transfer. Although integrating vectors have an inherent risk of inducing insertional mutagenesis, it is important to discriminate between the early γ-retroviral vectors that have been shown to cause leukaemia in some patients when used for bone marrow transduction [20], from the more advanced lentiviral vectors that have not shown evidence of insertional mutagenesis in clinical trials [21-23].

Lentiviral vectors have no natural lung tropism and, therefore, require pseudotyping with appropriate envelope proteins to facilitate lung gene transfer. The vesicular stomatitis virus G (VSV-G) protein is commonly used for this purpose and works well for bone marrow transduction *ex vivo*. However, for transduction of airway epithelium it is necessary to pre-condition the tissue with detergents which damage the epithelium and allow access to the basolateral membrane via intercellular spaces [24]. This raises safety concerns for translation into clinical trials, particularly in

CF patients with chronic lung infections. As a result, several groups, including our own, have investigated the use of other envelope proteins including the baculovirus protein GP64 [25], proteins from Ebola or Marburg filoviruses [26], the HA protein from influenza virus [27] and the F and HN protein from Sendai virus [28-30], (**Figure 3**), which are viruses that either have a broad tissue tropism (baculovirus), or a natural tropism for the lung (influenza and Sendai virus). It has been shown that a single dose of lentivirus leads to life-long stable gene expression in the murine lung (~ 2 years) and that repeated administration of the vector (10 daily doses, or three administrations at monthly intervals) is feasible [25;29-31] (**Figure 4**). To date there has been no report of insertional mutagenesis or other untoward toxicity in lungs of mice. A direct comparison between the lead non-viral vector GL67A which was used in the recently completed Phase IIb CF gene therapy trial (see above) and the F/HN-pseudotyped lentiviral vector, indicates that the virus is several log orders more efficient in transducing airway epithelial cells, which are the target cells for CF gene therapy.

In addition to the envelope proteins, promoter/enhancer elements that drive recombinant protein expression also require optimisation. The hCEF regulatory element, consisting of the elongation factor 1α promoter, coupled to the human CMV enhancer, leads to maximal levels of gene expression in murine lungs and human air liquid interface cultures (manuscript submitted). The efficiency, duration of expression, lack of toxicity and, uniquely, efficacy on repeated administration, support progression of the F/HN-pseudotyped lentivirus into a first-in-man phase I/IIa CF clinical trial which will start at the end of 2017.

Messenger RNA Therapy

Messenger RNA (mRNA) as a template for *CFTR* gene supplementation has long been appealing as an alternative to DNA-based gene delivery,

as it avoids the rate-limiting step of nuclear entry into non-dividing airway epithelial cells, being translated rapidly and efficiently directly in the cytoplasm[32].

Unfortunately, for many years researchers were unable to use *in vitro* transcribed (IVT) mRNAs to upregulate protein expression *in vivo*, as these transcripts were immediately recognized and destroyed by the immune system following injection[33]. Various nucleoside substitutions including incorporation of pseudouridine, N1-methyl-pseudouridine, thio-uridine and 5-methyl-cytidine have been made in IVT mRNA to improve stability and high performance liquid chromatography (HPLC) which removes residual double-stranded (ds)RNA has reduced immunogenicity [34].

With the above noted modifications, both single and multiple administrations of therapeutic mRNA transcripts become possible, overcoming issues of re-administration and representing a possibly safer alternative to the viral gene therapy approaches described above [33;34]. Proof-of-concept for the efficacy of repeated pulmonary delivery of chemically modified mRNA has been established in a murine model of Surfactant-Protein B deficiency [35]. In contrast, utilization of CFTR mRNA in CF knock-out animal models is still under investigation. Due to the comparatively long transcript, the development of CFTR mRNA therapy is more complex. As for gene therapy noted above, efficient delivery remains a key bottle-neck and more efficient, but non-immunogenic methods have to be found to deliver chemically-modified CFTR mRNA to the airways. Optimized chemical modification, assembly into nanoparticles, different administration routes and the possibility of linking CFTR mRNA to cell-specific aptamers are all being investigated [36;37]. Challenges related to regulation of protein expression and expression of CFTR in

non-target cells may have to be addressed. As a further extension of this technology, modified mRNAs can be utilized to encode CFTR site-specific endonucleases that might may be of use in the gene editing strategies discussed below [38]

Gene Editing

Gene editing exploits the ability of cellular DNA repair pathways to use a donor DNA molecules as a template to precisely alter the genomic DNA sequence in ~1 in 1,000 treated cells [39;40].

Rather than trying to increase editing efficiency, the focus of early studies was to develop strategies to isolate and enrich correctly edited cells by incorporation of selectable marker genes into the targeted locus. This approach led to the first gene edited animals [41]. These paved the way for the generation of a number of different CF animal models which are significantly increasing our understanding of CF pathophysiology [42-45].

The first attempts at increasing the efficiency of editing to potentially therapeutic levels, deliberately avoided the use of selectable markers on the basis that a donor molecule with a higher degree of homology would thermodynamically favour homologous pairing and thereby increase the probability for homologous exchange. This short fragment homologous replacement (SFHR) strategy was shown to precisely modify the human *CFTR* gene in ~1% of airway epithelial cells *in vitro* [46;47].

The next breakthrough showed that efficiency of donor-dependent gene editing increased by three orders of magnitude when cells were co-

treated with a DNA endonuclease capable of making a double stranded break (DSB) in the target gene [48]. However, the challenge was to develop DNA endonucleases which would create a DSB in a defined position (on-target), and prevent off-target breaks. The first "programmable" DNA endonucleases, successfully used to edit human cells, were zinc finger nucleases (ZFNs, **Figure 5**) with up to 20% of treated cells showing precise and permanent changes in the genome [49]. Murine studies with ZFNs provided proof-of principle for *in vivo* gene editing as a therapeutic approach for genetic disorders [50], and ZFNs were also used to successfully correct the F508del mutation in a human cell line [51]. A second class of programmable DNA endonucleases, the TAL-Effector Nucleases (TALENs), are easy to design and have very high specificity [52]. Human cells which have undergone *ex vivo* editing with ZFNs and TALENs have been administered to a small number of patients to successfully treat diseases affecting the haematopoietic system [53;54].

A radically different way to create a DSB and edit cells is the RNA-guided DNA-specific nuclease CRISPR/Cas9 editing system [55], which was used to demonstrate that correction of the F508del mutation resulted in restoration of CFTR function in human gut stem cells [56]. All three systems have subsequently been used to successfully edit the *CFTR* gene in human inducible pluripotent stem cells, which when differentiated into lung cells, provide new tools to model CF disease, identify novel drug targets and screen for lead compounds for clinical evaluation [57-60]. These models also raise the possibility of a gene-edited cell-based therapy for CF [61].

Another therapeutic option is direct editing of cells in the lungs *in vivo*. Proof-of-principle for this approach was established using SFHR in the lungs of normal mice [62], though no data are available with respect to editing efficiency. Neither programmable nor RNA-guided endonuclease

editing in CF lung *in vivo* has yet been reported. However, a combination of triplex-forming peptide nucleic acids and donor DNA delivered by nanoparticles has been shown to correct F508del in ~1% of mouse lung cells *in vivo* [63]. Direct editing has raised some concerns about off-target effects of the nuclease editing systems, but TALENs have an inherently high level of specificity as demonstrated by their ability to discern between two closely related human genes, CCR2 and CCR5, at a site which differs only by a single base pair [64], and recent modifications to Cas9 have reduced off-target effects to almost undetectable levels [65;66]. The use of donor DNA containing asymmetric homology arms [67], and careful choice of target regions [68], have both improved on-target editing.

The barriers to increasing the efficiency of gene editing *in vivo* are similar to those affecting gene transfer vectors described above. One might argue that gene editing should target airway progenitor cells, but these are buried beneath the surface epithelium and difficult to access with currently available vectors. However, the availability of efficient non-viral delivery methods to deliver cDNA/gene editing systems to the lung [69], robust systems to express ZFNs, TALENs and Cas9 with virus vectors [70;71], or directly as proteins (or ribonucleoprotein complexes) with modified lentivirus vectors [72;73] offers many opportunities to address these challenges. The use of modified mRNA that encodes *CFTR* site-specific endonucleases might also offer an opportunity to by-pass the barrier presented by the nuclear membrane.

The gene editing strategies described herein are dependent on DNA repair pathways that are most active in dividing cells, so in terminally differentiated, or slowly dividing cells, alternative editing techniques such as Obligare may have to be used [74]. This type of editing enables the direct insertion of DNA sequences to repair or replace mutant sequences using the non-homologous end joining (NHEJ) repair pathway, which is

independent of the cell cycle, and exploits the DNA overhangs created by ZFNs and TALENs (**Figure 5**). Whilst this approach could be difficult to adapt for use with Cas9 (which creates a blunt-end DSB), it may be possible to use Obligare in conjunction with the newly described RNA-guided nucleases such as Cpf1 [75].

Finally, in contrast to gene therapy, which in principle can genetically complement any CF-causing mutation, gene editing will require mutation-specific reagents, but in principle may be suitable to correct any mutation. Although there are ≥127 CF-causing mutations [5], 20 of these mutations (which account for 80% of CF alleles) are clustered in ten discrete locations each spanning ≤35 bp [76]. The small size suggests that all mutations in such a cluster could potentially repaired with a single Cas9/gRNA donor combination. As gene editing is permanent for the life time of the cell, and restores the gene under the control of its endogenous regulatory sequences, this may also be advantageous.

mRNA Repair

Over the last 20 years, there has been a marked increase in our knowledge of the role of RNA and how to manipulate RNA towards a therapeutic goal. For CF, repair of mutated mRNA is a viable therapeutic option and proof-of-concept was first established by Zamecnik *et al* [77]. Editing of RNA may be achieved through a number of different mechanisms including direct repair, exon exclusion and splice site changes and this approach has some attractive attributes. Repair of RNA can be achieved by oligonucleotides, short sequences of single- or double stranded RNA, usually 15-40 bases in length, which are modified for stability and to improve uptake into cells. Vectors or envelopes can be used, but are not necessary to achieve uptake into the cells. The RNA oligonucleotides are mutation-specific and targeting RNA for repair, potentially

removes the underlying cause of genetic diseases, but does not permanently alter cells. However, the effect only takes place in cells that express the gene that is causing the disease, obviating concerns about regulation or insertion into promoter regions.

There are two RNA oligonucleotides for the treatment of CF that have undergone extensive preclinical evaluation. One approach is based on targeting the CFTR splicing mutation 3849+10kb C-to-T mutation which results in the inclusion of an 84 base-pair (bp) cryptic exon containing a premature termination codon between exons 22 and 23. It has been shown that expression of splicing factors can modulate the splicing of the 84bp cryptic exon and lead to restored CFTR channel activity in a patient-derived cell line *in vitro* [78]. Subsequently, oligonucleotides were designed that target splicing motifs in the cryptic exon or at the exon-intron junctions which significantly increased translation of wild-type CFTR leading to increased CFTR channel function in human nasal epithelial cells when treated *ex vivo* [79]. It is likely that these oligonucleotides need to enter the nucleus to target the splicing motifs.

QR-010, developed by ProQR Therapeutics, targets the F508del mutation. The 33 mer oligonucleotide is chemically modified to enhance stability and cellular uptake (**Figure 6**). In contrast to the above, QR-010 likely acts in the cytoplasm and, therefore, bypasses the nuclear membrane barrier. The molecule has been shown to increase CFTR-specific chloride currents in CF PAC-1 cells and in primary human bronchial epithelial cells homozygous for the F508del mutation. More convincingly, QR-010 administered topically in the nares to F508del CF mice led to restoration of a normal nasal potential difference [80] (**Figure 7**). In addition, QR-010 administered intratracheally to F508del mice led to increased saliva secretion, which is reduced in CF mice. Importantly, this latter study also demonstrated that QR-010, administered via the

lungs, can be absorbed systemically and have extra-pulmonary effects. QR-010 is currently being studied in two clinical studies of individuals with CF. Study PQ-010-001 is a Phase Ib study of QR-010 administered via inhalation to adults homozygous for the F508del mutation to evaluate safety and tolerability, pharmacokinetics, and exploratory efficacy endpoints. [NCT02532764: https://clinicaltrials.gov/ct2/show/NCT02532764?term=ProQR&rank=1]. Study PQ-010-002 is a proof-of-concept study to evaluate the effect of QR-010 on nasal potential difference in adults with CF, either homozygous or compound heterozygous for the F508del mutation, with the QR-010 administered intranasally.

Outstanding questions relevant to all genetic medicines

How much CFTR expression do we need?

Patients with certain "mild" CF mutations, who retain approximately 10% of residual CFTR expression per cell do not suffer from lung disease, although other organs may be affected [81]. *In vitro* cell mixing experiments have shown that ~10% of non-CF cells restore CFTR-mediated chloride secretion when mixed with 90% of CF cells in a monolayer [82]. In a separate study it was shown that CFTR has to be expressed in at least 25% of cells grown in a monolayer to restore mucus transport [83]. However, these studies do not address whether complete correction of CFTR expression in 10% cells is equivalent to a low level (~10%) of CFTR expression in all cells. We currently also do not know whether the various forms of genetic medicines are more likely to achieve the former, or the latter.

Which cells do we need to target?

CFTR is expressed in various cell types in the lung including submucosal glands, ciliated epithelial cells and goblet cells. It is currently unknown which cells express the CFTR transgene following gene transfer. However, in order to transfect submucosal glands, gene transfer vectors and other oligonucleotides, when applied topically to the airways, will needs to negotiate ducts filled with mucus. It has been postulated that CFTR is also expressed in macrophages and neutrophils. Whether this leads to an intrinsic defect in host defence in the CF lung is still widely debated [3] and needs to be resolved before deciding whether gene replacement or gene editing targeted at *inflammatory cells* may lead to therapeutic benefit.

What are the risks of long-term CFTR expression and repeated administration of gene transfer agents?

The recently completed Multi-dose non-viral gene therapy trial described above, begins to address these important questions. Monthly repeat administration of pGM169/GL67A was safe and well tolerated, although we currently do not know whether these findings will translate into longer-term/life-long use of the formulation. Similarly, pre-clinical models have not raised concerns related to repeat administration of lentiviral vectors, but relevant human data will only be generated in clinical trials.

Should studies in CF models form a go-no-go decision point before progression into clinical trials?

CF mice do not acquire spontaneous airway infections or develop CF lung disease, but the nasal epithelium shows the characteristic CF chloride and sodium transport defects [84]. However, the relevance of measurement of CFTR function in the murine nose (via *in vivo* potential difference)

has been called into question by Ostrowski *et al* who showed that expression of human CFTR under the transcriptional control of a cilia-specific promoter did not correct ion transport in CF knockout mice [85]. In addition Grubb *et al* have suggested that the olfactory, rather than the respiratory, nasal epithelium mainly contributes to the ion transport defect in CF mice [86]. The CF mouse has been of limited value as a stepping-stone to human gene therapy trials [87] and we suggest should not be used as a go-no-go decision point for progression into gene therapy clinical trials. However, it remains to be seen whether CF mice prove useful in the context of other genetic medicine-based approaches. Whether correction of lung disease in CF knockout pigs or ferret is a better model to predict clinical success remains to be seen. Currently, these animals die shortly after birth due to intestinal disease and, therefore, are not yet available in large enough numbers to conduct clinically- predictive powered studies. Gut-corrected CF pigs have been generated [88] and, in time, these may be available in large enough numbers to assess the effects of gene therapy on disease pathophysiology. In addition, it is currently unclear whether the CF-like pathology is a close enough mimic of human disease to be used as a critical decision point for therapeutic development. Species-specific differences in CFTR nucleotide sequences also complicate gene and mRNA repair strategies.

Conclusions

Gene therapy for CF has been pursued since the cloning of the *CFTR* gene in 1989 and it is, therefore, not surprising that it is the most advanced of the genetic medicines discussed in this review, with ~27 clinical trials in ~600 patients having been completed. Most recently, the completion of a non-viral Phase IIb Multi-dose trial showed, for the first time, that gene therapy was able to alter the progression of CF lung disease. Approaches based on mRNA delivery and mRNA repair are making progress, with both strategies having the potential advantage of not needing

to negotiate the nuclear membrane barrier. To date, the strength of gene editing in the context of CF clearly lies in the development of pre-clinical animal and human *ex vivo* models to further advance all areas of CF research. It remains to be seen if these technologies are suited to *in vivo* pulmonary gene editing. The drug development path for mutation-specific gene editing or mRNA repair molecules may be challenging. The efficient *delivery* of the various nucleotide sequences to lung airway epithelial cells remains the common problem for all approaches. Given the data summarised above, we believe that genetic medicines will become a reality for CF patients, but continue to discourage the hype that typically accompanies any proposed novel treatment. Further, it is important to recognise the extended time lines that the development of these genetic medicines require, in comparison with small molecules.

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References

1. Wang, Y., J. A. Wrennall, Z. Cai, H. Li, and D. N. Sheppard. 2014. Understanding how cystic fibrosis mutations disrupt CFTR function: from single molecules to animal models. *Int.J.Biochem.Cell Biol.* 52:47-57.

2. Boucher, R. C. 2007. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu.Rev.Med.* 58:157-170.

3. Stoltz, D. A., D. K. Meyerholz, and M. J. Welsh. 2015. Origins of cystic fibrosis lung disease. *N.Engl.J.Med.* 372:1574-1575.

4. Shamsuddin, A. K. and P. M. Quinton. 2014. Native small airways secrete bicarbonate. *Am.J.Respir.Cell Mol.Biol.* 50:796-804.

5. Sosnay, P. R., K. R. Siklosi, G. F. Van, K. Kaniecki, H. Yu, N. Sharma, A. S. Ramalho, M. D. Amaral, R. Dorfman, J. Zielenski, et al. 2013. Defining the disease liability of variants in the cystic fibrosis transmembrane conductance regulator gene. *Nat.Genet.* 45:1160-1167.

6. Griesenbach, U., K. M. Pytel, and E. W. Alton. 2015. Cystic Fibrosis Gene Therapy in the UK and Elsewhere. *Hum.Gene Ther.* 26:266-275.

7. Stern, M., K. Ulrich, D. M. Geddes, and E. W. Alton. 2003. Poly (D, L-lactide-co-glycolide)/DNA microspheres to facilitate prolonged transgene expression in airway epithelium in vitro, ex vivo and in vivo. *Gene Ther.* 10:1282-1288.

8. Yonemitsu, Y., C. Kitson, S. Ferrari, R. Farley, U. Griesenbach, D. Judd, R. Steel, P. Scheid, J. Zhu, P. K. Jeffery, et al. 2000. Efficient gene transfer to airway epithelium using recombinant Sendai virus. *Nat.Biotechnol.* 18:970-973.

9. Schuster, B. S., A. J. Kim, J. C. Kays, M. M. Kanzawa, W. B. Guggino, M. P. Boyle, S. M. Rowe, N. Muzyczka, J. S. Suk, and J. Hanes. 2014. Overcoming the cystic fibrosis sputum barrier to leading adeno-associated virus gene therapy vectors. *Mol.Ther.* 22:1484-1493.

10. Xia, E., M. A. Munegowda, H. Cao, and J. Hu. 2014. Lung gene therapy-How to capture illumination from the light already present in the tunnel. *Genes Dis.* 1:40-52.

11. Hyde, S. C., K. W. Southern, U. Gileadi, E. M. Fitzjohn, K. A. Mofford, B. E. Waddell, H. C. Gooi, C. A. Goddard, K. Hannavy, S. E. Smyth, et al. 2000. Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis. *Gene Ther.* 7:1156-1165.

12. McLachlan, G., H. Davidson, E. Holder, L. A. Davies, I. A. Pringle, S. G. Sumner-Jones, A. Baker, P. Tennant, C. Gordon, C. Vrettou, et al. 2011. Pre-clinical evaluation of three non-viral gene transfer agents for cystic fibrosis after aerosol delivery to the ovine lung. *Gene Ther.* 18:996-1005.

13. Alton, E. W., M. Stern, R. Farley, A. Jaffe, S. L. Chadwick, J. Phillips, J. Davies, S. N. Smith, J. Browning, M. G. Davies, et al. 1999. Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet* 353:947-954.

14. Ruiz, F. E., J. P. Clancy, M. A. Perricone, Z. Bebok, J. S. Hong, S. H. Cheng, D. P. Meeker, K. R. Young, R. A. Schoumacher, M. R. Weatherly, et al. 2001. A clinical inflammatory syndrome attributable to aerosolized lipid-DNA administration in cystic fibrosis. *Hum.Gene Ther.* 12:751-761.

15. Hyde, S. C., I. A. Pringle, S. Abdullah, A. E. Lawton, L. A. Davies, A. Varathalingam, G. Nunez-Alonso, A. M. Green, R. P. Bazzani, S. G. Sumner-Jones, et al. 2008. CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. *Nat.Biotechnol.* 26:549-551.

16. Alton, E. W., A. C. Boyd, S. H. Cheng, J. C. Davies, L. A. Davies, A. Dayan, D. R. Gill, U. Griesenbach, T. Higgins, S. C. Hyde, et al. 2014. Toxicology study assessing efficacy and safety of repeated administration of lipid/DNA complexes to mouse lung. *Gene Ther.* 21:89-95.

17. Alton, E. W., A. Baker, E. Baker, A. C. Boyd, S. H. Cheng, R. L. Coles, D. D. Collie, H. Davidson, J. C. Davies, D. R. Gill, et al. 2013. The safety profile of a cationic lipid-mediated cystic fibrosis gene transfer agent following repeated monthly aerosol administration to sheep. *Biomaterials* 34:10267-10277.

18. Alton, E. W., A. C. Boyd, D. J. Porteous, G. Davies, J. C. Davies, U. Griesenbach, T. E. Higgins, D. R. Gill, S. C. Hyde, and J. A. Innes. 2015. A Phase I/IIa Safety and Efficacy Study of Nebulized Liposome-mediated Gene Therapy for Cystic Fibrosis Supports a Multidose Trial. *Am.J.Respir.Crit Care Med.* 192:1389-1392.

19. Alton, E. W., D. K. Armstrong, D. Ashby, K. J. Bayfield, D. Bilton, E. V. Bloomfield, A. C. Boyd, J. Brand, R. Buchan, R. Calcedo, et al. 2015. Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: a randomised, double-blind, placebo-controlled, phase 2b trial. *Lancet Respir.Med.* 3:684-691.

20. Rivat, C., G. Santilli, H. B. Gaspar, and A. J. Thrasher. 2012. Gene therapy for primary immunodeficiencies. *Hum.Gene Ther.* 23:668-675.

21. Aiuti, A., G. Cossu, F. P. de, M. C. Galli, G. Narayanan, M. Renner, A. Stahlbom, C. K. Schneider, and C. Voltz-Girolt. 2013. The committee for advanced therapies' of the European Medicines Agency reflection paper on management of clinical risks deriving from insertional mutagenesis. *Hum.Gene Ther.Clin.Dev.* 24:47-54.

22. Biffi, A., E. Montini, L. Lorioli, M. Cesani, F. Fumagalli, T. Plati, C. Baldoli, S. Martino, A. Calabria, S. Canale, et al. 2013. Lentiviral Hematopoietic Stem Cell Gene Therapy Benefits Metachromatic Leukodystrophy. *Science*.

23. Palfi, S., J. M. Gurruchaga, G. S. Ralph, H. Lepetit, S. Lavisse, P. C. Buttery, C. Watts, J. Miskin, M. Kelleher, S. Deeley, et al. 2014. Long-term safety and tolerability of ProSavin, a lentiviral vector-based gene therapy for Parkinson's disease: a dose escalation, open-label, phase 1/2 trial. *Lancet* 383:1138-1146.

24. Cmielewski, P., D. S. Anson, and D. W. Parsons. 2010. Lysophosphatidylcholine as an adjuvant for lentiviral vector mediated gene transfer to airway epithelium: effect of acyl chain length. *Respir.Res.* 11:84.

25. Sinn, P. L., A. L. Cooney, M. Oakland, D. E. Dylla, T. J. Wallen, A. A. Pezzulo, E. H. Chang, and P. B. McCray, Jr. 2012. Lentiviral vector gene transfer to porcine airways. *Mol.Ther.Nucleic Acids* 1:e56.

26. Kobinger, G. P., D. J. Weiner, Q. C. Yu, and J. M. Wilson. 2001. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. *Nat.Biotechnol.* 19:225-230.

27. Patel, M., A. M. Giddings, J. Sechelski, and J. C. Olsen. 2013. High efficiency gene transfer to airways of mice using influenza hemagglutinin pseudotyped lentiviral vectors. *J.Gene Med.* 15:51-62.

28. Kobayashi, M., A. Iida, Y. Ueda, and M. Hasegawa. 2003. Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *J.Virol.* 77:2607-2614.

29. Mitomo, K., U. Griesenbach, M. Inoue, L. Somerton, C. Meng, E. Akiba, T. Tabata, Y. Ueda, G. M. Frankel, R. Farley, et al. 2010. Toward Gene Therapy for Cystic Fibrosis Using a Lentivirus Pseudotyped With Sendai Virus Envelopes. *Mol.Ther.* 18:1173-1182.

30. Griesenbach, U., M. Inoue, C. Meng, R. Farley, M. Chan, N. K. Newman, A. Brum, J. You, A. Kerton, A. Shoemark, et al. 2012. Assessment of F/HN-pseudotyped lentivirus as a clinically relevant vector for lung gene therapy. *Am.J.Respir.Crit Care Med.* 186:846-856.

31. Sinn, P. L., A. C. Arias, K. A. Brogden, and P. B. McCray, Jr. 2008. Lentivirus vector can be readministered to nasal epithelia without blocking immune responses. *J.Virol.* 82:10684-10692.

32. Griesenbach, U. and E. W. Alton. 2009. Gene transfer to the lung: lessons learned from more than 2 decades of CF gene therapy. *Adv.Drug Deliv.Rev.* 61:128-139.

33. Kariko, K., H. Muramatsu, F. A. Welsh, J. Ludwig, H. Kato, S. Akira, and D. Weissman. 2008. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol.Ther.* 16:1833-1840.

34. Kariko, K., H. Muramatsu, J. Ludwig, and D. Weissman. 2011. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res.* 39:e142.

35. Kormann, M. S., G. Hasenpusch, M. K. Aneja, G. Nica, A. W. Flemmer, S. Herber-Jonat, M. Huppmann, L. E. Mays, M. Illenyi, A. Schams, et al. 2011. Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. *Nat.Biotechnol.* 29:154-157.

36. Mahiny, A. J., A. Dewerth, L. E. Mays, M. Alkhaled, B. Mothes, E. Malaeksefat, B. Loretz, J. Rottenberger, D. M. Brosch, P. Reautschnig, et al. 2015. In vivo genome editing using nuclease-encoding mRNA corrects SP-B deficiency. *Nat.Biotechnol.* 33:584-586.

37. Mays, L. E., S. mmon-Treiber, B. Mothes, M. Alkhaled, J. Rottenberger, E. S. Muller-Hermelink, M. Grimm, M. Mezger, S. Beer-Hammer, S. E. von, et al. 2013. Modified Foxp3 mRNA protects against asthma through an IL-10-dependent mechanism. *J.Clin.Invest* 123:1216-1228.

38. Rogan, M. P., D. A. Stoltz, and D. B. Hornick. 2011. Cystic fibrosis transmembrane conductance regulator intracellular processing, trafficking, and opportunities for mutation-specific treatment. *Chest* 139:1480-1490.

39. Smithies, O., R. G. Gregg, S. S. Boggs, M. A. Koralewski, and R. S. Kucherlapati. 1985. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* 317:230-234.

40. Thomas, K. R., K. R. Folger, and M. R. Capecchi. 1986. High frequency targeting of genes to specific sites in the mammalian genome. *Cell* 44:419-428.

41. Thompson, S., A. R. Clarke, A. M. Pow, M. L. Hooper, and D. W. Melton. 1989. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell* 56:313-321.

42. Wilke, M., R. M. Buijs-Offerman, J. Aarbiou, W. H. Colledge, D. N. Sheppard, L. Touqui, A. Bot, H. Jorna, H. R. de Jonge, and B. J. Scholte. 2011. Mouse models of cystic fibrosis: phenotypic analysis and research applications. *J.Cyst.Fibros.* 10 Suppl 2:S152-S171.

43. Keiser, N. W. and J. F. Engelhardt. 2011. New animal models of cystic fibrosis: what are they teaching us? *Curr.Opin.Pulm.Med.* 17:478-483.

44. Shah, V. S., D. K. Meyerholz, X. X. Tang, L. Reznikov, A. M. Abou, S. E. Ernst, P. H. Karp, C. L. Wohlford-Lenane, K. P. Heilmann, M. R. Leidinger, et al. 2016. Airway acidification initiates host defense abnormalities in cystic fibrosis mice. *Science* 351:503-507.

45. Tuggle, K. L., S. E. Birket, X. Cui, J. Hong, J. Warren, L. Reid, A. Chambers, D. Ji, K. Gamber, K. K. Chu, et al. 2014. Characterization of defects in ion transport and tissue development in cystic fibrosis transmembrane conductance regulator (CFTR)-knockout rats. *PLoS.One.* 9:e91253.

46. Kunzelmann, K., J. Y. Legendre, D. L. Knoell, L. C. Escobar, Z. Xu, and D. C. Gruenert. 1996. Gene targeting of CFTR DNA in CF epithelial cells. *Gene Ther.* 3:859-867.

47. Goncz, K. K., K. Kunzelmann, Z. Xu, and D. C. Gruenert. 1998. Targeted replacement of normal and mutant CFTR sequences in human airway epithelial cells using DNA fragments. *Hum.Mol.Genet.* 7:1913-1919.

48. Elliott, B., C. Richardson, J. Winderbaum, J. A. Nickoloff, and M. Jasin. 1998. Gene conversion tracts from double-strand break repair in mammalian cells. *Mol.Cell Biol.* 18:93-101.

49. Urnov, F. D., J. C. Miller, Y. L. Lee, C. M. Beausejour, J. M. Rock, S. Augustus, A. C. Jamieson, M. H. Porteus, P. D. Gregory, and M. C. Holmes. 2005. Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature* 435:646-651.

50. Li, H., V. Haurigot, Y. Doyon, T. Li, S. Y. Wong, A. S. Bhagwat, N. Malani, X. M. Anguela, R. Sharma, L. Ivanciu, et al. 2011. In vivo genome editing restores haemostasis in a mouse model of haemophilia. *Nature* 475:217-221.

51. Lee, C. M., R. Flynn, J. A. Hollywood, M. F. Scallan, and P. T. Harrison. 2012. Correction of the DeltaF508 Mutation in the Cystic Fibrosis Transmembrane Conductance Regulator Gene by Zinc-Finger Nuclease Homology-Directed Repair. *Biores.Open.Access.* 1:99-108.

52. Li, T., S. Huang, X. Zhao, D. A. Wright, S. Carpenter, M. H. Spalding, D. P. Weeks, and B. Yang. 2011. Modularly assembled designer TAL effector nucleases for targeted gene knockout and gene replacement in eukaryotes. *Nucleic Acids Res.* 39:6315-6325.

53. Tebas, P., D. Stein, W. W. Tang, I. Frank, S. Q. Wang, G. Lee, S. K. Spratt, R. T. Surosky, M. A. Giedlin, G. Nichol et al. 2014. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N.Engl.J.Med.* 370:901-910.

54. Poirot, L., B. Philip, C. Schiffer-Mannioui, C. D. Le, I. Chion-Sotinel, S. Derniame, P. Potrel, C. Bas, L. Lemaire, R. Galetto, et al. 2015. Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell Immunotherapies. *Cancer Res.* 75:3853-3864.

55. Jinek, M., K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, and E. Charpentier. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816-821.

56. Schwank, G., B. K. Koo, V. Sasselli, J. F. Dekkers, I. Heo, T. Demircan, N. Sasaki, S. Boymans, E. Cuppen, C. K. van der Ent, et al. 2013. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell* 13:653-658.

57. Ramalingam, S., N. Annaluru, K. Kandavelou, and S. Chandrasegaran. 2014. TALEN-mediated generation and genetic correction of disease-specific human induced pluripotent stem cells. *Curr.Gene Ther.* 14:461-472.

58. Crane, A. M., P. Kramer, J. H. Bui, W. J. Chung, X. S. Li, M. L. Gonzalez-Garay, F. Hawkins, W. Liao, D. Mora, S. Choi, et al. 2015. Targeted correction and restored function of the CFTR gene in cystic fibrosis induced pluripotent stem cells. *Stem Cell Reports.* 4:569-577.

59. Firth, A. L., T. Menon, G. S. Parker, S. J. Qualls, B. M. Lewis, E. Ke, C. T. Dargitz, R. Wright, A. Khanna, F. H. Gage, et al. 2015. Functional Gene Correction for Cystic Fibrosis in Lung Epithelial Cells Generated from Patient iPSCs. *Cell Rep.* 12:1385-1390.

60. Suzuki, S., R. G. Sargent, B. Illek, H. Fischer, A. Esmaeili-Shandiz, M. J. Yezzi, A. Lee, Y. Yang, S. Kim, P. Renz, et al. 2016. TALENs Facilitate Single-step Seamless SDF Correction of F508del CFTR in Airway Epithelial Submucosal Gland Cell-derived CF-iPSCs. *Mol.Ther.Nucleic Acids* 5:e273.

61. Butler, C. R., R. E. Hynds, K. H. Gowers, D. D. Lee, J. M. Brown, C. Crowley, V. H. Teixeira, C. M. Smith, L. Urbani, N. J. Hamilton, et al. 2016. Rapid Expansion of Human Epithelial Stem Cells Suitable for Airway Tissue Engineering. *Am.J.Respir.Crit Care Med.*

62. Goncz, K. K., A. Colosimo, B. Dallapiccola, L. Gagne, K. Hong, G. Novelli, D. Papahadjopoulos, T. Sawa, H. Schreier, J. Wiener-Kronish, et al. 2001. Expression of DeltaF508 CFTR in normal mouse lung after site-specific modification of CFTR sequences by SFHR. *Gene Ther.* 8:961-965.

63. McNeer, N. A., K. Anandalingam, R. J. Fields, C. Caputo, S. Kopic, A. Gupta, E. Quijano, L. Polikoff, Y. Kong, R. Bahal, et al. 2015. Nanoparticles that deliver triplex-forming peptide nucleic acid molecules correct F508del CFTR in airway epithelium. *Nat.Commun.* 6:6952.

64. Mussolino, C., R. Morbitzer, F. Lutge, N. Dannemann, T. Lahaye, and T. Cathomen. 2011. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. *Nucleic Acids Res.* 39:9283-9293.

65. Slaymaker, I. M., L. Gao, B. Zetsche, D. A. Scott, W. X. Yan, and F. Zhang. 2016. Rationally engineered Cas9 nucleases with improved specificity. *Science* 351:84-88.

66. Kleinstiver, B. P., V. Pattanayak, M. S. Prew, S. Q. Tsai, N. T. Nguyen, Z. Zheng, and J. K. Joung. 2016. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature* 529:490-495.

67. Richardson, C. D., G. J. Ray, M. A. DeWitt, G. L. Curie, and J. E. Corn. 2016. Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat.Biotechnol.* 34:339-344.

68. Miyaoka, Y., J. R. Berman, S. B. Cooper, S. J. Mayerl, A. H. Chan, B. Zhang, G. A. Karlin-Neumann, and B. R. Conklin. 2016. Systematic quantification of HDR and NHEJ reveals effects of locus, nuclease, and cell type on genome-editing. *Sci.Rep.* 6:23549.

69. Munye, M. M., A. D. Tagalakis, J. L. Barnes, R. E. Brown, R. J. McAnulty, S. J. Howe, and S. L. Hart. 2016. Minicircle DNA Provides Enhanced and Prolonged Transgene Expression Following Airway Gene Transfer. *Sci.Rep.* 6:23125.

70. Lombardo, A., P. Genovese, C. M. Beausejour, S. Colleoni, Y. L. Lee, K. A. Kim, D. Ando, F. D. Urnov, C. Galli, P. D. Gregory, et al. 2007. Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat.Biotechnol.* 25:1298-1306.

71. Holkers, M., I. Maggio, J. Liu, J. M. Janssen, F. Miselli, C. Mussolino, A. Recchia, T. Cathomen, and M. A. Goncalves. 2013. Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells. *Nucleic Acids Res.* 41:e63.

72. Cai, Y., R. O. Bak, and J. G. Mikkelsen. 2014. Targeted genome editing by lentiviral protein transduction of zinc-finger and TAL-effector nucleases. *Elife.* 3:e01911.

73. Choi, J. G., Y. Dang, S. Abraham, H. Ma, J. Zhang, H. Guo, Y. Cai, J. G. Mikkelsen, H. Wu, P. Shankar, et al. 2016. Lentivirus pre-packed with Cas9 protein for safer gene editing. *Gene Ther*.

74. Maresca, M., V. G. Lin, N. Guo, and Y. Yang. 2013. Obligate ligation-gated recombination (ObLiGaRe): custom-designed nuclease-mediated targeted integration through nonhomologous end joining. *Genome Res.* 23:539-546.

75. Zetsche, B., J. S. Gootenberg, O. O. Abudayyeh, I. M. Slaymaker, K. S. Makarova, P. Essletzbichler, S. E. Volz, J. Joung, O. J. van der, A. Regev, et al. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163:759-771.

76. Bobadilla, J. L., M. Macek, Jr., J. P. Fine, and P. M. Farrell. 2002. Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening. *Hum.Mutat.* 19:575-606.

77. Zamecnik, P. C., M. K. Raychowdhury, D. R. Tabatadze, and H. F. Cantiello. 2004. Reversal of cystic fibrosis phenotype in a cultured Delta508 cystic fibrosis transmembrane conductance regulator cell line by oligonucleotide insertion. *Proc.Natl.Acad.Sci.U.S.A* 101:8150-8155.

78. Nissim-Rafinia, M., O. Chiba-Falek, G. Sharon, A. Boss, and B. Kerem. 2000. Cellular and viral splicing factors can modify the splicing pattern of CFTR transcripts carrying splicing mutations. *Hum.Mol.Genet.* 9:1771-1778.

79. M.Irony-Tur Sinai, S.Wilton, and B.Kerem. Restoration of the CFTR function by antisense oligonucleotide splicing modulation. J Cystic Fibrosis S2[WS1.5]. 2014.
Ref Type: Abstract

80. Beumer et al. QR-010, an RNA Therapy, Restores CFTR Function in DeltaF508-CFTR Mice. Pediatr.Pulmonol. 49[S38], 227-228. 2014.
Ref Type: Abstract

81. Chu, C. S., B. C. Trapnell, S. Curristin, G. R. Cutting, and R. G. Crystal. 1993. Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. *Nat.Genet.* 3:151-156.

82. Farmen, S. L., P. H. Karp, P. Ng, D. J. Palmer, D. R. Koehler, J. Hu, A. L. Beaudet, J. Zabner, and M. J. Welsh. 2005. Gene transfer of CFTR to airway epithelia: low levels of expression are sufficient to correct Cl- transport and overexpression can generate basolateral CFTR. *Am.J.Physiol Lung Cell Mol.Physiol* 289:L1123-L1130.

83. Zhang, L., B. Button, S. E. Gabriel, S. Burkett, Y. Yan, M. H. Skiadopoulos, Y. L. Dang, L. N. Vogel, T. McKay, A. Mengos, et al. 2009. CFTR delivery to 25% of surface epithelial cells restores normal rates of mucus transport to human cystic fibrosis airway epithelium. *PLoS.Biol.* 7:e1000155.

84. Griesenbach, U., S. N. Smith, R. Farley, C. Singh, and E. W. Alton. 2008. Validation of Nasal Potential Difference Measurements in Gut-corrected CF Knockout Mice. *Am.J.Respir.Cell Mol.Biol.* 39:490-496.

85. Ostrowski, L. E., W. Yin, P. S. Diggs, T. D. Rogers, W. K. O'Neal, and B. R. Grubb. 2007. Expression of CFTR from a ciliated cell-specific promoter is ineffective at correcting nasal potential difference in CF mice. *Gene Ther.* 14:1492-1501.

86. Grubb, B. R., T. D. Rogers, R. C. Boucher, and L. E. Ostrowski. 2009. Ion transport across CF and normal murine olfactory and ciliated epithelium. *Am.J.Physiol Cell Physiol* 296:C1301-C1309.

87. Griesenbach, U., S. G. Sumner-Jones, E. Holder, F. M. Munkonge, T. Wodehouse, S. N. Smith, M. Y. Wasowicz, I. Pringle, I. Casamayor, M. Chan, et al. 2010. Limitations of the murine nose in the development of nonviral airway gene transfer. *Am.J.Respir.Cell Mol.Biol.* 43:46-54.

88. Stoltz, D. A., T. Rokhlina, S. E. Ernst, A. A. Pezzulo, L. S. Ostedgaard, P. H. Karp, M. S. Samuel, L. R. Reznikov, M. V. Rector, N. D. Gansemer, et al. 2013. Intestinal CFTR expression alleviates meconium ileus in cystic fibrosis pigs. *J Clin.Invest* 123:2685-2693.

89. Alton, E. W., A. C. Boyd, S. H. Cheng, J. C. Davies, L. A. Davies, A. Dayan, D. R. Gill, U. Griesenbach, T. Higgins, S. C. Hyde, et al. 2013. Toxicology study assessing efficacy and safety of repeated administration of lipid/DNA complexes to mouse lung. *Gene Ther.*

90. Rosenfeld, M. A., K. Yoshimura, B. C. Trapnell, K. Yoneyama, E. R. Rosenthal, W. Dalemans, M. Fukayama, J. Bargon, L. E. Stier, L. Stratford-Perricaudet, et al. 1992. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell* 68:143-155.

91. Hyde, S. C., D. R. Gill, C. F. Higgins, A. E. Trezise, L. J. MacVinish, A. W. Cuthbert, R. Ratcliff, M. J. Evans, and W. H. Colledge. 1993. Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature* 362:250-255.

92. Zabner, J., L. A. Couture, R. J. Gregory, S. M. Graham, A. E. Smith, and M. J. Welsh. 1993. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell* 75:207-216.

93. Crystal, R. G., N. G. McElvaney, M. A. Rosenfeld, C. S. Chu, A. Mastrangeli, J. G. Hay, S. L. Brody, H. A. Jaffe, N. T. Eissa, and C. Danel. 1994. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat.Genet.* 8:42-51.

94. Caplen, N. J., E. W. Alton, P. G. Middleton, J. R. Dorin, B. J. Stevenson, X. Gao, S. R. Durham, P. K. Jeffery, M. E. Hodson, C. Coutelle, et al. 1995. Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nat.Med.* 1:39-46.

95. Wagner, J. A., A. H. Messner, M. L. Moran, R. Daifuku, K. Kouyama, J. K. Desch, S. Manley, A. M. Norbash, C. K. Conrad, S. Friborg, et al. 1999. Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. *Laryngoscope* 109:266-274.

96. Moss, R. B., D. Rodman, L. T. Spencer, M. L. Aitken, P. L. Zeitlin, D. Waltz, C. Milla, A. S. Brody, J. P. Clancy, B. Ramsey, et al. 2004. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 125:509-521.

Figure legends

Figure 1: Schematic presentation of "genetic medicine" strategies that are currently being developed for CF

The basic concepts of gene therapy (**A**) and mRNA therapy (**B**), as well as gene editing (C) and mRNA repair (D) are outlined. In addition to chloride (Cl^-) ions, CFTR also conducts a number of other anions, including bicarbonate. For simplicity these molecules are not shown in the Figure.

Figure 2: Stabilisation of lung function after repeated administration of the non-viral formulation pGM169/GL67A

Cystic fibrosis patients were treated monthly for 12 months with either active drug or the placebo. Lung function (FEV_1=forced expiratory volume in 1 second) was measured at each treatment visit before administration of study drugs. Data are expressed as relative change from baseline in % predicted FEV_1. Error bars show the standard error of the mean. (A) All patients. There was a significant, albeit modest, treatment effect in the pGM169/GL67A group versus placebo at 12 months' follow-up (3·7%, p=0·046). (B) Patients with more severe lung function at start of treatment (Baseline FEV_1 = 50-70%), (C) Patients with less severe lung function at start of treatment (Baseline FEV_1=70-90%). The figure is adapted from [89] as part of a CCBY license.

Figure 3: Generation of F/HN-pseudotyped lentiviral vector

Molecular techniques enable the replacement of the gp120 envelope glycoprotein, which supports lentivirus entry into T-cells but is not suitable

for entry into airway epithelial cells, with the F (Fusion) and HN (Hemagglutinin-Neuraminidase) proteins from Sendai virus which support efficient entry into airway epithelial cells. This process of pseudotyping leads to the generation of a chimeric pseudotyped F/HN lentiviral vector. The Sendai virus F and HN proteins were chosen because they are, in part, responsible for the high transduction efficiency of Sendai virus in lungs [8].

Figure 4: F/HN-pseudotyped lentivirus transduction leads to persistent gene expression in mouse airways

Mice were transduced with F/HN-pseudotyped lentivirus expressing a luciferease reporter gene by nasal sniffing (or received PBS (negative controls). Luciferase expression was visualised using bioluminescence imaging, 2 to 22 months after transduction.

Figure 5: Programmable and RNA-guided DNA endonucleases

Schematic representation of the four most commonly used programmable and RNA-guided DNA endonucleases. ZNF= Zinc finger nucleases, ZF= Zinc finger, TALEN= TAL-Effector Nucleases, PAM= protospacer adjacent motif, RVD= repeat variable di-residue

Figure 6: Structure of the chemically modified QR-010 oligonucleotide used for mRNA repair

QR-010 is a single stranded, RNA oligonucleotide (33 nucleotides) with 2'O methyl base modifications and a phosphorothioate (PS) backbone to facilitate intracellular delivery and stability. QR-010 is designed to bind to sequences adjacent to the deleted F508 region in the CFTR mRNA resulting in the production of fully functional CFTR protein.

Figure 7: Restoration of ion transport in nasal epithelium of F508del mice after treatment with QR-010

F508del mice were treated with six doses of QR-010, or remained untreated, and nasal potential difference (NPD) was measured. Representative NPD traces of wild-type, untreated and QP-010 treated F508del mice are shown.

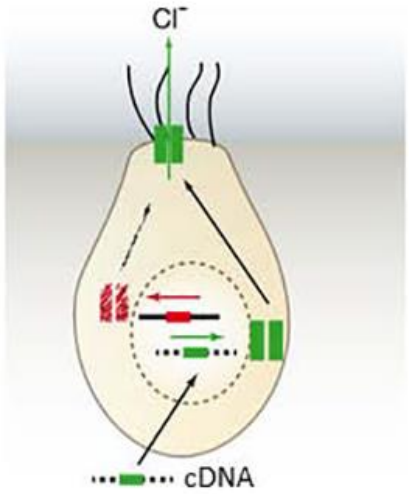
Tables

Table 1: Gene therapy milestone studies

- Three years after cloning of *CFTR*, Rosenfeld *et al* provided evidence of successful CFTR mRNA and protein expression after adenovirus-mediated *CFTR* cDNA transfer into cotton rats [90].
- Four years after cloning of *CFTR* Hyde *et al* showed that non-viral *CFTR* cDNA transfer was able to partially correct the chloride transport in tracheal epithelium of CF knockout mice [91].
- In the same year, Zabner *et al* performed the first, albeit small and not placebo-controlled, CF gene therapy trial in 3 patients. A first generation adenoviral vector carrying the *CFTR* cDNA was administered to the nasal epithelium and shown to partially restore cAMP-mediated chloride transport [92].
- Five years after cloning, of *CFTR* Crystal *et al* performed the first Phase I dose-escalation CF gene therapy study. This was first and foremost a safety study and showed transient inflammatory responses only at the highest dose (5x1e9 plaque forming units (PFU)/patient) [93].
- Six years after cloning of *CFTR*, Caplen *et al* provided first evidence that a non-viral gene transfer agent (DC-Chol:DOPE) complexed with *CFTR* cDNA could partially correct cAMP-mediated chloride transport in the nasal epithelium of CF patients [94].
- Ten years after cloning of *CFTR*, the first of six AAV2 trials was published [95]. These trials initially looked encouraging [96], but ultimately were discontinued due to lack of efficacy

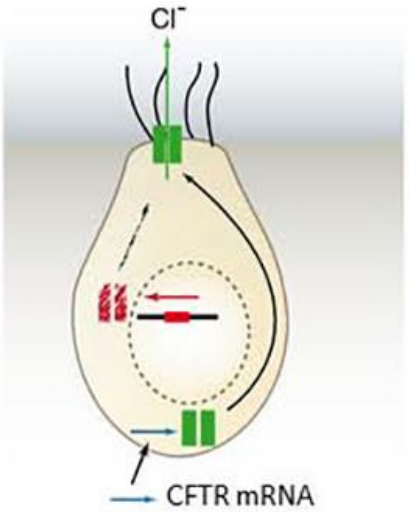
- The same year, Alton *et al* demonstrated that a non-viral gene transfer agent (GL67A) complexed with a plasmid DNA carrying the *CFTR* cDNA could partially correct cAMP-mediated chloride transport in the lungs of CF patients[13].
- 26 years after cloning of *CFTR*, Alton *et al* demonstrated that repeated administration of GL67A complexed with a plasmid DNA carrying the *CFTR* cDNA significantly, albeit modestly, stabilised lung function in CF patients [19].

A



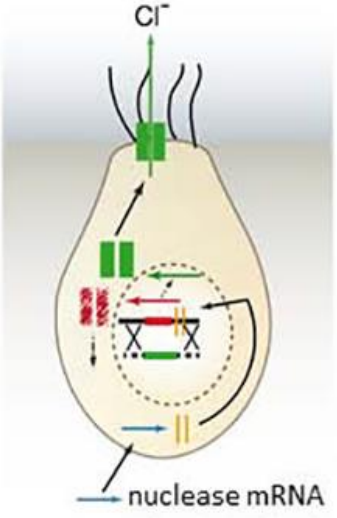
Gene therapy: a correct version of the CFTR DNA sequence is delivered to the nucleus and normal protein is made

B



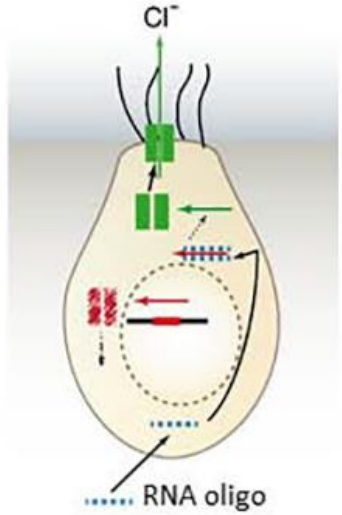
mRNA therapy: a correct version of the CFTR mRNA is delivered to the cytoplasm and normal protein is made

C

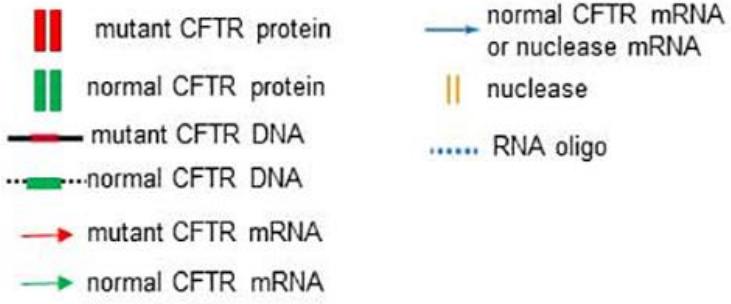


Gene repair: a correct version of the CFTR DNA sequence is delivered to the nucleus. In addition mRNA encoding a nuclease which causes a break in the DNA, which triggers recombination, is delivered. The CFTR DNA is repaired and Normal protein is made.

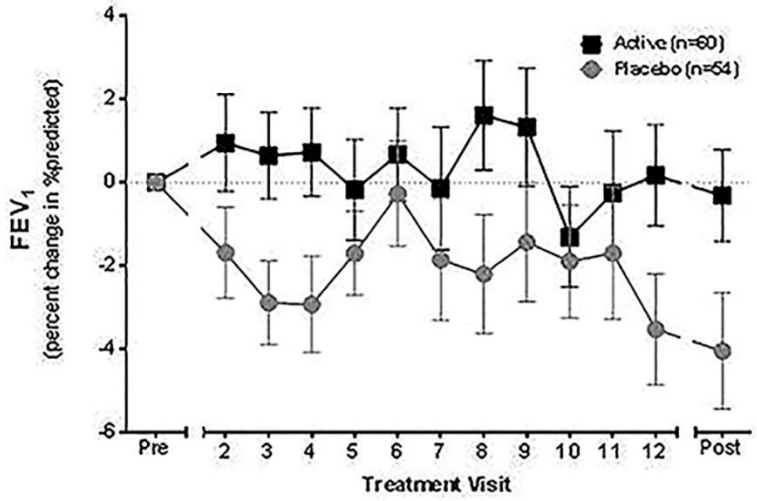
D



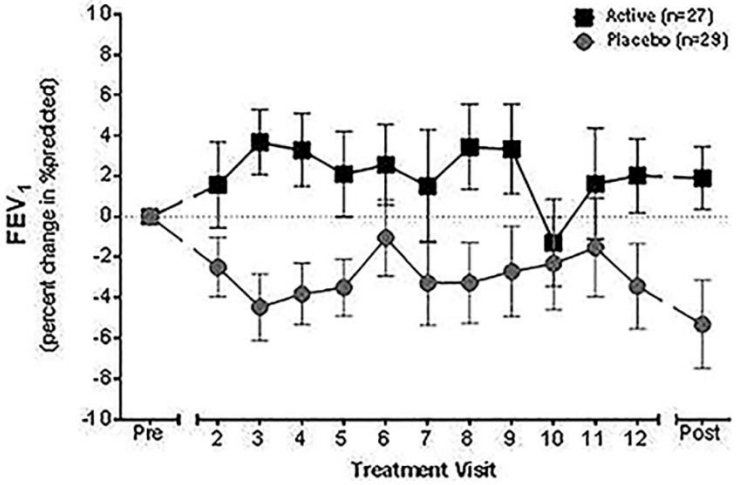
mRNA repair: a CFTR RNA oligonucleotide is delivered to the cytoplasm and repairs the CFTR mRNA leading to normal protein production



A.


Active (n=60)
Placebo (n=54)
FEV1
(percent change in %predicted)
4
2
0
-2
-4
-6
Pre
2 3 4 5 6 7 8 9 10 11 12
Post
Treatment Visit

B.


Active (n=27)
Placebo (n=29)
FEV1
(percent change in %predicted)
10
8
6
4
2
0
-2
-4
-6
-8
-10
Pre
2 3 4 5 6 7 8 9 10 11 12
Post
Treatment Visit

C.

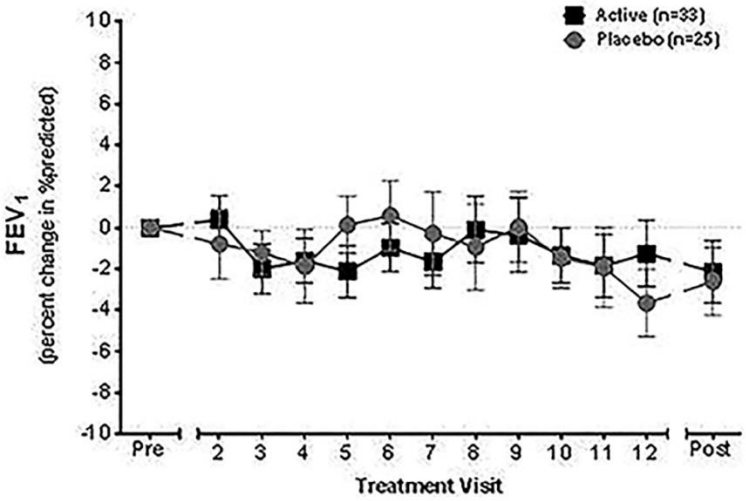

Active (n=33)
Placebo (n=25)
FEV1
(percent change in %predicted)
10
8
6
4
2
0
-2
-4
-6
-8
-10
Pre
2 3 4 5 6 7 8 9 10 11 12
Post
Treatment Visit

Figure 2

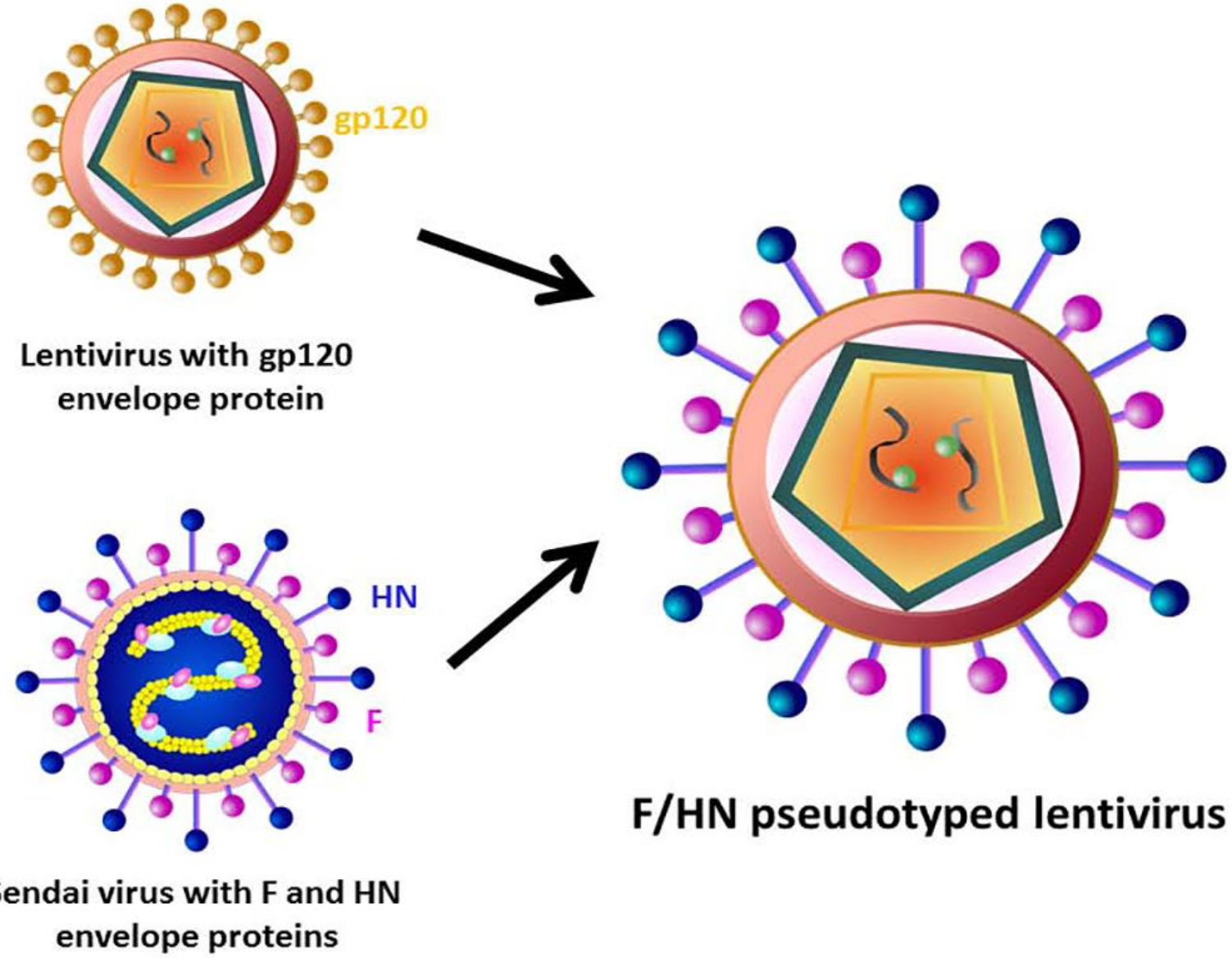

gp120
Lentivirus with gp120 envelope protein
HN
F
Sendai virus with F and HN envelope proteins
F/HN pseudotyped lentivirus

Figure 3

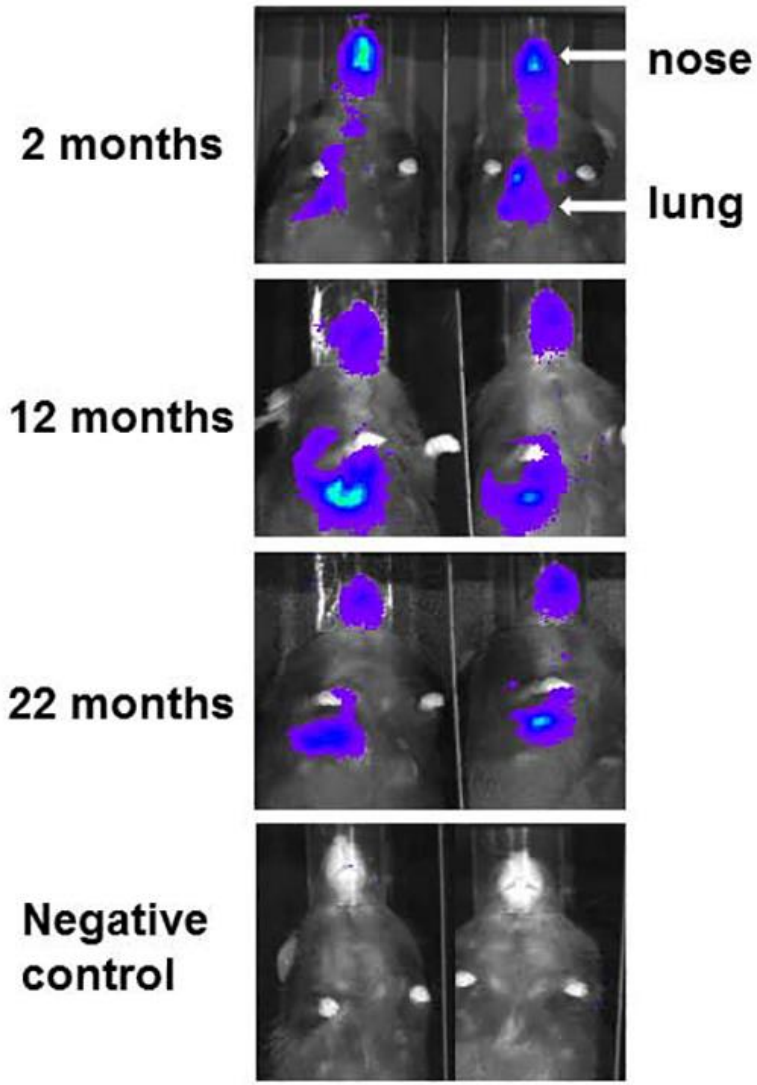
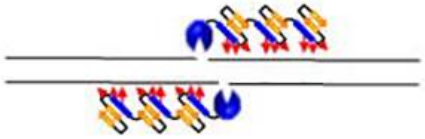
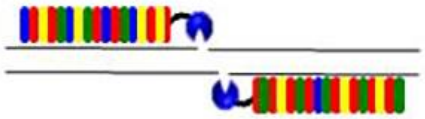


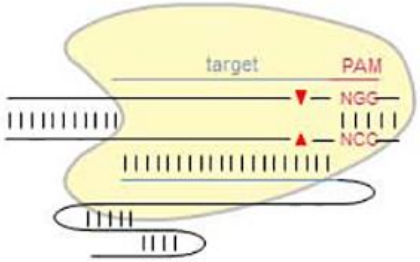
Figure 4



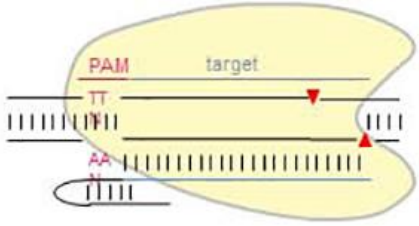
ZFNs
- Obligate dimer
- Recognition site 18-24 bp
- 4 bp overhang
- Each ZF unit binds 3 bp



TALENs
- Obligate dimer
- Recognition site 29-35 bp
- 4 bp overhang
- Each RVD unit binds 1 bp



Cas9
- RNA-guided monomer
- Recognition site 20 bp
- Blunt end cut
- Multiple PAM options



Cpf1
- RNA-guided monomer
- Recognition site 23 bp
- 5 bp overhang
- TTN or TTTN PAM

Figure 5

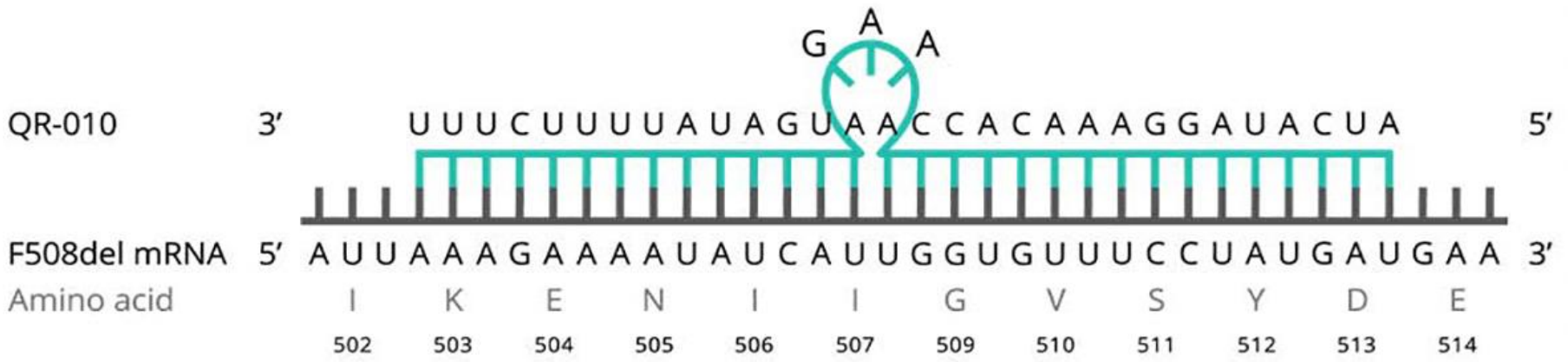

G A A
QR-010 3′ U U U C U U U U A U A G U A A C C A C A A A G G A U A C U A 5′
F508del mRNA 5′ A U U A A A G A A A A U A U C A U U G G U G U U U C C U A U G A U G A A 3′
Amino acid I K E N I I G V S Y D E
502 503 504 505 506 507 509 510 511 512 513 514

Figure 6

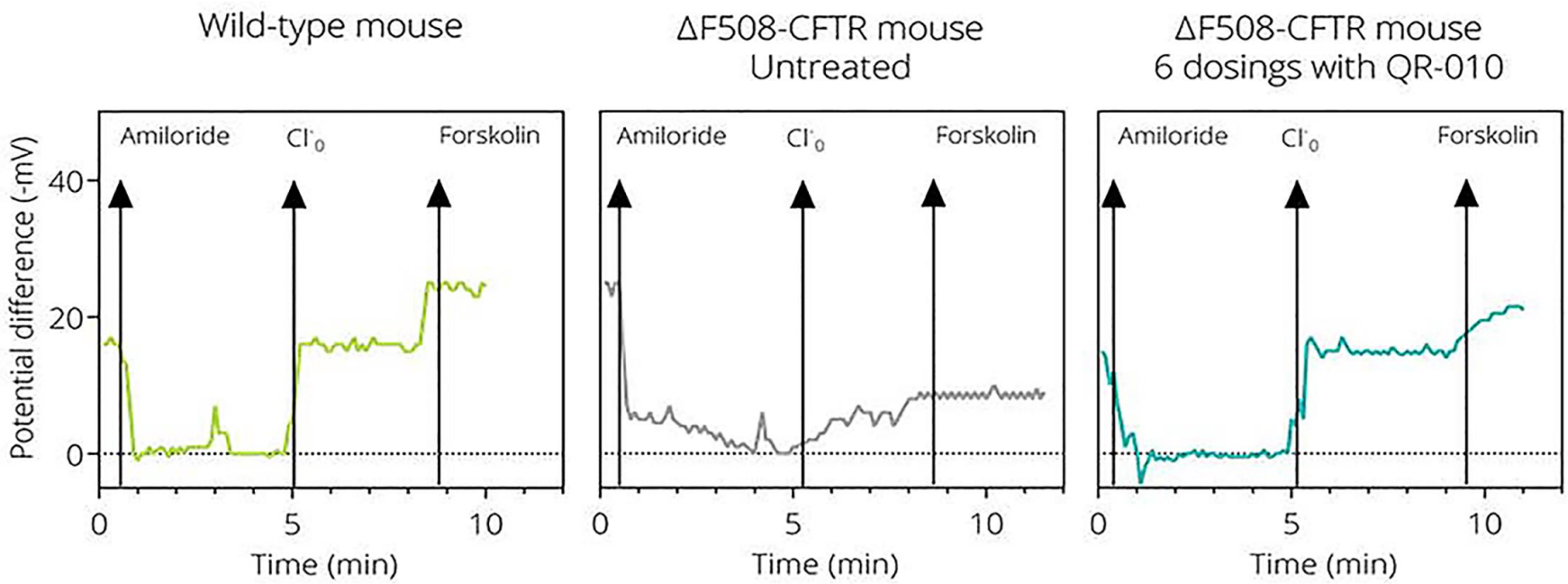

Wild-type mouse
ΔF508-CFTR mouse
Untreated
ΔF508-CFTR mouse
6 dosings with QR-010
Amiloride
Cl-0
Forskolin
Potential difference (-mV)
40
20
0
0
5
10
Time (min)

Figure 7