REVIEW

Roadmap for an early gene therapy for cystic fibrosis airway disease

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Abstract

Gene therapy provides a mutation-independent approach to treat or even cure CF airway disease. To develop a clinical candidate for CF gene therapy, a thorough examination of preclinical efficacy in relevant cell and animal models is a prerequisite. For a long time, the CF field was struggling with a lack of appropriate animal models for CF airway pathology. Since 2008, many different and complementary animal models have been generated that develop hallmarks of CF airway disease, including the CF pig, ferret, and rat. With this, a new era has arisen that allows investigating the efficacy of gene therapy beyond molecular and electrophysiological end-points. Successful gene therapy most likely requires an appropriate time window. CF lung pathology progresses with age and therefore an early treatment would be beneficial to prevent irreversible damage. In that regard, newborn screening programs and prenatal diagnosis already provide a basis to facilitate future preventive gene-based treatment. If successful, gene therapy for CF airway disease would markedly reduce the treatment burden and improve life quality and life expectancy of CF patients.

1 | INTRODUCTION

During the last decades, medical knowledge moved forward significantly, providing better care and nursing. We live longer than ever before, still most remedies do not tackle the underlying disease cause, but merely treat symptoms. Gene therapy holds promise to cure a wide range of genetic and acquired disorders and has become a valuable treatment for genetic disorders, such as adenosine deaminase deficiency, X-linked severe combined immunodeficiency, congenital blindness, hemophilia B, spinal muscular atrophy, and lipoprotein lipase deficiency.^{1–6} However, the unparalleled clinical potential of gene therapy is yet to be proven for cystic fibrosis (CF).

CF is a chronic autosomal recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), resulting in multi-organ pathology. Lung disease however is the major cause of mortality. Despite better symptomatic care, the median age at death remains early thirties.⁷ The pathophysiology of CF relates to mutations in CFTR (>2000, www.genet.sickkids.on.ca), a chloride/bicarbonate channel. This channel also regulates other epithelial ion transporters, such as non-CFTR chloride channels and the epithelial sodium channel, ENaC.⁸ CFTR directly regulates the airway surface

liquid (ASL) height through chloride and water transport but is also thought to play an important role in the release and unfolding of mucus granules from the submucosal glands and surface epithelium through bicarbonate secretion.⁹ Both these processes are disturbed in CF and lead to impeded ciliary activity, mucostasis, and viscous secretions. Additionally, reduced ASL pH caused by defective bicarbonate secretion leads to abnormal innate immunity of the airways, as seen in studies in neonatal CF pigs and ferrets.^{10,11} Combined, these defects result in the inability of CF airways to efficiently eradicate pathogens, contributing to recurrent and chronic infection, excessive inflammation, and lung damage.

Since the discovery of the *CFTR* gene in 1989,^{12–14} 27 clinical trials have been conducted with viral vectors or non-viral vehicles (reviewed in Sondhi et al¹⁵ and Alton et al¹⁶). The safety and tolerability of different vectors have been proven, but none have led to a persistent clinical benefit. The most recent study by Alton et al demonstrated that liposome delivery of *CFTR* cDNA stabilized lung function (forced expiratory volume in 1 second) in treated patients, whereas a further decline was observed in the placebo group.¹⁷ Although the benefit was modest, these results fuel further research on CF gene therapy. This review therefore sets out a roadmap for successful clinical gene therapy. We discuss the progress made and phrase the remaining

questions to be answered in the broad portfolio of available cell and animal models of CF lung disease.

2 | FROM GENE ADDITION TO GENE EDITING—EFFICIENCY OF GENE DELIVERY MATTERS

Gene therapy in its broadest context means transfer of therapeutic DNA or RNA into a target tissue to correct the function of a protein that, if dysfunctional, leads to a hereditary or acquired disorder. The most straightforward procedure is gene addition therapy, a strategy used since the early nineties for many monogenetic disorders including CF.¹⁵ The aim is to introduce the correct genetic information of a mutated gene by introducing the coding sequence of this gene and delivering it to affected organ(s). Gene correction by gene editing on the other hand refers to the process of correcting the endogenous mutated gene in its genomic location using “nuclear scissors”, eg, Cas9, Zinc-finger, or TALE nucleases.^{18,19} Additional delivery of a repair template allows the original DNA sequence of the affected gene to be restored by homology directed repair. The latter is exemplified by correction of the most common CF-causing mutation (F508del) using CRISPR/Cas9 in patient-derived intestinal organoids.²⁰ The major obstacles for transferring gen(om)e editing strategies to the clinic are insufficient fidelity,^{21,22} low efficacy in vivo, and existing challenges for stem cell engraftment after transplantation.^{23,24} Nevertheless, it is a highly promising gene therapeutic strategy, and future work will undoubtedly extend the current limits of this approach.

Efficient correction of genetic disorders depends on the efficacy of gene delivery, which remains the major hurdle for clinical success. Nevertheless, considerable progress has been made in viral and non-viral vectors since recombinant DNA technology hit the stage in the 1970s.^{25–28} Non-viral vectors include cationic liposomes, which fuse to the plasma membrane of the cell,²⁹ or poly(lactic-co-glycolic) acid nanoparticles which can be modified with targeting ligands or cell penetrating peptides for enhanced uptake.³⁰ Advantages include incorporation of large transgenes and low immunogenicity, allowing repeated administration. Poor transduction efficiency is the main limitation as transport from cytoplasm to nucleus is rate limiting.²⁶ By contrast, viral vectors have an inherent mechanism to efficiently enter the host cell and nucleus. Monthly administrations of liposome-*CFTR* cDNA complexes to CF patients' airways in a phase IIb trial showed that gene delivery was well tolerated, but the efficacy modest,¹⁷ underscoring the need for more potent gene therapeutic strategies. Adenoviral vectors (AdV) were the first vectors tested clinically for CF in the early 1990s.^{31,32} *CFTR* expression was however transient, and repeated doses did not increase the clinical benefit due to high immunogenicity.^{33,34} A new generation of helper-dependent (HD) AdV has been developed, devoid of viral genes.³⁵ Current preclinical work focuses on the longevity of transgene expression in airways, one of the major obstacles in the past.³⁶

In parallel, recombinant adeno-associated viral vectors (rAAV) for CF gene therapy were extensively tested clinically. rAAV have low immunogenicity, (mainly) are non-integrating, and devoid of viral

What is already known about this topic?

- 27 gene therapy clinical trials for cystic fibrosis (CF) have demonstrated safety and tolerability, but none a persistent clinical benefit.
- Progress in vector technology and animal models with human CF-like lung disease hold promise to develop and reliably test an effective gene therapy for CF.
- Preclinical studies on fetal gene therapy point out specific advantages compared with postnatal gene therapy, but further comparative studies are needed to substantiate these findings.

What does this study add?

- This review sets out a roadmap for successful gene therapy in the clinic. We discuss the progress made and list the remaining questions to be answered in cell and animal models of CF lung disease.
- To prevent irreversible lung damage early gene therapy is key. The advantages of starting gene therapy in the neonatal or even fetal period are discussed and are weighted against the inherent risk for complications.

genes.³⁷ Their packaging capacity of approximately 5 kb potentially limits its use for larger genes, such as *CFTR* (4.4 kb). In 2012, the EMA approved the first rAAV gene therapy (Glybera, UniQure) for lipoprotein lipase deficiency (www.ema.europa.eu). The first and best studied serotype (rAAV2/2) was used in initial trials to deliver full-length *CFTR* cDNA to the respiratory tract under the control of a weak inverted terminal repeat (ITR) promoter.^{38–42} Whereas an improvement in lung function was observed in a first trial,⁴⁰ there was no clinical benefit in a larger follow-up study.⁴¹ In retrospect, absence of receptors for this serotype at the apical side of the airway epithelium, the weak promoter or effective barriers in CF patients' lungs might have contributed to inefficient gene transfer.⁴³ Nevertheless, these studies confirmed the favorable safety profile of rAAV with only a few adverse effects potentially related to vector treatment. Current research focuses on improving transduction efficacy by using alternative, airway-tropic rAAV serotypes^{44–47} and promoters^{47–49} and/or optimizing the expression cassette to accommodate a *CFTR* minigene.^{45,50–52}

Lentiviral vectors (LV) have been studied only preclinically.^{53–55} LV have shown long-term gene expression in mouse models.^{54,56} It is yet to be shown whether sustained gene expression arises from vector integration into stem cells, or transduction of long-living terminally differentiated epithelial cells.⁵⁷ A complete overview of all preclinical and clinical progress on CF gene therapy is given by Sondhi and colleagues.¹⁵

3 | WHAT HAVE CF MOUSE MODELS TAUGHT US ABOUT GENE THERAPY?

CF mice were the only available animal models to study gene delivery to the airways until the first CFTR^{-/-} pig was produced in 2008.^{58,59} An overview of all CF mice and their phenotypic similarities and discrepancies compared with humans is given in Wilke et al⁶⁰ and Conese et al⁶¹. Although CF mice do not spontaneously develop lung disease, they mimic the abnormalities in ion transport in the upper airways (nose). Also, cell composition is similar to human airways.⁶² Therefore, the mouse nose can be used as a read-out for CFTR function. A protocol for nasal potential differences (NPD), a CFTR biomarker in humans, has been adapted for use in CF mice.^{63,64} We demonstrated rescue of CFTR activity by NPD in the CF mouse nose after rAAV2/5 delivery of a shortened CFTR (CFTRΔR⁴⁹), lacking a portion of the regulatory domain, which retains regulated channel activity.⁴⁵ Also, other vectors, such as LVs or a gene editing nanoparticle formulation, could partially restore CFTR function in CF mice as measured by improvements in NPD.^{65,66} Furthermore, mouse models have been instrumental in addressing generic outcomes of gene therapy, ie, transduction efficiency, cell-specific tropism, immune responses to vector and transgene, and longevity of gene expression (reviewed in Mingozi and High⁶⁷). In non-CF mouse airways, we have shown sustained reporter expression till 15 months after rAAV2/5 delivery, a substantial proportion of the lifespan of a laboratory mouse.⁴⁶ Nevertheless, this question remains to be

re-investigated in an animal model spontaneously developing lung disease, such as the β-ENaC overexpressing mouse or the CF rat, ferret, and pig.^{59,68-70} This because chronic inflammation leads to enhanced turnover of respiratory epithelium and transduction efficacy of many vectors is hampered by their inability to penetrate excessive mucus combined with infiltrating immune cells.^{71,72} On the other hand, misfolded mutant CFTR, eg, F508del, leads to endoplasmic reticulum stress, which renders the cell less equipped to block viral vector trafficking. As such, transduction of F508del overexpressing cells with rAAV2-eGFP was significantly enhanced compared with wild type (WT)-CFTR cells.⁷³ Similarly, proteasome inhibitors co-delivered with rAAV in mice, dogs, and ferrets led to enhanced transduction.^{74,75} They are postulated to enhance transduction by preventing degradation of rAAV capsids, although their exact mechanism remains unknown.⁷⁶ These studies point out the complexity of predicting in vivo efficacy as many factors influence this process, including inflamed CF airways and subcellular stress caused by specific CFTR mutations or infections.

4 | A NEW ERA OF CF ANIMAL MODELS AND READ-OUTS TO STUDY THE HALLMARKS OF CF AIRWAY DISEASE

The CF rat, ferret, and pig represent human physiology and CF pathology more faithfully compared with CF mice and hence are a great

TABLE 1 Overview of clinically most relevant lung disease parameters of available CF animal models

	Mouse ^{a,b}	Rat	Ferret	Pig
CF-like bioelectric properties	Yes ^{60,62c}	Yes ⁷⁷	Yes ^{70,83,128}	Yes ^{79,84,85}
Altered pH of ASL	No ⁸⁶	Yes ⁸⁷	Yes ¹¹	Yes ^{10,79}
Dehydrated airways (reduced PCL and ASL height)	Yes ⁸⁸	Yes ⁷⁷	Yes ¹¹	Mixed results ^{84,108}
Changes in mucus composition (eg, increase in mucus solids)	NR	NR	NR	Yes ⁸⁹
Defective mucociliary clearance	No ^{60,90}	Yes ⁸⁷	Yes ^{11,128}	Yes ^{80,82,108}
Mucus accumulation/obstruction	No ⁶⁰	NR	Yes ¹⁰⁵	Yes ^{80,113}
Abnormalities in submucosal gland function resulting in defective fluid and/or mucus secretion	Yes ^{92d, e}	NR	Yes ⁷⁰	Yes ^{80,82,91}
Defective innate immunity (eg, reduced activity of antimicrobials, immune cell defects)	Yes ^{60,93,94}	NR	Yes ¹¹	Yes ^{10,79}
Spontaneous infection	No ⁶⁰	NR	Yes ^{11,128}	Yes ^{10,95,109}
Anatomical defects of the airways (eg, tracheomalacia, sinus hypoplasia)	Yes ⁹⁶	Yes ⁷⁷	NR	Yes ^{78,95,97,98}
Human-relevant CFTR mutations	Yes ^{60,62}	NR	NR	Yes ^{58,99}
In utero presence of lung abnormalities (eg, tracheomalacia, abnormal lung development ^f)	NR	NR	NR	NR

Legend: This overview covers all models related to the *Cftr* gene, including knock-out and knock-in models where a specific mutation is introduced into the endogenous *Cftr* gene of the model species used.

Abbreviations: ASL, airway surface liquid; NR, not reported; PCL, periciliary liquid height.

^aThe β-ENaC overexpressing mouse presents many features of CF airway disease, including a significant reduction in PCL height and mucociliary clearance, mucus retention and plugging, and finally asphyxia and death.⁶⁸

^bFor a full overview of all lung-related phenotypes of the many different CF mouse models available, see reviews.^{60,100}

^cLoss of cAMP-induced CFTR chloride currents in nasal epithelium of *Cftr*^{-/-} mice is genetic background dependent⁶⁰; in trachea and bronchi, this loss is compensated by calcium activated chloride channels (CaCC, TMEM16A).^{62,101,102} Mouse CFTR in nasal epithelium is expressed principally in brush cells, not in ciliated cells.⁶⁰

^dSubmucosal glands only present in nose and proximal trachea.

^eMouse serous cells lack cAMP-mediated Ca²⁺ signaling to activate basolateral membrane K⁺ conductance, resulting in weak cAMP-driven serous cell fluid secretion compared with human glands.¹⁰³

^fCFTR has been implicated in normal lung organogenesis in utero from studies in sheep and humans,^{104,105} but abnormal lung development in subjects with CF remains to be demonstrated.

advance in modeling this disease.^{58,59,70,77,78} An important feature of these models is the presence of submucosal glands (SMGs) throughout the proximal airways. CF airway pathology is largely attributed to defects in the SMGs as CFTR dysfunction is associated with abnormal mucus secretion and defective innate immunity.^{11,79-82} SMGs are therefore proposed as one of the targets for gene therapy. An overview of the main hallmarks of each model related to airway disease, including the CF mouse models, is presented in Table 1.

CF animal models that spontaneously develop lung disease allow investigating the efficacy of gene therapy beyond molecular and electrophysiological end-points. In 2016, 2 gene therapy studies conducted in the newborn gut-corrected CFTR^{-/-} pig reported that early disease manifestations of CF could be treated with a gp64-pseudotyped LV or rAAV2H22. These are the first studies reporting on the ability of gene therapy to improve clinically relevant lung disease features in the neonatal period, such as increased bacterial killing and ASL pH.^{55,106} Read-outs on key features of progressing lung disease are emerging, including particle tracking⁸⁰ or micro-optical coherence tomography^{77,107} for identifying changes in mucociliary clearance. Computed tomography (CT) or μ CT can reveal structural lung damage (bronchiectasis, airway wall thickening, bronchial obstruction, collapse of lung segments)¹⁰⁸⁻¹¹¹ as well as abnormalities in lung function (airflow obstruction, air trapping) with inspiratory versus expiratory CT.¹¹² Pulmonary function tests inform on vital capacity, resistance, and compliance of the respiratory system. Forced expiratory volumes (FEV) can be obtained in animals, but is not yet routinely used.^{113,114} The human equivalent of this, FEV in 1 second (FEV₁), is the primary end-point in most clinical trials. These read-outs will allow detailed evaluation of novel gene therapies: how and to what extent do individual factors improve lung disease phenotypes.

5 | AN EARLY INTERVENTION TO PREVENT PROGRESSIVE LUNG DISEASE

There is a growing consensus that treating CF patients early is crucial to prevent irreversible lung damage. Evidence is given in other disease areas, for instance, in congenital blindness trials where rAAV2/2 gene therapy resulted in the greatest increase in eye sight in youngest patients.¹ For an inherited lysosomal storage disorder, disease progress could be prevented in pre-symptomatic patients, further supporting the concept of an early treatment.¹¹⁵ Initiating treatment early in children with CF depends on a timely diagnosis. Traditionally, diagnosis relied on recognition of characteristic clinical symptoms, but in many countries, newborn screening is now a common practice.¹¹⁶ Also, many populations are screened for maternal carrier status. Via prenatal ultrasound screening, meconium ileus, peritonitis, and bowel obstruction can be identified, caused by CFTR dysfunction in the gastro-intestinal tract.¹¹⁶ Starting symptomatic treatment just after diagnosis via positive newborn screening has shown to improve outcomes compared with treatment initiation after diagnosis via symptoms.¹¹⁷ For therapies targeting the underlying CFTR defect, the anticipated impact of preventive therapy is even bigger. Due to better symptomatic care, classical lung function parameters such as FEV₁ remain in the normal

range throughout childhood in many patients, whereas radiographic and bronchoalveolar lavage read-outs (eg, bronchiectasis, increased neutrophil counts) are abnormal, suggesting progression of pulmonary disease despite a normal FEV₁.^{109,118,119} Clearly, more sensitive outcome parameters are needed to monitor discrete changes in lung disease, especially in early stages of CF. Many groups are investigating the use of multiple breath inert-gas washout to determine the lung clearance index (LCI) for identifying pulmonary abnormalities, such as mucus obstruction.¹¹⁹ Other early manifestations of CF airway disease are decreased ASL, abnormal ciliary beating, reduced mucociliary clearance, and impaired bacterial killing, features present even a few hours after birth in neonatal piglets, ferrets and rats.^{10,11,77,80,107}

Theoretically, poor transduction and poor clinical outcome in postnatal gene therapy clinical trials for CF could be overcome by gene therapy in the neonatal or fetal period. The advantages of gene transfer in utero are (1) administration before irreversible organ damage, (2) easier access to target cells, (3) longer contact time of the vector, (4) a higher vector to target cell ratio, (5) a less pronounced immune response against transgene and vector with even the possibility of tolerance induction, and (6) the presence of expanding stem and progenitor populations (reviewed in Shangaris and David¹²⁰). Initial attempts to transfer the therapeutic gene for CF in mice in utero were done in late 1990s. In a study by Larson and colleagues, gene transfer of AdV-CFTR to CF mouse fetuses restored their severe gastrointestinal phenotype.¹²¹ Interestingly, CFTR expression was not detected 72 hours after gene transfer suggesting that transient CFTR expression during prenatal development was sufficient to permanently reverse gastrointestinal pathology. This suggests a crucial role for CFTR during prenatal development in mice. In 2008, 2 independent groups failed to replicate these findings, although the original experimental setup was meticulously mimicked,^{122,123} providing no further support for this intriguing hypothesis.^{122,123}

CFTR is naturally expressed in ciliated cells of the surface epithelium as well as in the SMG ducts and serous cells of SMG acini.⁸¹ SMGs are considered a difficult target because of their anatomic location, but in the developing lung, these cell populations might be more easily accessible.¹²⁴ SMG development has been extensively characterized in mice¹²⁵ but also studied in humans by fetal tracheal xenograft models in mice.¹²⁶ In the fully developed SMGs, progenitor cells reside in the SMG ducts, which give rise to cells that form either SMGs or surface epithelium.¹²⁷ These stem cells are likely more accessible during the process of SMG development, which in humans already starts in utero.^{124,126} Evidence for this hypothesis was provided by Keswani et al where rAAV2/2 and rAAV2/8-LacZ targeted fetal SMGs a lot more efficiently than in a postnatal setting and this in a human tracheal xenograft mouse model.¹²⁴ Even the surface epithelium, however, which in theory seems an easy target, is not as easy transduced by gene delivery agents as expected based on its location.^{72,124} One of the major challenges in a postnatal setting is the accumulation of excessive mucus in CF due to recurrent infection and inflammation. As the fetal lungs are typically sterile, this process only starts after birth.^{10,128} Although vector penetration of CF sputum samples has already been assessed for various vector systems postnatally,⁷² it will be of great interest to extend these data to comparative studies between fetal and postnatal, CF and WT. In that regard, the human

tracheal xenograft mouse model¹²⁴ and human explant lungs¹²⁹ would be appropriate models to compare fetal versus postnatal transduction efficiencies of target cells for CF. Fetal vector delivery furthermore allows efficient and homogenous distribution of injected particles which is likely promoted by their fluid filled nature. This was for example demonstrated in WT pigs and mice after fetal intratracheal delivery of fluospheres or rAAV2/6.2.^{47,130,131} Finally, tolerance to transgenes after fetal or neonatal transduction has been demonstrated for human FIX in mice and dogs.¹³²⁻¹³⁴ There is however no evidence for tolerance induction against the viral vector itself, probably due to the limited half-life of the viral proteins once injected into the body.¹³² Furthermore, we have learnt from fetal gene therapy studies in mice that vector delivery to an immature immune system allows a single repeated administration due to immunological ignorance against the rAAV capsid, but that later readministrations are hampered by anti-capsid neutralizing antibodies.¹³⁵⁻¹³⁷ Translation to humans however remains to be investigated.

Despite several indications for a fetal gene therapy, CF is not associated with serious morbidity or mortality in utero, one of the criteria stated by the National Institutes for Health Recombinant DNA Advisory Committee for considering a fetal intervention.¹³⁸ Furthermore, the benefits of the intervention must be weighed against the possible risk for complications, which could range from prelabour rupture of the membranes, vector spread to germline cells or maternal circulation or developmental aberrations for the fetus.^{139,140} Based on current progress in preclinical fetal gene therapy for CF, a neonatal intervention would currently be most realistic for clinical translation. However, if more substantial evidence becomes available on the clear benefits of fetal versus postnatal gene therapy in relevant animal and human airway models, these findings could provide a basis for translating fetal gene therapy to the clinics in the future.

6 | ROADMAP TO SUCCESSFUL GENE THERAPY FOR CF AIRWAY DISEASE

The complexity of the lung, the need for more efficient vector systems, the tightly controlled CFTR expression and activity, and until recently the absence of animal models developing human-like CF airway disease represent the main barriers to clinical benefit for gene therapy. Many of these obstacles have been addressed in the numerous animal and cell models present to date and are by now better understood. In Figure 1, a schematic overview is given of the contribution of each model in predicting the expected benefit in CF patients. Mouse models are instrumental to study proof-of-principle efficacy by assessing CFTR expression at the transcript or protein level and CFTR function using NPD. Additionally, mechanistic processes can be unraveled relating to vector-mediated gene delivery in a live host, such as immune responses to vector and transgene. Nevertheless, mouse models do not always predict human responses as exemplified by the unexpected cytotoxic T-cell responses to the rAAV capsid in hemophilia B trials which limited the expression of the therapeutic protein.¹⁴¹ The larger CF animal models that develop spontaneous lung disease have shown their first application for neonatal gene therapy, where early parameters of CF lung disease (reduced ASL pH and bacterial killing) were corrected by rAAV or LV.^{55,106} Follow-up studies will likely focus on preventing irreversible lung damage by studying the effect of gene therapy on mucociliary clearance, mucus viscosity, mucus plugging, and lung function. These models will furthermore allow correlating clinical lung disease improvements to the level of CFTR rescue and the cell types corrected in the surface epithelium and/or SMGs. Finally, researchers will need to study the optimal time for gene therapy initiation (fetal, neonatal, adult) to achieve efficient airway transduction allowing prevention of irreversible lung disease.

FIGURE 1 Overview of the benefits of different cell and animal models used in gene therapy research for cystic fibrosis (CF). Abbreviations: CFTR: Cystic fibrosis transmembrane conductance regulator, NPD: Nasal potential difference, ASL: Airway surface liquid, MCC: Mucociliary clearance, NHP: Non-human primates, SMGs: Submucosal glands

Apart from studying CF read-outs, large animal species such as sheep and non-human primates (NHP) have shown their application in vector toxicity, biodistribution, and tropism determination as one of the final steps to clinical translation due to similar lung size and structure as humans (reviewed in Alton et al²⁹ and Flotte et al¹⁴²). For example, sheep have been used to assess acute and chronic vector toxicity to repeated liposome delivery of hCFTR. Regarding NHP, chimpanzees are the closest genetic relatives to humans (reviewed in Flotte et al¹⁴²). The proposed indication for this species is to determine rAAV tropism and capsid immunogenicity, which influence transduction efficiency and longevity of gene expression.¹⁴² However, they are a protected species and no longer approved for invasive research in many countries.¹⁴³ The rhesus macaque and marmoset have been used to assess safety and toxicity of vector delivery as well as molecular end-points of CFTR expression.^{144,145} Human bronchial epithelial (HBE) cells from lung explants have been the golden standard for CF drug development.¹⁴⁶ Also, for clinical gene therapy development, HBEs are of value as they present the model most closely mimicking the human airways. The development of human tracheal xenograft mouse models¹²⁵ and human explant lungs¹²⁹ will be invaluable to gather further evidence on a solid comparison of fetal versus postnatal transduction efficiencies of target cells for CF. This will be a starting point to further unravel additional benefits of fetal gene therapy on the longevity of expression, possibility to readminister the vector, and extent to which CF lung disease can be prevented.

In conclusion, the wide array of cell and animal models presenting specific aspects of CF lung disease will contribute to unraveling key questions on the development of an optimal gene therapy protocol for CF. Figure 2 illustrates the proposed roadmap from preclinical to clinical gene therapy for CF. Progressing beyond molecular and electrophysiological end-points will likely increase the success of providing CF patients with a gene therapy treatment that can substantially improve their quality of life by preventing irreversible lung damage.

A first step in the development of a gene therapy for CF is the design of a viral or non-viral vector capable of restoring CFTR expression and function *in vitro*. Proof-of-principle studies in mice then allow identifying if the vector formulation allows targeting the airways in a live host. This gives a first idea on vector tropism, transduction efficiency at different doses, and longevity of gene expression. Of importance is to confirm as soon as possible in human primary airway cultures the ability of the vector to restore CFTR expression and function. As transduction efficiencies can differ between the fetal and adult period,¹⁴⁷ human tissues should be collected from the desired period for vector testing. If vectors test negative in this step, improved vector design is necessary. Next, CF animal models that develop spontaneous human-like CF lung disease allow assessing if the vector can restore or even prevent the development of early and progressive lung disease features. Only at this point will it be possible to correlate the level of CFTR expression and function with a clinically meaningful benefit. Also, the time point of vector delivery (fetal, neonatal, adult) can be

FIGURE 2 Proposed roadmap for an early gene therapy for cystic fibrosis airway disease

fine-tuned and correlated with the status of lung disease severity. Finally, large animal models closely related to humans regarding lung anatomy, physiology, and cell composition allow assessing if the vector is well tolerated even after repeated administrations, before progressing into early clinical development.

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DISCLOSURE CONFLICT OF INTEREST

No conflicts of interest.

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