Chapter

Recent Advances in Targeted Genetic Medicines for Cystic Fibrosis

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Abstract

The cystic fibrosis transmembrane conductance regulator (*CFTR*) gene was discovered just over 30 years ago, and soon after, gene therapy for cystic fibrosis (CF) has been rapidly and continually developing. Recently, novel gene therapy strategies have been developed, including mRNA delivery, genome editing, and mRNA repair; all these strategies are collectively named "genetic medicines." The last quarter of the century showed a significant boost in the development of viral and nonviral vectors to deliver genetic treatment. This chapter will provide a brief overview of the *CFTR* gene and its different classes of mutations as well as a review of the different genetic therapeutic options that are under research. Later in this chapter, drugs that target different *CFTR* mutation classes and are currently approved to treat CF patients will be briefly presented.

Keywords: cystic fibrosis, CFTR, gene therapy, CRISPR/Cas9, mRNA therapy, gene editing, gene delivery, viral vectors, nonviral vectors, CF animal models, CF drugs

1. Introduction

CF is an autosomal recessive genetic disorder and is caused by mutations in both copies of *CFTR*. The *CFTR* gene is found on chromosome 7, on the long arm at position q31.2 from bp 116,907,253 to bp 117,095,955. *CFTR* consists of 27 exons, whereas the CFTR protein has 1480 amino acids with a molecular mass of 168,138 Da [1].

The *CFTR* gene encodes a protein that is an ATP-gated chloride and bicarbonate channel. It is located only on the apical membrane of the airway, intestinal, and exocrine glands epithelium. The CFTR protein undergoes different steps of post-translational modifications and trafficking inside the epithelial cells (**Figure 1**). The CFTR protein structure consists of four main domains: an extracellular domain, a transmembrane domain, a nuclear binding domain (NBD), and the regulatory domain (R domain) (**Figure 2**) [2].

1. **Extracellular domain**: It comprises of small loops that connect the transmembrane proteins, e.g., (M1 and M2), (M3 and M4), (M5 and M6), etc.

2. **Transmembrane domain**: It consists of two groups; each of them consists of six membrane-bound regions that are each connected to a nuclear binding

domain (NBD). It was found that it plays a major role in the pore function of the membrane.

3. **NBD domain (NBD; NBD1 and NBD2)**: It is responsible for ATP binding.

4. **Regulatory domain (R domain)**: It consists of numerous charged amino acids, and it is phosphorylated and activated by protein kinase A.

The CFTR transport mechanism depends on two membrane-spanning domains (MSD) and two nucleotide-binding domains (NBD). The cycle of the transport of the chloride ions starts with the phosphorylation of the R domain that activates the channel. This step will start the ATP ligation to the NBD and the subsequent conformational changes and dimerization. This step will provide the energy for the release of the chloride ions across the cellular membrane. Once ATP is hydrolyzed, the NBD is destabilized, releasing ADP and phosphates; this results in the protein regaining its basal state. This cycle is called the ATP switch model of CFTR [3].

The *CFTR* mutations can be classified into six main classes based on their effect on the synthesis and/or function of the encoded protein. More recently, a Class VII has been added (**Figure 3**) [5, 6].

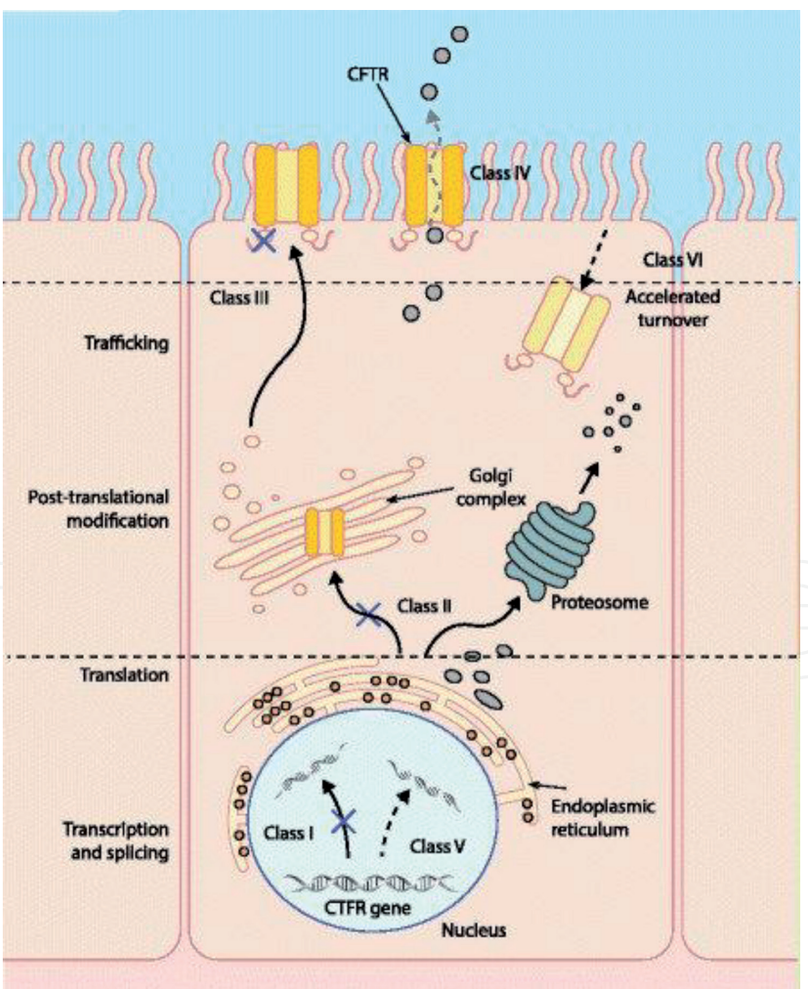

Figure 1.
The physiological process of CFTR *transcription and cellular processing of the protein inside the cells. The process starts with the mRNA transcription in the nucleus, and then the mRNA leaves the nucleus and is translated by ribosomes in the endoplasmic reticulum to protein. Chaperone proteins facilitate folding of the new CFTR proteins. The CFTR protein next undergoes post-translational modifications in the Golgi apparatus such as glycosylation, ubiquitination, SUMOylation, and phosphorylation and is then transported to the epithelial cell surface [4].*

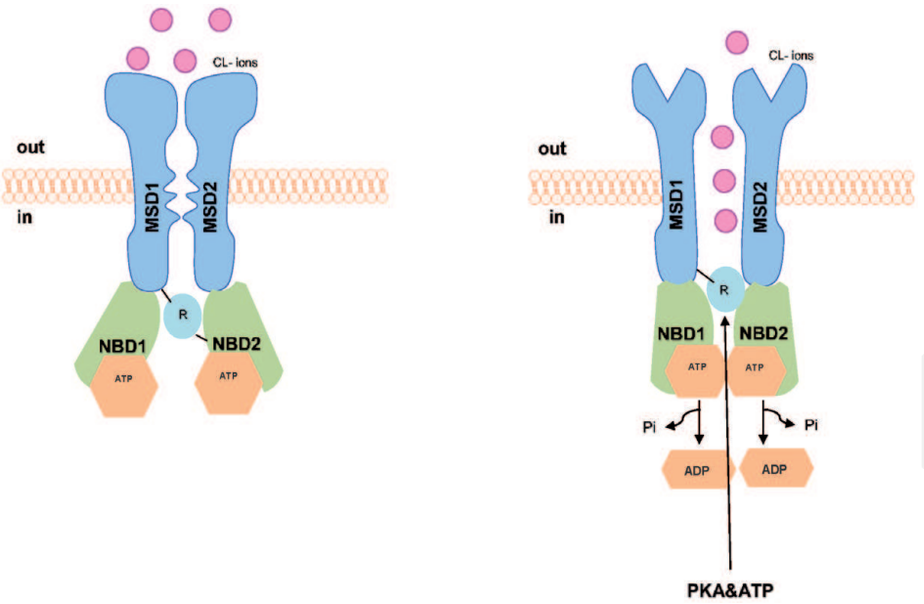

Figure 2.
This figure illustrates the composition of the CFTR chloride channels (at rest and when activated) in the apical epithelial membranes. It is composed of different domains including the MSD, NBD, and the R domains.

Class I mutations result in a partial or complete lack of production of a functional CFTR protein. Those mutations are due to the introduction of a premature termination codon (PTC). This class includes mutations such as p.Gly542X, p.Arg553X, and p.Trp1282X. The p.Gly542X mutation is the most common mutation of this class worldwide.

Class II mutations are associated with abnormal trafficking of the CFTR protein due to misfolding of the protein. These mutations occur in any domain of the CFTR protein and can lead to either a partial reduction (p.Leu206Trp) or complete absence of the mature CFTR protein (p.Arg1066Cys). The F508del (p.Phe508del) is the most common mutation worldwide, and it has been demonstrated that it leads to instability of the NBD1 domain and alters the CFTR assembly.

Class III mutations are missense mutations frequently located in the ATP binding domains (NBD1 and NBD2). They are also known as gating mutations since in this type there is a defective channel gating. There is production of CFTR, which is efficiently transported to the cell membrane at normal levels, but the protein is resistant to activation by protein kinase A and cannot exhibit channel gating function (e.g., p.Gly178Arg and p.Gly551Asp).

Class IV mutations are missense mutations located in the membrane domains, which are responsible for the formation of the channel pores. The protein can still efficiently reach the membrane but with reduced channel conductance (e.g., p.Arg117His and p.Arg334Trp).

Class V mutations reduce the amount of functional CFTR protein. Nonfunctioning proteins are produced due to alternative splicing. Moreover, as a result of amino acid substitution, there is less protein maturation, reducing the amount of functional CFTR that reaches the cell surface. Consequently, the reduced numbers of CFTR channels lead to the subsequent loss of chloride transport (e.g., c.3272-26A>G). Direct RNA analysis is not routinely performed and this in turn leads in underestimation of the number of mutations causing splicing defects.

Class VI mutations result in a protein that is unstable, degrades easily, and has abnormally fast turnover rates due to the truncated C terminus of the protein (e.g., p.Lys684fs and p.Gln1412X).

Finally, **Class VII mutations** are a subtype of Class I mutations with no messenger RNA (mRNA) transcription [7]. The outcome is the same as that of class I mutations, i.e., complete absence of CFTR protein which cannot be treated by the CFTR correctors.

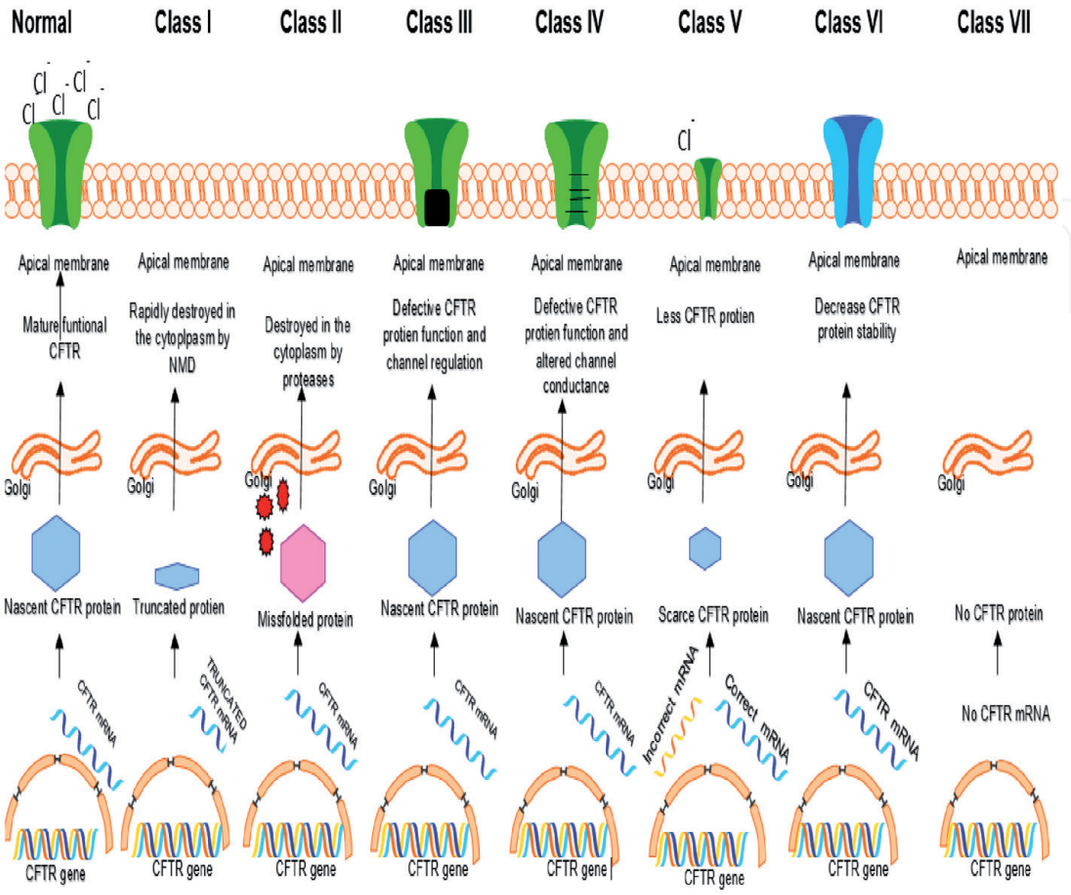

Figure 3.
This figure describes the different classes of CF according to the production of the encoded CFTR protein.

2. Genetic medicine

Although there are some approved drugs for specific patients who harbor certain mutations, genetic medicine is important as it offers the ultimate treatment for all CF mutations and can benefit every CF patient [8]. There are multiple genetic strategies that are currently under investigation for the treatment of CF. They can be summarized as follows (**Figure 4**).

1. **Gene therapy**: Here, the correct copy of the *CFTR* gene is delivered to the diseased CF cells using either viral or nonviral vectors such as nanocomplexes.

2. **Gene editing (repair)**: This technique aims to correct the mutant *CFTR* allele by cutting the double strand DNA and correct the existing mutations inside the cells at the DNA level.

3. **mRNA-based therapeutics**: RNA oligonucleotides are delivered to the cytoplasm and repair the defective CFTR mRNA.

4. **mRNA therapy**: Wild-type CFTR mRNA is delivered to the cytoplasm of the cell, resulting in the production of normal CFTR protein [8].

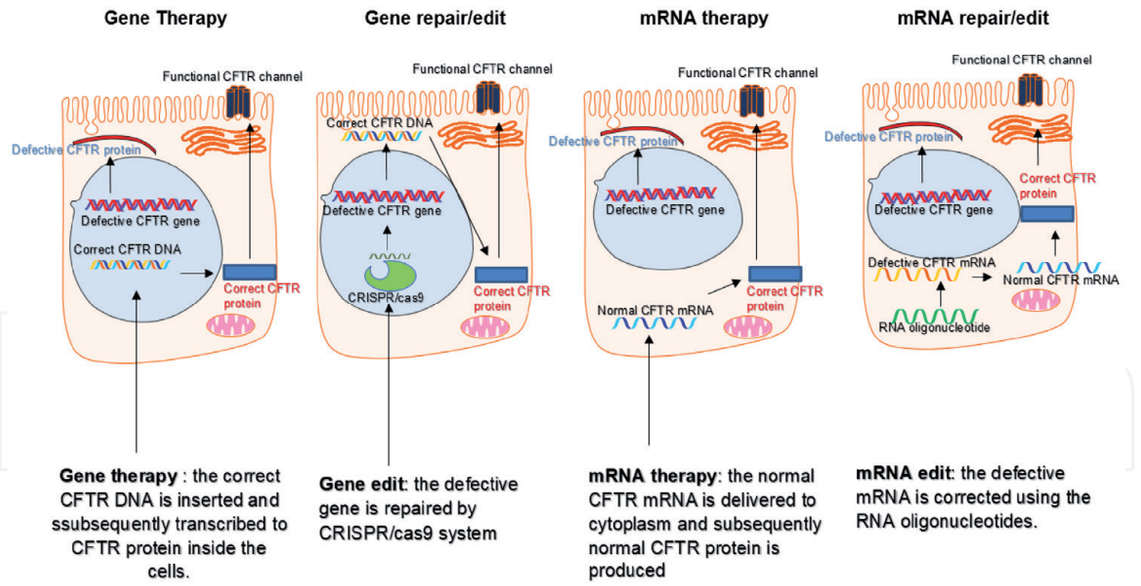

Figure 4.
This figure illustrates the different genetic medicine strategies for gene treatment of the CF mutations. It includes gene therapy, gene editing, mRNA repair, and mRNA therapy.

2.1 Gene therapy

Gene therapy is currently the most advanced and promising field of CF genetic medicine. For a long time, the main obstacle of this approach has been the absence of an efficient delivery system for the lung. The barriers (intracellular or extracellular) that are there to protect us from viruses and bacteria also prevent the uptake of different gene treatments via inhalation. The barriers also include the nuclear membrane which prevents the passage of the genetic materials from the cytoplasm to the nucleus. Other obstacles include airway mucus, mucociliary clearance, CF mucopurulent sputum, and the humoral and cellular immune responses. All these hinder the efficiency and the effectiveness of gene therapy as a treatment for CF [8].

Vectors can be classified broadly into two categories: viral and nonviral [9]. Viral vectors include adenoviruses and adeno-associated viruses (AAV). Both viruses can infect the lung cells efficiently and carry specific proteins in their cell surface to overcome the lung's natural defense systems [10]. However, any preexisting immunity toward the viruses will render them useless. Even if there is no previous immunity, the repeated administration of the virus will eventually lead to the development of immunity toward it and limit its success. However, recent preclinical studies in animals showed that multiple administrations of lentiviral vectors in immunocompetent lungs are effective [10]. Although some adenoviral clinical trials showed partial correction of the chloride transport in CF nasal epithelium by measuring the potential difference between the outer and inner cell membranes, this correction was only recorded after the nasal epithelium was damaged and removed during delivery [10].

Due to the simple structure of the nonviral vectors, they do not usually induce immune reactions inside the body [8]. The UK CF Gene Therapy Consortium (GTC) was formed from three groups in Edinburgh, London, and Oxford. Their aim was to share expertise to assess gene therapy and its ability to stop the progression of CF lung disease. After extensive research, they concluded that the nonviral cationic lipid formulation GL67A combined with the modified pGM169 plasmid (which encodes a CpG-free and codon-optimized CFTR) can produce some improvements in spirometry assessments in animals and even longer duration of response of up to 1 month [8]. In a randomized double-blind phase IIb trial, conducted on 120 patients with different mutations in the UK, it was found that

pGM169/GL67A was associated with a small but statistically significant stabilization of lung function in the patients [11]. In addition, the safety of this nonviral system was validated following 12 monthly administrations.

Another promising viral vector that has been investigated is the lentivirus. However, because this virus lacks the lung tropism, it must be combined with another virus in order to transduce the lung cells. The VSV-G protein is commonly used for this purpose, but others like the HA protein from the influenza virus and the F and HN proteins from the Sendai virus (**Figure 5**) have also been used [12].

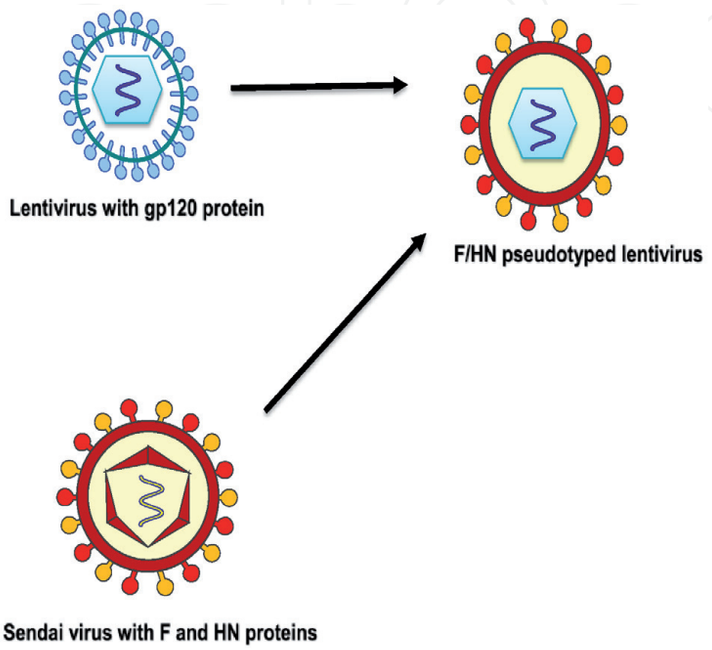

Figure 5.
This figure shows the F/HN pseudotyped lentiviral vector. The virus loses its gp120 protein which originally enables it to enter the T-cells but it gains the HN envelope proteins from the Sendai virus to facilitate its transduction inside the lung epithelial cells.

It has been reported in murines that one dose of lentivirus leads to life-long stable expression of luciferase (almost for 2 years). In addition, repeated administrations of the vector (10 daily doses, or three administrations at monthly intervals) did not cause a significant immune response. In a comparison between the GL67A/pGM169 and the lentivirus, it was found that the lentivirus is a much more effective form of gene therapy [8, 10].

At the end of 2017, the preparation for a clinical trial of a F/HN-pseudotyped lentivirus was announced [8]. This clinical trial will be a single-dose, double-blinded, dose-escalating phase I/IIa safety, and efficacy study. In a preliminary study, for the preparation of this clinical trial, it was predicted that only between 5 and 25% of the lung epithelial cells will need to be corrected in order to provide a clinical level of correction [13].

The human bocavirus virus-1 (HBoV1) is a parvovirus which efficiently infects the human airway epithelium. It was successfully recombined with an adenovirus to give a chimeric rAAV2/HBoV1 virus that was able to deliver a full-length *CFTR* gene coding sequence in CF human epithelial cells [14].

Marked progress in the development of vectors for airway gene delivery, along with a better understanding of CF pathophysiology and the presence of new animal models, has increased the possibility and the hope of gene therapy for CF. However, some obstacles to overcome include the percentage of the lung epithelial cells that need to be corrected to restore physiological function, as well as the limited life

span of the ciliated epithelium of the lung. In addition, repeated dosing will require a better understanding of the immune system and the use of immune modulators. Regardless of the strategy, the benefit of a gene therapy approach will ultimately be realized in well-designed CF clinical trials [11].

2.2 Gene editing

Gene editing is an advanced form of genetic engineering which enables the insertion, deletion, or change of the nucleotide sequence of any living organism. It certainly gives the promise of providing therapy for diseases that were previously considered untreatable or difficult to treat. The field of genome-editing technologies is rapidly evolving and progressing, and the newer techniques seem to be more promising [15]. Gene editing was originally developed in the 1980s by Capecchi, Evans, and Smithies (awarded the 2007 Nobel Prize in Physiology or Medicine) but was mainly used in mice and pigs. The outstanding discovery that editing efficiency is increased at the site of double-stranded breaks (DSBs) made it possible to use the technique in larger studies of animal models and human cells. However, a method to create specific breaks at a certain genomic location with minimal off-target effects, insertions, and deletions in the DNA sequence had yet to be discovered [15].

In 2005, the development of fully programmable zinc finger nucleases (ZFNs) and their ability to perform this exact task led to its use in research extensively, but the limitation was the inefficiency and high cost of the ZFNs technology [16]. In 2009, the emergence of TAL-effector nucleases (TALENs) increased the gene editing specificity and the ease of design and production [16]. However, in 2013, the development of the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system has revolutionized gene editing as a research method that can be used by many groups worldwide [16].

CRISPR is an adaptive immunity function in bacteria like *Streptococcus pyogenes* through which they can defend themselves against the bacteriophage virus' DNA or RNA. The main function of the CRISPR system inside the bacteria is to act as a molecular immunity protective mechanism to keep a copy of previous bacteriophage infections, in the form of a short sequence target of DNA or RNA molecules, inside the cytoplasm of the bacteria, allowing a more rapid identification and elimination of foreign DNA from the cytoplasm [17].

2.2.1 CRISPR/Cas9 system

Generally, the CRISPR/Cas9 system is composed of (**Figure 6**) the following:

1. The cas9 endonuclease that is capable of binding and unwinding the DNA helix and cleave any sequence complimentary to the guide RNA attached to it.

2. The guide RNA molecule (gRNA) that is designed to bind to the desired sequence and direct the Cas9 endonuclease. Usually, it is a short segment about 20 nucleotides long.

3. A template DNA, to achieve the repair of the DSB with homology directed repair (HDR) rather than nonhomologous end joining repair (NHEJ).

The ribonucleoprotein complex of Cas9 and sgRNA first scans the DNA, anneals to the complementary DNA sequence and then makes a double strand cut before the sequence of the protospacer-associated motif (PAM) (it is a part of the DNA sequence ~2–6 base pair long immediately downstream of the sequence targeted by the Cas9 nuclease and it is essential for the Cas9 endonuclease function) [18].

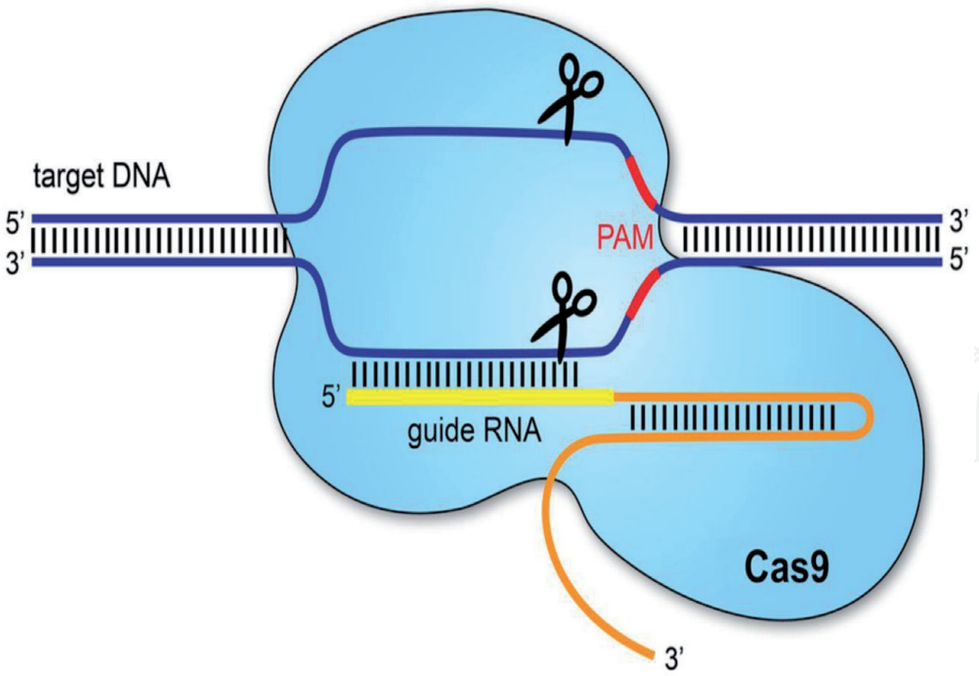

Figure 6.
This figure illustrates the composition of the CRISPR/Cas 9 system [19].

The application of this system for the editing of the genomes is quite simple, efficient, multiplexed, applicable in many species, and relatively affordable compared to other forms of gene editing. In addition, this system can be modified to perform activation or repression of certain genes, and the Cas enzymes can be fused to epigenetic modifiers to create programmable epigenome-engineering tools [20].

A more advanced approach of genome editing is the base editing technique (BE), a newer approach to gene editing that achieves the direct and programmable conversion of one DNA base pair to another DNA base pair chemically, using specific enzymes, without inducing a DSB [21]. It was proposed that different base editors were needed to make more efficient and specific conversion of nucleotides with minimal off-target effects, e.g., the conversion of G: C to A: T by using the third-generation base editor (BE3) [21].

Typically, BE3 contains (**Figure** 7):

1. A catalytically inactive dCas9 that binds only to DNA but is not able to cut the strand. It is only capable of creating a DNA bubble at a guide RNA-specified region.

2. A cytidine deaminase enzyme that changes cytidine to uracil within a 3–5 nucleotide window of the single-stranded DNA bubble, e.g. APOBEC1 (Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 1 enzyme).

3. A uracil glycosylase inhibitor (UGI) that inhibits the automatic cellular repair mechanisms by inhibiting the base excision, therefore improving the efficiency of the BE technique.

4. Nickase activity: to make a cut only in one strand of the DNA in order to achieve manipulation of the cellular mismatch repair innate mechanisms of the cells to replace the G-containing DNA strand.

These components combine to achieve a permanent C to T (or G to A) conversion in the cells with minimal or lack of in-del formation [21].

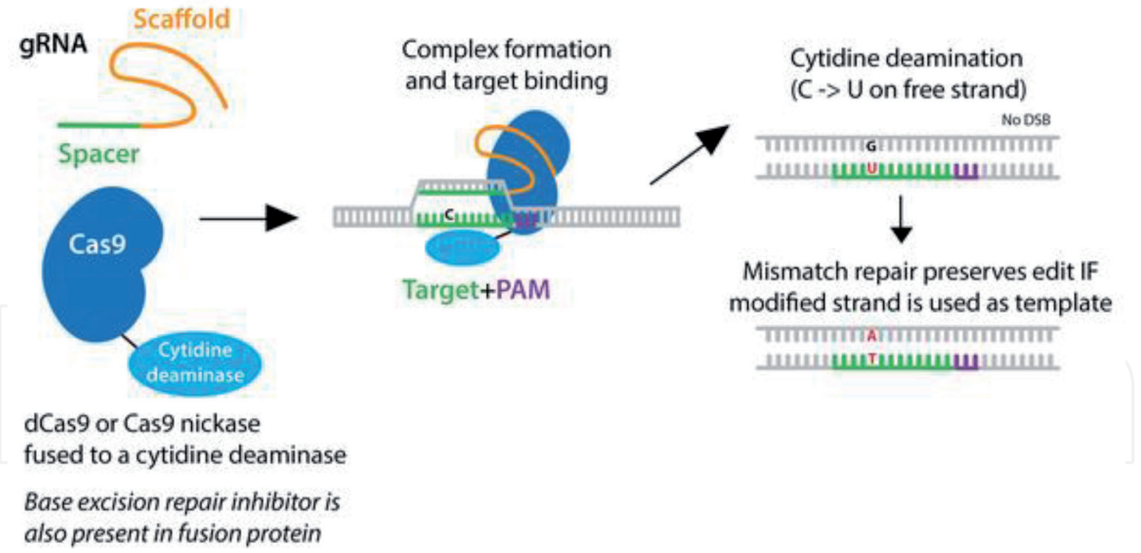

Figure 7.
This figure describes the different components and the mechanism of action of base editing converting G: C to A: T [22].

Moreover, additional modifications have been made to BEs to limit off-target effects (e.g., Hypa-BE3), decrease bystander effects (e.g., YE1-BE3, YE2-BE3), increase the editing window (BE-PLUS), and improve intracellular expression (BE4max) [23].

A recent technique (late 2017) is the use of Adenine base editor (ABE) which is able to convert A: T to G: C by using an adenine deaminase enzyme such as E. coli TAD-A, human ADAR2, mouse ADA, and human ADAT2 [22]. The adenine base is converted to inosine by deamination. Inosine is then treated as guanine by cell polymerases, therefore pairing it with cytidine in the opposite strand and ultimately converting A: T to G: C. The ABE also consists of a guide RNA, a catalytically impaired Cas9 and an adenine deaminase enzyme such as E. coli TAD-A (**Figure 8**).

Therefore, these base editors (both ABEs and BEs) revolutionize the field of genome editing and can position all the transitional DNA bases at specific loci in different cells with a minimum of harmful by-products [24].

In CF, CRISPR/Cas9 was used to correct the F508del mutation, which resulted in recovery of the functions of CFTR in human gastrointestinal tract stem cells in

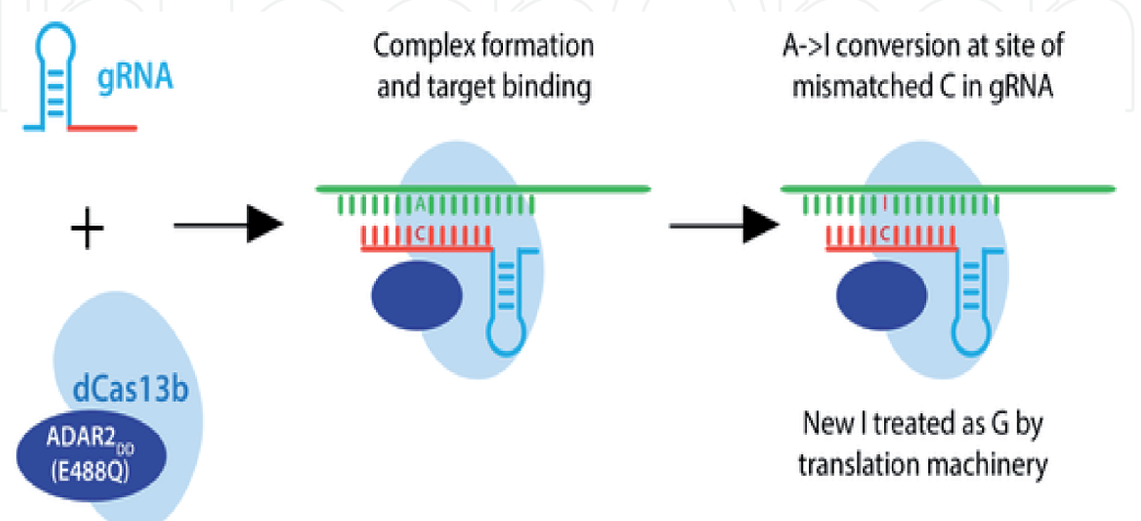

Figure 8.
This figure illustrates the composition and the mechanism of action of adenine base editing converting A: T to G: C [25].

an intestinal organoid model [20]. CRISPR/Cas9 has also been used to edit *CFTR* in human-induced pluripotent stem cells (iPSCs). This approach provided new models for CF disease, and it helped in the identification of novel drug targets [26].

Added advantages of gene editing over gene therapy are the use of the endogenous cell machinery and the fact that the modifications are permanent for the cell's life time. In addition, the reagents used for one mutation can also be used for any other CF mutation [8].

One of the main concerns when using the CRISPR/Cas9 system is the possibility of off-target effects; therefore, multiple modifications have been made to Cas9 to reduce such effects, e.g., the use of the nickase Cas9-D10A [27] in yeast achieved precise editing with completely undetectable off-target events. Moreover, both the meticulous choice of the target regions and the use of donor DNA templates with asymmetric homology arms have improved the on-target editing [28].

Other obstacles needed to be overcome in order to increase the gene editing efficiency in vivo are similar to those that affect gene transfer vectors such as the delivery mechanism to the stem basal cells of the lungs through the mucus-obstructed CF lung epithelium. Ideally, the target for the gene editing should be the basal airway progenitor cells, but unfortunately, these are "buried" beneath the surface epithelium and it is difficult to reach with the vectors available currently [29]. On the other hand, there is some optimism using different approaches to deliver CRISPR components: either as mRNA or directly as a protein or ribonucleoprotein complexes with modified lentiviral vectors [30].

Another dilemma, unique to CF, is which cells need to be corrected in the airway epithelium to achieve normal lung function and whether the lung stem cells should be targeted. Furthermore, unrestrained high CFTR expression across all the cells of the lung epithelium might have adverse effects, since normally the expression of the CFTR is controlled with tight activation and repression mechanisms [31, 32].

2.3 mRNA-based therapeutics

For CF, the repair of mRNA is a valuable therapeutic technique that was first investigated by Zamecnik et al. [33]. The mRNA repair could be done by either direct repair, exclusion of the defective exon, or a splice site change. The repair of the RNA is done using short double-stranded RNA oligonucleotides, targeting an mRNA sequence between 15 and 40 nucleotides. These oligonucleotides are designed to be specific for every mutation; hence, they might repair or remove the defective RNA [8]. In other studies, the oligonucleotide was designed to target the CFTR splicing mutation 3849 10 kb C-to-T, and it was shown that the defective splicing can be changed to include a cryptic exon and regain the CFTR function [34].

Moreover, ProQR Therapeutics developed QR-010 which targets the F508del mutation. It does not need to cross the nuclear membrane, since it acts in the cytoplasm. QR-010 showed that it can increase the CFTR Cl^- channel activity in homozygous F508del HBE cells. Also, when administered intranasally to mice, it restored the normal potential difference of the lung epithelium [34–37]. QR-010 is currently in a Phase Ib clinical study given as an inhalational drug to treat the homozygous F508del mutation in adults to evaluate its tolerability and its pharmacokinetics [37].

Small interfering RNA (siRNA) is one of the mRNA therapies that is used to silence the epithelial sodium channel, ENaC. It has been shown that upregulation of ENaC in CF leads to dehydration of the airway and formation of thickened mucus [38]. Due to the lack of a proper delivery system, the use of siRNA to transfect epithelial lung CF cells is difficult. However, ENAC silencing by siRNA when formulated with lipid-peptide nanocomplexes was recently reported both in vitro and in vivo [38].

2.4 mRNA therapy

Messenger RNA as a gene therapy approach has several advantages over DNA (as it does not require nuclear localization or transcription) and viruses, since it does not integrate in the genome once inside the cell. For years, scientists have been investigating the possibility of injecting the wild form of the CFTR mRNA to the cytoplasm to act as a template to produce wild-type CFTR protein [9]. Nevertheless, the unstable nature of RNA and its capacity to elicit innate immune responses pose limitations for in vivo applications. However, recent advances in synthetic biology helped alleviate these limitations by modifying the exogenously synthesized mRNAs to mimic their endogenous counterparts. These modifications have led in both an increase in mRNA transfection efficiency, as well as longer protein expression [39].

The immune system has evolved to recognize exogenous RNA, as it can also be found in viruses and other pathogens. Viral single- and double-stranded RNA can induce immune stimulation by interacting with pattern recognition receptors (PRR) tasked with identifying pathogen-associated molecular patterns. Endogenous RNAs evade immune response since they contain modified nucleotides that affect PRR engagement. For example, the incorporation of nucleotide analogs in the RNA sequence, such as 2-thiouridine (2-SU), 5-methylcytidine (5-meC), and 1-methylpseudouridine (m1Ψ), enables them to prevent recognition [40].

Furthermore, to optimize their translational efficiency and stability, the in vitro synthesized mRNAs incorporate a 5′-end modified cap (anti-reverse analogue [modified ARCA]) and a 3′-end poly(A) tail, eventually resembling fully-processed endogenous mRNA molecules [40]. In conclusion, as a result of extensive research, a variety of different chemical modifications of the mRNA in conjunction with its encapsulation into nanoparticles are currently under investigation [41, 42]. A recent study in bronchial epithelial cells has even demonstrated the restoration of chloride secretion using lipid nanoparticles (LNPs) to package and deliver chemically modified CFTR mRNA [43].

3. Drugs for the treatment of cystic fibrosis mutations

There are several drugs that were investigated for the treatment of CF mutations. According to the class of the mutation, different drugs with different mechanisms of action are used. CFTR modulators are small molecule drugs that improve CFTR protein function by a variety of mechanisms [44]. However, those molecules do not treat the main mutation defect of the *CFTR* gene. They can be classified into four categories (**Figure 9**) [45]:

1. The potentiators that increase the gating function and the opening probability of the CFTR Cl^- gates, e.g., Ivacaftor.

2. The correctors that promote protein folding, assisting the transition of the CFTR protein through the cytoplasm to the cell surface, and the rescue of the CFTR protein, e.g., Lumacaftor.

3. The read-through drugs that enable the overriding of the premature termination codons and subsequently lead to complete translation and production of the full length protein, e.g., ataluren.

4. The amplifiers that increase the amount of the CFTR inside the cells and are usually given with other modulators (mentioned above) to increase their efficiency.

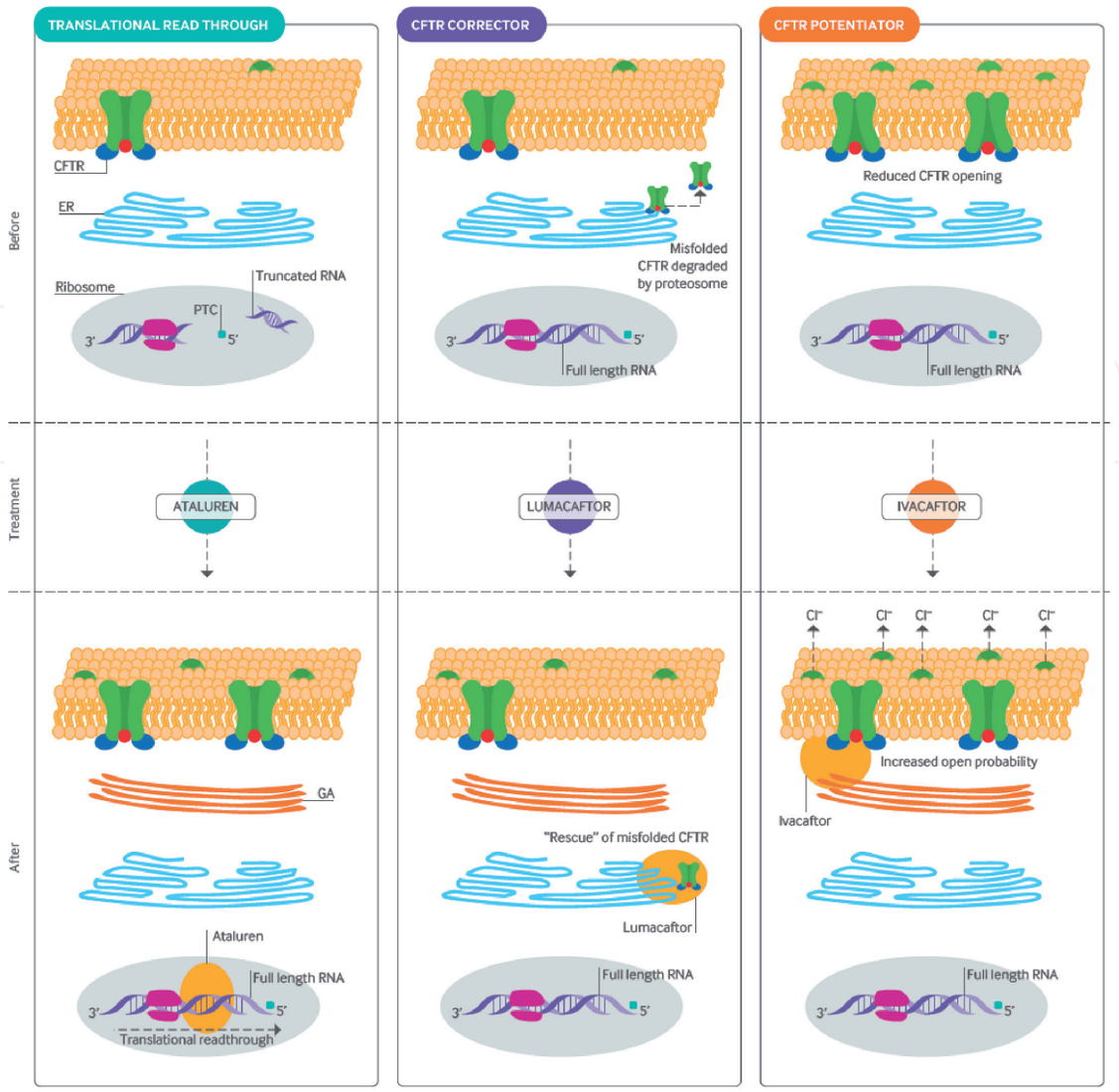

Figure 9.
Different mechanisms of action of drugs that are used to treat the different classes of CF mutations [45]. There is a fourth category of drugs, the amplifiers, which are not depicted here. ER, endoplasmic reticulum; GA, Golgi apparatus; PTC, premature termination codon.

F508del accounts for ~69% of CF-causing alleles [46]. To address this mutation defect, two different forms of drugs are used: CFTR correctors to increase the amount of correctly-folded CFTR protein and CFTR potentiators that improve the gating mechanism of the apical CFTR protein [47]. When combined together, they restore the Cl^- transport and improve the airway mucociliary clearance [48]. The commercially available formulations of these two drugs are the corrector Lumacaftor (VX-809) and the potentiator Ivacaftor (VX-770). When administered alone in patients homozygous for F508del, Lumacaftor lead to a modest, yet statistically significant reduction of ≥10 mmol/L in the sweat chloride concentration, but no other improvements in lung function (FEV_1) and quality of life (CFQ-R) were observed [49]. On the other hand, in patients with the G551D mutation, Ivacaftor lead to an all-around improvement. In detail, after 48 weeks, the treated patients demonstrated an overall increase in BMI and quality of life markers, a 10.6% increase in FEV_1, as well as a decrease of 48.1 mmol/L in sweat chloride levels, making Ivacaftor the first agent to achieve a reduction to values below the diagnostic threshold for CF (60 mmol/L). As a result, Ivacaftor was approved for the treatment of the Class III CF mutations in 2012 [50, 51]. The combination of both, which is called Orkambi, is currently available for CF patients as it proved beneficial for homozygous F508del

mutation treatment. To elaborate, in addition to a significant general improvement in the clinical picture of the disease, such as increased BMI and CFQ-R, and a decreased rate of exacerbations, a 5% improvement of FEV_1, when compared to the placebo, was observed [52, 53]. In 2019, a triple combination therapy consisting of the correctors Elexacaftor and Tezacaftor and the potentiator Ivacaftor (called Trikafta and developed by Vertex Pharmaceuticals) was tested in a double blind, randomized phase 3 clinical trial, demonstrating remarkable results. Among others, a significant increase in CFQ-R and a favorable safety profile. Moreover, there was a 10.4% increase in FEV_1 and a considerable improvement in sweat chloride concentration, with a mean decrease of 43.4 mmol/L, achieving values below the diagnostic threshold for CF. Subsequently, Trikafta was approved by the FDA as a treatment among patients aged 12 years or older with the F508del mutation [53].

Furthermore, a number of proteins based on proteostasis modulation have been identified as useful drug targets for CF therapy [54–56]. Hsp90 and AHA1 are thought to have a role in CFTR folding and degradation. It was found that treatment with Hsp90-AHA1 inhibitors combined with Lumacaftor was more effective than Lumacaftor alone [56].

Ataluren is another drug that was used to facilitate the read-through of nonsense mutations in Duchene Muscular Dystrophy. However, a randomized clinical phase II trial showed no significant efficacy of Ataluren in the treatment of CF [57]. ELX-O2 is another drug that is recently developed by ELoxx Pharmaceuticals for its read-through effects. It is currently in a phase 2 clinical trial involving CF patients [58].

Another possible drug target is endoplasmic reticulum-associated degradation (ERAD), including chaperone proteins and ubiquitin complexes. RNF5 (also known as RMA1) was found to be important in the protein folding and NBD domain synthesis [59].

Interestingly, due to the presence of more than 2000 mutations in *CFTR*, the use of "theratyping" for the patient becomes of value. The term "theratype" is described as classifying the CFTR variants according to their response to the corrector and potentiator drugs. More recently, this term is used to classify the mutations according to their characterization and their response to CFTR modulators across many model systems, which include functional and biochemical characterization [45]. Theratyping is also used to predict the clinical outcome of the patient toward the drug by the in vitro studies [45].

4. Nonviral delivery vectors

For a long time, viral vectors dominated the fields of gene therapy and vector development, mainly due to their very high efficiency. However, over the last years, novel approaches in vector design and recent advances in microfluidics have turned nonviral vectors into a promising method of drug and gene delivery [60, 61]. There are multiple materials that can be used to create nonviral vectors, including liposomes, which allow the delivery of the nucleic acids inside the lung epithelial cells. Liposomes are spherical vesicles composed of two layers of phospholipids with a hydrophilic core. They are normally formulated with natural lipids and possess no immunogenicity [62].

Nonviral vectors have the advantages of simple large-scale production and a large capacity for nucleic acids as cargos. Furthermore, low host immunogenicity and the ability to maintain their efficiency even after repeated administration render them a popular alternative to their viral counterparts. In addition, recent advances in vector technology have yielded molecules and techniques with even higher transfection efficiencies [60, 61]. These new vectors can be used to deliver

small molecules such as siRNAs, miRNAs, or even small therapeutic molecules and drugs, as well as bigger molecules like mRNA, minicircle, and plasmid DNA.

The cationic lipid-based vectors are an effective delivery approach for the CRISPR/Cas9 system but only after local administration [62]. However, the main problem about liposomes as drug delivery vectors for the treatment of CF or any chronic obstructive disease remains the development of inhalational formulations which can be delivered by nebulization. The nebulizer can alter the stability of the liposomes and cause their aggregation [63]. Therefore, several methods have been developed to stabilize the liposomal formulations such as lyophilisation [64] or use of dry powder inhaler (DPI) liposomal formulations which have shown promising results for drug administration in the lung, but those are still in an early development stage [65]. Targeted liposome-peptide nanocomplexes have been successfully nebulized, offering another alternative [66, 67].

Additionally, mucus-penetrating nanoparticles have emerged as a suitable vector to deliver various drugs and nucleic acids across the thick mucus barrier in cystic fibrosis. In CF, targeted mucus-penetrating nanocomplexes successfully delivered siRNA against ENAC in the airway epithelium and decreased the Na^+ reabsorption, thus restoring the clearance of the mucus and regaining the function of cilia [38, 68]. Mucus-penetrating NPs have a small size which leads to a lower mucus surface tension and easy penetrance. Also, they are coated with polyethylene glycol (PEG) which is electrically neutral and lead to an enhancement of the penetrance of the thick mucus of CF [69]. PEGylated nanoparticles loaded with Ivacaftor were formulated to test the drug uptake capacity of CF artificial mucus (CF-AM) on human bronchial epithelial (16-HBE) cells [70]. It was found that there was a higher release and uptake of Ivacaftor by 12% compared to Ivacaftor alone. In light of these results, the PEGylated mucus-penetrating NPs are considered a good vehicle to deliver the CFTR modulators through pulmonary administration to treat CF patients [70]. However, in order to be effective, the size of mucus-penetrating NPs should be small enough to penetrate mucus and big enough to prevent rapid exhalation and expulsion from the lung. Moreover, in order to increase their efficacy, certain parameters must be considered such as the nanoparticle morphology and their surface properties [71].

In summary, both viral and nonviral vectors are used to introduce different nucleic acids into the cell. Though the design of viral vectors has improved in the last few years and they have become more efficient, the immunogenicity and safety concerns still remain a big issue. On the other hand, the nonviral vectors offer safe and low-cost therapies with increased transfection efficiencies. Further improvements and optimization of these therapies and delivery vehicles could lead to a great outcome for CF [72].

5. CF animal models, organoids and iPSCs

Having an animal model is a crucial step to understand the disease pathogenesis, progression, and to test new drugs. The CFTR-knockout pigs and ferrets were generated approximately 15 years ago [73]. These species have a similar lung biology to humans because their submucosal glands are in their cartilaginous parts of the lung. On the contrary, rats and mice have their submucosal glands in the trachea, and rabbits do not have glands at all [74].

Among other in vitro cell culture models, one that has particular value in CF is the use of organoids, which have become a very useful model for CF research [75]. Organoids are 3D cultures of the lung progenitor cells grown in the presence of appropriate medium. They grow also with supporting cells that organize in a very similar way as the in vivo organs. In CF research, organoids of the intestinal and respiratory systems

are currently used to screen and test the newest drugs for CF [20]. Moreover, the intestinal organoids have been used as a model for the CRISPR/Cas9 technique [20].

These models could also be used potentially for testing gene editing-based therapeutics in CF [8, 76]. Another therapeutic option is to directly edit the progenitor cells in the lung epithelium in vivo, but a CRISPR editing system in CF lung in vivo has yet to be reported [8].

Human embryonic stem cells (ESCs) and iPSCs are newer models that can be used in CF. iPSCs are obtained by somatic cell reprogramming and differentiating these cells into specific human tissues [77]. The iPSCs can produce cell lines with the different rare CF mutations. The CRISPR/Cas9 technique was efficiently used to correct the CFTR F508del in patient-derived iPSCs that were differentiated to proximal airway cells [78].

6. Conclusion

Cystic fibrosis is a good example of how a deeper understanding of the genetics of disease can lead to personalized therapy for each patient. Continued efforts to develop better viral and nanoparticle-based nonviral vectors and produce novel gene editing with CRISPR/Cas9 are always investigated. Along with the advancement in the production of CF animal and in vitro human models and the presence of different electrophysiological methods such as transepithelial potential difference (TPD), all these give the promise and hope for the future of CF patients. Certainly, the recent use of organoids will be essential to personalized genetic medicine. This chapter has presented the past and current research that shows that the concept of genetic medicine can become a reality for CF patients in the near future.

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