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Chapter 15

In vivo Gene Correction of Cystic Fibrosis

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Abstract

The cystic fibrosis transmembrane conductance regulator (CFTR) gene was cloned over two decades ago and a vast number of pre-clinical and clinical studies have been performed since that time. Despite this progress, a true "cure" for the disease has not been achieved, partly because the lung is a major barrier for intruders, making it exceedingly difficult for new pharmaceutical formulations to penetrate target cells. Safety-engineered viral vectors, such as adeno-associated viral vectors (AAVs) or integrase-defective lentiviruses, have been used with moderate success in temporarily supplementing the expression of critical proteins. However, stability and safety concerns often dampen the effects of these approaches. With emerging technologies, such as modified messenger (mRNA) and new genome editing strategies, scientists are now exploring the possibility of not only supplementing defective proteins, but instead, correcting the genetic defects at their source. This chapter will highlight the theoretical possibilities and primary data in pre-clinical models supporting the efforts toward *in vivo* gene correction of cystic fibrosis (CF).

Keywords: Cystic fibrosis (CF), gene correction, messenger RNA (mRNA), zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), clustered regularly interspaced short palindromic repeats (CRISPR), CRISPR/Cas9, dimeric CRISPR RNA-guided *Fok*I

1. Introduction

Cystic fibrosis (CF) is the most prevalent life-shortening autosomal recessive disorder in Caucasian populations [1]. Occurring in 1 out of every 3,500 newborns in the United States,

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and 1 out of every 2,000–3,000 in the European Union, CF affects more than 70,000 individuals worldwide [2]. Chronic lung disease is the major factor contributing to morbidity and mortality among CF patients, as abnormal airway secretions and chronic endobronchial infection lead to progressive airway obstruction. In addition to the respiratory tract, the disease may also affect the pancreas, liver, kidneys, intestine, and reproductive system [3].

Disease severity varies greatly among those with CF, depending largely upon the degree to which the lungs are affected. However, eventual deterioration of the lungs leading to airway obstruction and death is inevitable, and for many years the average CF patient was not expected to reach adulthood [2]. Over the course of the past three decades, advancements in modern medicine have allowed physicians to postpone debilitating changes to the lungs, slowing the progression of disease and allowing many individuals with CF to live well into their 50s or 60s. Despite these advances in current therapy, the median age of survival remains only 33.4 years [2], emphasizing the need for novel therapeutic approaches to further improve patient outcomes in CF.

2. The pathophysiology of cystic fibrosis

Cystic fibrosis is the direct result of a mutation in both alleles of *CFTR*. This gene is responsible for encoding the CFTR protein, a chloride ion channel anchored in the plasma membrane of lung cells, pancreatic cells, sweat and other exocrine glands. Functionally, CFTR is important for the production and movement of sweat, digestive fluids, and mucus across the membrane, where mutations in the encoding gene may result in impaired anion secretion and hyper-absorption of sodium across epithelia [4–6].

Over 1500 different mutations have been described in the CFTR gene, each leading to different defects in the CFTR protein itself [7]. In the most common mutation, the deletion of phenylalanine (F) from position 508 (ΔF508), improper protein folding results in the degradation of CFTR by the cell, which limits the amount of CFTR that reaches the epithelial cell surface. ΔF508-CFTR accounts for approximately 70% of CF cases worldwide and 90% of those occurring in the United States [7].

Alternative mutations in CFTR may result in truncation of the protein via premature stop codons, prevention of proper processing, folding, or trafficking to the plasma membrane, or interference with the chloride channel's ion transport ability, leading to poor gating or conductance [8]. A patient's specific CFTR gene mutation often dictates the severity of his or her disease, as well as the availability of drugs designed to target their particular protein defect.

In addition to mutations within *CFTR* itself, polymorphisms in other genes may also modify disease severity in patients with CF [9,10]. For instance, genetic variation in the gene encoding transforming growth factor β1 (*TGFβ1*) has been associated with more severe pulmonary phenotypes predictive of poorer long-term outcomes [9]. Polymorphisms in the histone-deacetylase-dependent transcriptional co-regulator, *IFRD1*, have also been shown to modulate the pathogenesis of CF lung disease through the regulation of neutrophil effector function [10,11].

Traditional management strategies for CF typically involve the use of antibiotics to treat infection as well as agents or mechanical devices to improve mucus clearance and prevent damage to the lungs. Non-CFTR ion channel agents, for instance, are small molecules designed to normalize the transport of sodium and chloride by targeting non-CFTR ion channels expressed by epithelial cells. Osmotic agents, or inhaled hypertonic solutions, have also been employed to restore airway surface liquid by drawing liquid out of the airway epithelium and into the mucus [12].

In more recent years, the characterization of *CFTR* mutations and genetic modifiers have provided numerous targets for the development of novel therapies aimed at treating the underlying cause, rather than symptoms, of the disease. These agents are designed to directly compensate for *CFTR* mutations in one of three main ways:

- Overcoming the specific functional defect in the patient's CFTR protein (Figure 1),
- Supplementing the cell with a functional copy of the CFTR protein (Figure 2), or
- Repairing the *CFTR* gene mutation at its source (Figure 3).

3. Targeting functional defects in CFTR Protein: Small molecule CFTR modulators

A number of small molecules have been designed to overcome the functional defects in CFTR protein caused by upstream gene mutations (Figure 1). These CFTR modulators can be classified into three families according to the specific functional defect that they target: premature stop codon suppressors, correctors, and potentiators [13].

Premature stop codon suppressors, otherwise known as production correctors or read-through agents, encourage the cell to overlook any premature stop codons transcribed within the *CFTR* mRNA (Figure 1A). They instruct cellular ribosomes to read-through these premature termination codons as the mRNA is being translated into protein. This encourages the production of full-length CFTR. Several *CFTR* mutations interfere with proper protein processing, resulting in misfolded CFTR that is degraded by the cell. **Correctors** focus on improving the processing and transport of CFTR protein to the cell surface (Figure 1B). By ensuring that CFTR is processed and folded correctly, the protein can be trafficked to the plasma membrane where it functions. Additional *CFTR* mutations allow this chloride ion channel to arrive at the plasma membrane, but cause defects in its gating or conductance ability. **Potentiators** work on these defects, to enhance opening of the channel or increasing the flow of chloride ions (Figure 1C) [13].

Lumacaftor (VX-809, Vertex Pharmaceuticals), for instance, is a CFTR corrector that increases trafficking of ΔF508-CFTR to the epithelial cell surface [14]. Lumacaftor has also been used in combination with ivacaftor (VX-770, Vertex Pharmaceuticals, trade name Kalydeco), a CFTR potentiator that improves the transport of chloride through CFTR channels rendered dysfunctional by G551D or R560T missense mutations. By binding to the channels and inducing

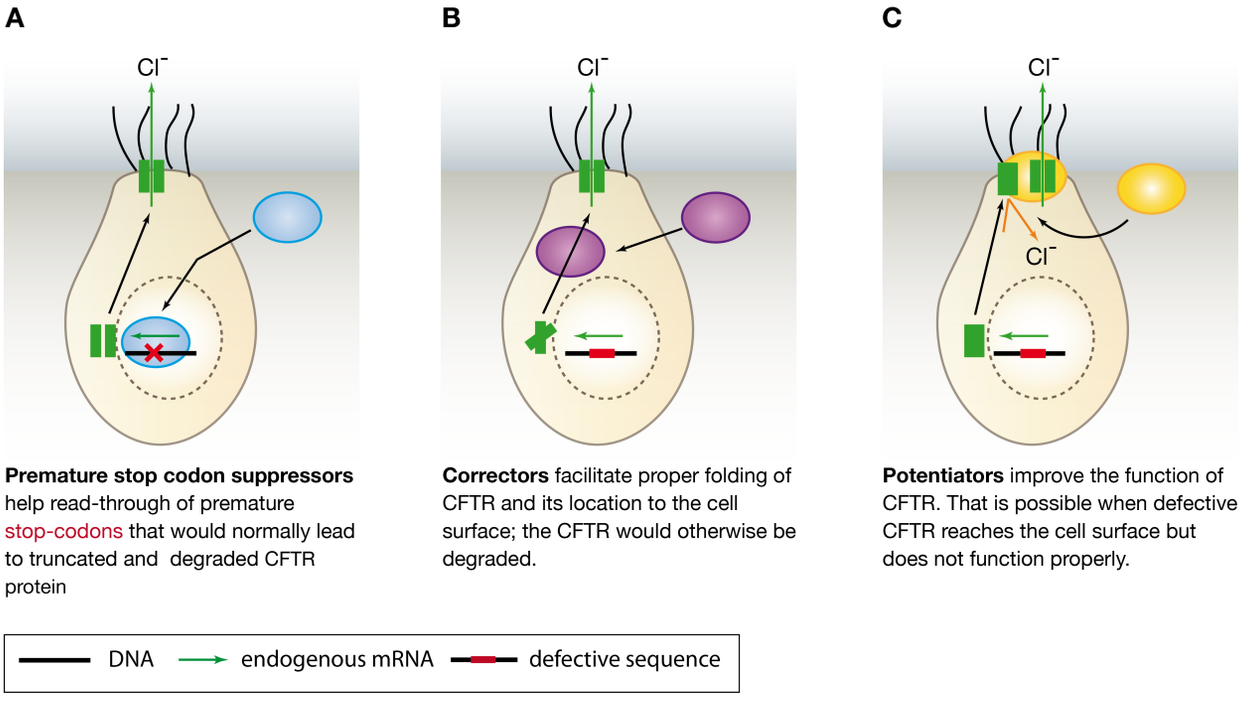

Figure 1. Small molecule CFTR modulators target functional defects within the CFTR protein. Three types of CFTR modulators have been developed: premature stop codon suppressors **(A)**, correctors **(B)**, and potentiators **(C)**. These small molecules act by targeting the transcription, translation, protein processing, membrane trafficking, and ion transport functionality of the CFTR protein, respectively.

a non-conventional mode of gating, ivacaftor increases the probability that the chloride channel is open [14]. Another small molecule CFTR modulator, ataluren (PTC124, PTC Therapeutics, trade name Translarna in the EU), is a production corrector that makes ribosomes less sensitive to G542X or W1282X nonsense mutations [15]. Overcoming these premature stop codons allows the synthesis of full-length, functional CFTR. Together, these small molecule modulators focus on addressing the functional defects in a patient's own CFTR protein.

Gene therapy: Gene supplementation and transcript supplementation therapy

Instead of addressing the functional deficit in a patient's endogenous CFTR, another approach involves supplementing the cell with an exogenous, functional copy of the protein (Figure 2). This can occur in one of several ways: through gene, transcript, or protein replacement therapy. By delivering a functional copy of the *CFTR*, subsequent mRNA transcript, or protein itself, the cell may regain enough CFTR function to halt the progression of disease. Protein replacement strategies have met limited success, as the therapeutic protein is often metabolized before it can enter the target tissue. Gene and transcript therapy approaches, however, continue to

be investigated. Unlike CFTR modulators, these approaches have the potential to be used for all CF patients, regardless of the type of mutation they carry.

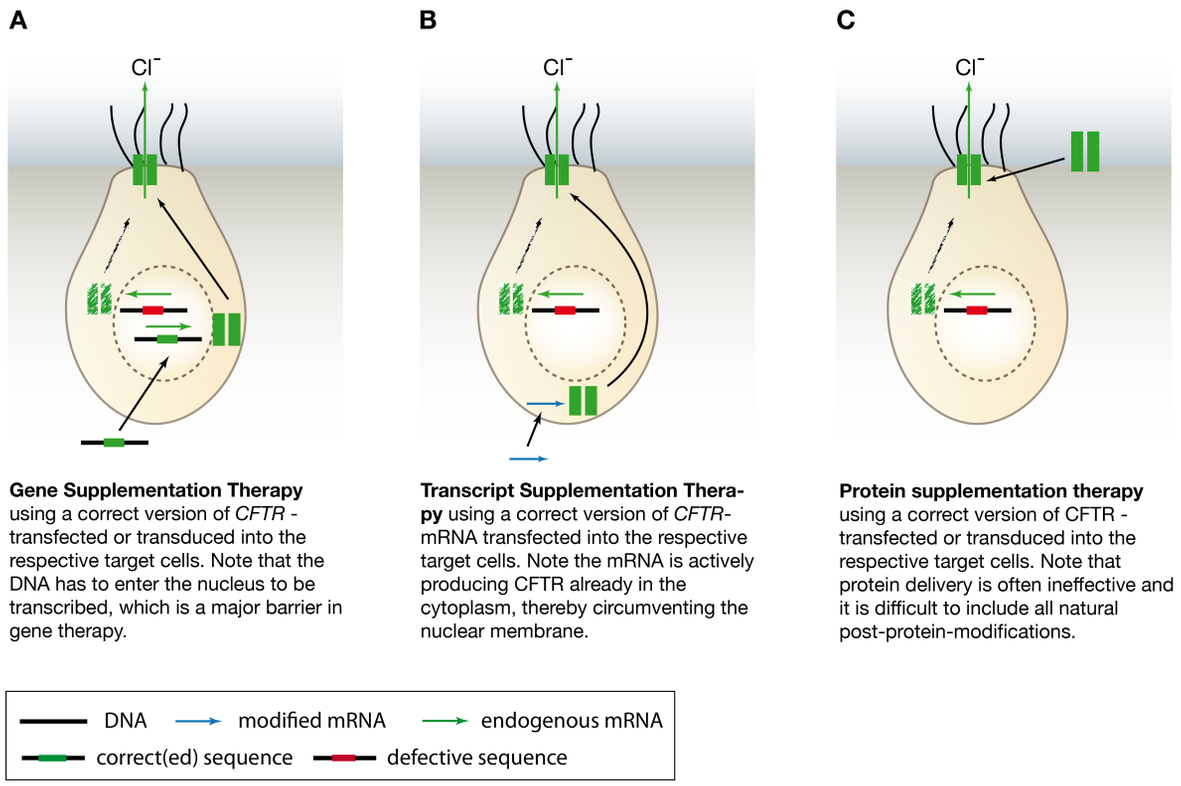

Figure 2. Gene, transcript, and protein replacement therapy supplement cells with a functional copy of the CFTR protein. Supplementing the cell with functional *CFTR* cDNA **(A)**, mRNA transcripts **(B)**, or CFTR protein **(C)** is another method of overcoming the genetic defects underlying Cystic Fibrosis.

4.1. Gene supplementation therapy

The two main forms of gene supplementation therapy are defined by the vehicle used to deliver functional cDNA to the cell: this consists of either viral or non-viral vectors (Figure 2A).

Non-viral vectors are typically comprised of plasmid DNA (pDNA) complexed with carrier molecules, such as cationic lipids or polymers. By binding to the negatively charged pDNA, these molecules either condense or encapsulate the DNA, forming lipoplexes or polyplexes that are then thought to be endocytosed by the cell [16]. In the absence of non-human, viral protein components, it is believed that non-viral vectors may incite minimal immune activation and increase the opportunity for repeat administration. However, even pDNA expression is often limited by CpG motifs that induce strong immune responses through innate immune receptors, such as Toll-like receptor 9 (TLR9) [17]. In addition, non-viral vectors are typically much less efficient than viral vectors at transfecting slowly dividing mammalian cells. This is due to the fact that viruses have evolved efficient strategies for improving cell entry, endosomal escape, cytoplasmic trafficking, and nuclear uptake, all of which make them naturally skilled vehicles for delivering therapeutic cDNA to the cell nucleus [16].

Viral vectors have been designed to harness these evolutionary advantages, while removing components of the viral genome that may cause harm. The ideal viral vector should be replication defective, non-immunogenic, and avoid integrating into actively transcribed genes. Random integration events into an oncogene or tumor suppressor may cause insertional mutagenesis leading to cell death or cancer.

Adenoviral vectors (Ad) engineered to be devoid of the viral genome were the first to be utilized for CF gene supplementation therapy. These vectors have the advantage of being non-integrating, with a natural tropism for the lung. In clinical trials using Ad-CFTR, low levels of gene transfer and partial correction of chloride transport in nasal epithelium were observed in some patients [16]. However, issues such as dose-dependent lung inflammation and humoral and cellular immune responses preventing repeat administration remained limiting factors.

Adeno-associated viral vectors (AAV) also remain largely episomal inside the nucleus, minimizing the threat of insertional mutagenesis. Over 130 serotypes of AAV have been identified, with each viral capsid demonstrating its own unique transduction profile [18]. Capsids from AAV1, 5, 6, 8, and 9 may be the most efficient for transducing cells of the airway epithelium [19]. In addition, the creation of hybrid AAV capsids, such as AAV6.2, may allow the customization of vectors optimized for transducing the desired target cell. Early phase I trials with AAV2.CFTR showed limited efficacy, due in part to the use of a non-lung-tropic AAV2 serotype, limited packaging space for an optimal promoter (*CFTR* cDNA uses 4.7kb of the vector's ~5kb packaging capacity), as well as AAV capsid-specific immune responses limiting repeat administration [16]. Strategies aimed at minimizing adaptive immunity to AAV vectors or reducing the need for repeat administration continue within the field. Removing CpG motifs from AAV vectors or designing hybrid AAV capsids has been shown to reduce innate and adaptive immune responses following intramuscular delivery [20,21]. Targeting AAV delivery to progenitor cells in mouse lung also shows promise as a means of avoiding lung cell turnover and circumventing the need for redelivery [22].

Lentiviral vectors based on recombinant human (HIV), simian (SIV), feline (FIV), and equine (EIV) immunodeficiency viruses have also been investigated for gene replacement therapy [16]. Lentiviral vectors are pseudotyped with the envelope proteins from various viruses to increase tissue tropism. The vesicular stomatitis virus G (VSV-G) envelope glycoprotein has most commonly been incorporated, although the F and HN proteins from murine parainfluenza virus type 1, or Sendai virus (SeV), may improve airway transduction. Studies with SeV-pseudotyped lentiviral vectors have accommodated repeat administration to murine airways in pre-clinical studies [23]. Should repeat administration also be feasible in human subjects, the two major remaining limitations to lentiviral use include safety concerns over genomic integration and scale-up of vector production. The concern over vector integration came to the forefront in 2003, when the integration of a retroviral vector used to treat X-linked severe combined immunodeficiency (X-SCID) triggered unexpected activation of a proto-oncogene leading to leukemia in nearly half of the trial's participants [24,25].

4.2. Transcript supplementation therapy

In recent years, transcript supplementation therapy has been introduced as an alternative to gene replacement therapy (Figure 2B). As mRNA transcripts are not capable of integrating into the chromosome, the threat of insertional mutagenesis is completely void. Following uptake via receptor-mediated endocytosis and lysosomal trafficking, mRNA also completely avoids the rate-limiting step of nuclear entry, being translated rapidly and efficiently directly in the cytoplasm [26]. With its naturally short half-life, mRNA transcripts are particularly useful for applications where short bursts of protein expression are desired. However, the addition of chemical modifications mimicking endogenous mRNA modification schemes has increased expression and stability, while decreasing immune responses. One major benefit to the use of chemically modified mRNA is the ability to readminister the vector as necessary.

The use of mRNA itself has long been appealing as an alternative to gene-based delivery vehicles. Unfortunately, for many years researchers were unable to use *in vitro* transcribed mRNAs to upregulate protein expression *in vivo*, as these transcripts were immediately recognized and destroyed by the immune system following injection [27]. Recent work has shown that by completely substituting uridine with pseudouridine during mRNA synthesis, the binding affinity of mRNA to innate immune receptors can be reduced, making systemic *in vivo* application possible [28,29]. More recent work has shown that partial substitution of combinations of various nucleotide modifications, more closely mimicking those observed in endogenous transcripts, can yield mRNA transcripts with further increased stability, specifically in murine lung [30,31]. This emphasizes that the design of mRNA may have substantially different effects in specific organs *in vivo* compared with *in vitro* use.

In one recent study, transcript therapy with chemically modified *surfactant protein B (SP-B)* mRNA exhibited success in achieving therapeutic levels of protein expression in a murine model of SP-B deficiency [30]. Repeated intratracheal administration of modified *Foxp3* mRNA to murine lung was also shown to alleviate asthma symptoms in two different models of experimental asthma [31]. Both of these models demonstrate the efficacy of nucleotide modified mRNA in achieving therapeutic levels of protein expression in the lung following repeated, *in vivo* delivery. As a vehicle for delivery, modified mRNA may present a safer alternative to viral and non-viral DNA-based approaches, as immune activation can be efficiently prevented and the possibility of genomic integration is eliminated. Importantly, however, due to the short half-life of mRNA, the benefits of modified mRNA transcripts may be better utilized outside of direct transcript supplementation.

5. Repairing the *CFTR* mutation: Gene correction with genome-editing nucleases

With residual limitations in optimizing gene and transcript supplementation therapies for CF lung disease, a new field has begun to emerge: aiming to correct, rather than supplement, the defective gene. Compared to gene or transcript replacement approaches, "gene correction"

aims to replace the defective portion of the CFTR gene with a normal allele at its natural chromosomal location (Figure 3). The repair of a mutant gene directly at its original locus has two major advantages. Most importantly, the corrected gene remains under control of its endogenous promoter, hence assuring life-long expression and native regulation in the cell. Moreover, depending on the delivery vehicle(s) used, gene correction has the potential to avoid the involvement of foreign DNA, thus reducing the risk of insertional mutagenesis.

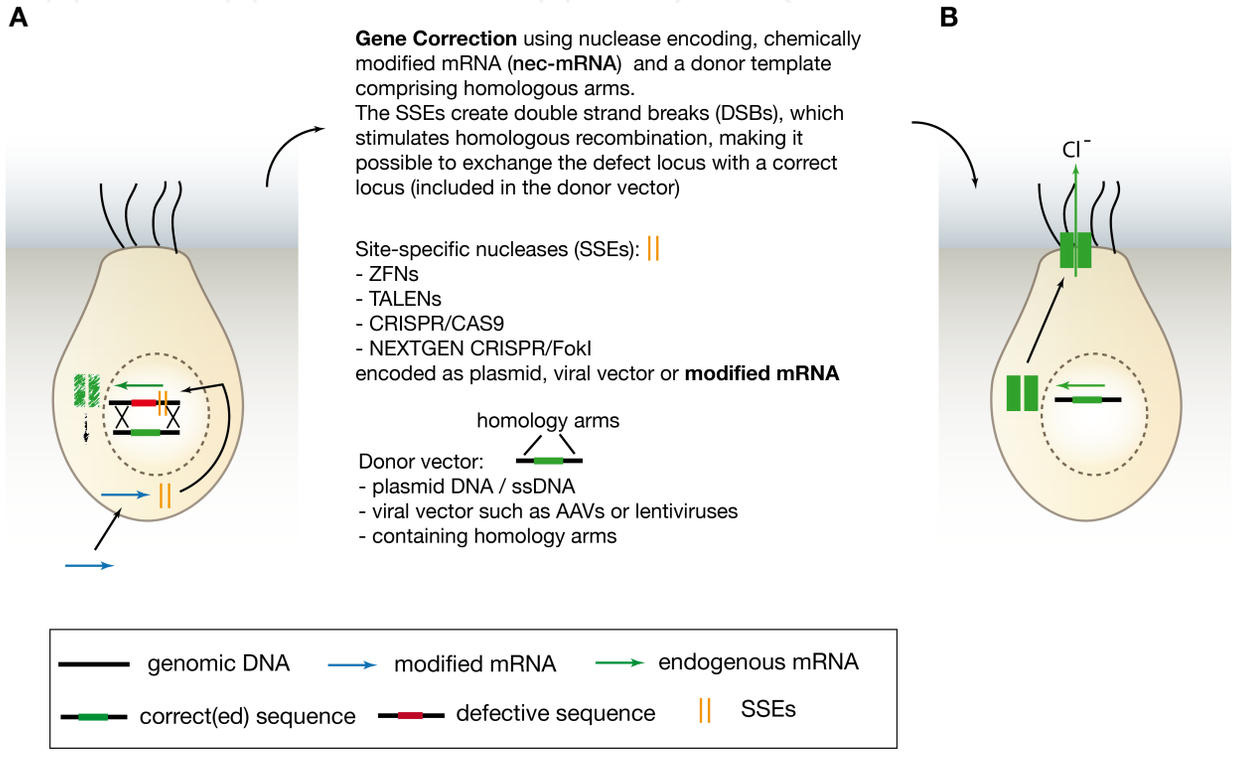

Figure 3. Gene correction approaches aim to repair the *CFTR* gene mutation directly at the endogenous chromosomal locus. The use of mRNA-encoded site-specific nucleases (SSE) can introduce double-strand breaks (DSB) near the genetic defect(s). If a donor vector with corrected sequence is also delivered to the cell, it can be utilized as a template for homologous repair (HR) as the cell works to repair the DSB (**A**). Successful homology-directed repair (HDR) will result in a corrected chromosomal locus, even after the SSE and donor template delivery vehicles are no longer expressed (**B**).

Several lines of investigation into viable gene correction approaches have been pursued. These involve the use of genome-editing nucleases, such as ZFNs, TALENs, or CRISPR-based systems, to take advantage of the cell's natural damage repair pathways. In this strategy, delivery of a site-specific-endonuclease (SSE) or SSE pair elicits a double-strand break (DSB) in the defective gene near the site of an unwanted mutation or sequence, initiating cellular repair mechanisms including homologous recombination (HR) and non-homologous end-joining (NHEJ).

NHEJ, an error-prone process, can be utilized to initiate mutations that essentially disrupt or knock out an undesirable gene. As NHEJ repairs the DSB by ligating the broken strands together, this process commonly results in small insertions or deletions of base pairs, known as indels. The generation of indels at the repair site can cause frame-shift mutations that prevent the protein from being properly transcribed and translated. This concept was dem-

onstrated in 2008, when investigators utilized ZFNs and NHEJ to disrupt the HIV co-receptor, *CCR5*, rendering human $CD4^+$ T cells more difficult for the HIV virus to transfect [32]. Through this mechanism, multiple nuclease pairs can also be utilized to create two DSBs, where NHEJ may completely cut out large segments of unwanted genomic sequence [33,34].

Alternatively, a donor template can be delivered to the cell in addition to the nuclease(s), and used as a guide for directing HR, in a process referred to as homology-directed repair (HDR). In HDR, an extra-chromosomal donor fragment or "repair template" contains regions of significant homology up- and downstream of the DSB site. In between the homology arms, the repair template houses the desired, corrected sequence. Once the nuclease has cleaved, the regions of homology will be used as a template for rebuilding the site. As a result, the mutation-free sequence housed between homology arms is incorporated into the chromosome (Figure 3).

In cases where the patient is homozygous for the target allele, the uncleaved copy of the allele may be favored as a template for HR, decreasing efficiency. As such, it is important to provide the repair template in excess, to ensure that the target cell favors the repair template over the sister chromatid. It is also important to note that even when a repair template is provided in excess for HR, SSE binding and cleavage can also occur at off-target sites, which may initiate NHEJ. In the case of an off-target cutting event, NHEJ can cause unexpected mutations that may be harmful to the cell. As a result, potential off-target binding sites of the SSE should be predicted *in silico* and subsequently sequenced to monitor for deleterious off-target effects. Overall, HDR can be utilized to correct individual point mutations, as well as to insert larger fragments, such as complete copies of functional cDNA, into desired sites. To date, gene correction has been achieved *in vitro*, *ex vivo*, as well as directly in murine liver and lung [35–37].

Each of the available nuclease technologies utilizes a different method for recognizing specific sites and initiating DSB cleavage. In the following section, we will discuss the various mechanisms of action, as well as the pros and cons of each technology. It is important to note that in order to be safe for use in a clinical setting, nuclease technology must meet the following criteria:

- Minimal off-target activity:
  - High binding specificity
  - Transient nuclease expression
- High cleavage efficiency
- Delivery to the cell using vectors that minimize insertional mutagenesis risk

5.1. Zinc finger nucleases

Zinc fingers are a common DNA-binding protein that can be found in nearly half of all transcription factors in the human genome [38]. These naturally abundant proteins can be re-engineered to recognize and bind specific target sequences. ZFN technology takes advantage of this by attaching a DNA-cleaving nuclease to the zinc finger-binding domain. The result is a site-specific binding protein that can cleave a strand of DNA at a precise location.

ZFNs utilize the non-specific DNA cleavage domain from the *Fok*I restriction endonuclease to confer their cleavage activity [39]. Their design traditionally incorporates the wild-type *Fok*I cleavage domain; however, more recent studies have utilized variants with improved cleavage activity or specificity [40–42]. Functioning as a dimer, the *Fok*I domain requires two ZFN constructs, working together as a pair: one ZFN binds to a sequence immediately upstream of the intended cut site, while the other targets sequence immediately downstream of the cut site on the complementary strand (Figure 4A). This alignment places the C-terminal nucleases at a desired distance apart across the cut site, where they dimerize and create a DSB. Proper spacing of binding and cleavage domains is critical for optimal DSB induction.

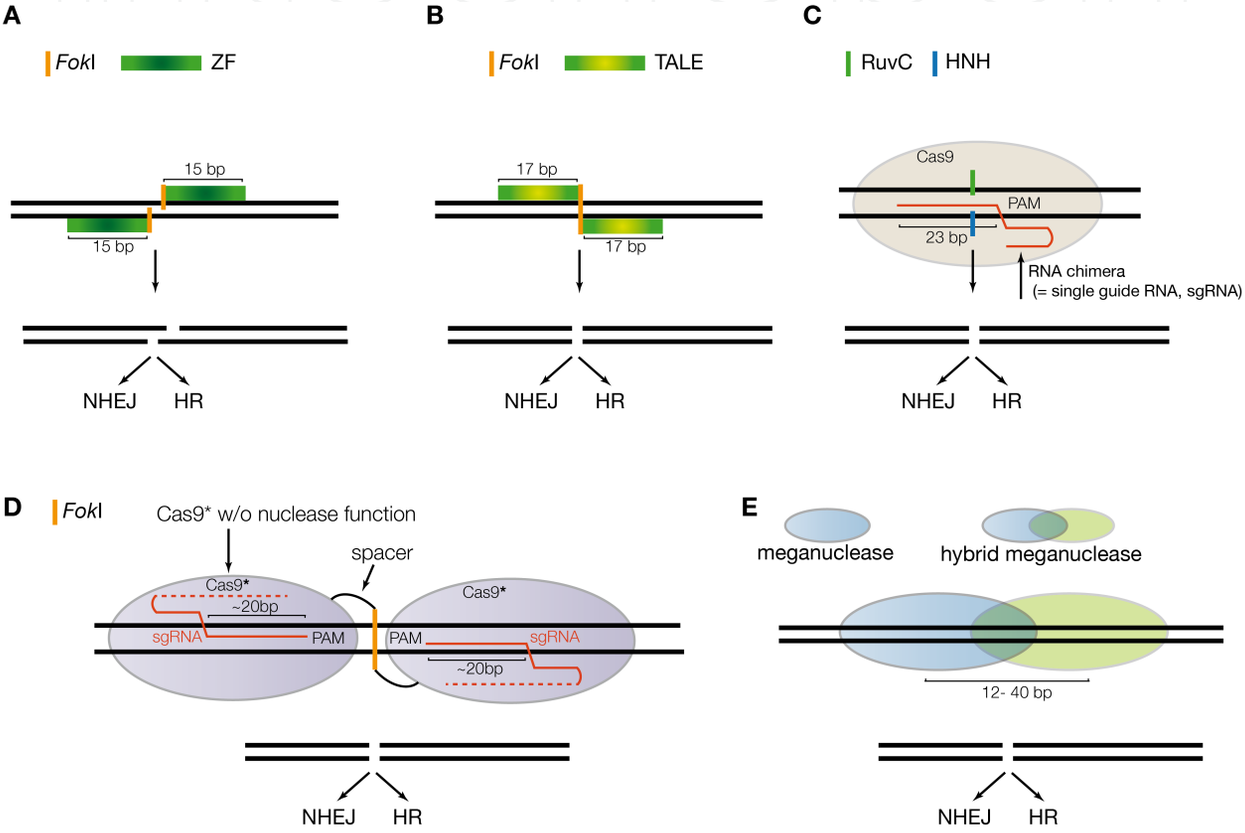

Figure 4. Genome-editing nucleases: Mechanisms of action. An overview of the mechanisms of DNA binding and cleavage used by ZFNs (**A**), TALENs (**B**), CRISPR/Cas9 (**C**), the dimeric CRISPR RNA-guided *Fok*I system (**D**), and meganucleases (**E**). PAM, protospacer adjacent motif; sgRNA, single guide RNA; RuvC, nuclease domain in Cas9 that cleaves the non-target strand of DNA; HNH, nuclease domain in Cas9 that cleaves the target strand of DNA.

Numerous evidence has supported the use of ZFNs for targeted gene editing in multiple species, including mice, rats, rabbits, pigs, plants, and zebrafish [38]. The use of this platform has also extended to the manipulation of stem cell populations *ex vivo*. In one seminal study, ZFNs facilitated targeted disruption of *CCR5*, a co-receptor involved in HIV entry [32]. By introducing the *CCR5*-Δ32 mutation into *ex vivo* expanded $CD4^+$ T cells, followed by engraftment into HIV-1 infected mice, these target cells no longer expressed functional CCR5 entry receptors, making them more resistant to infection. Further studies utilized a dual strategy to target both of the HIV entry co-receptors, CCR5 and CXCR4 [43]. The use of ZFNs was also

successfully demonstrated *in vivo* in murine liver, to correct a model of hemophilia B, or factor IX (FIX) deficiency [36].

Despite this, ZFNs are not without their limitations. First, they are relatively difficult to engineer and expensive to purchase commercially, leaving them inaccessible to the majority of investigators. Secondly, not all sequences can be targeted by ZFNs, restricting their use in certain applications. And lastly, the specificity of ZFN pairs is not 100%, resulting in the potential for off-target cleavage events and related damage to occur. In the event of low specificity, off-target DSB induction may overwhelm cellular repair machinery leading to chromosomal rearrangements and/or cell death. These instances may also support random integration of donor DNA into undesirable locations, which has the potential to interfere with tumor suppressors, proto-oncogenes, or other actively transcribed genes [24,25].

5.2. Transcription activator-like effector nucleases

Transcription activator-like effector (TAL effector, or TALE) proteins are secreted by *Xanthomonas* bacteria upon infecting various species of plant. They function by binding to promoter sequences in the host to upregulate plant genes that are beneficial to bacterial infection. Similar to the concept of ZFNs, TALENs are built by fusing the DNA binding domain of a TAL effector to a DNA cleavage domain with nuclease activity [44–47]. The DNA binding domain consists of multiple repeats of a 33-34 amino acid sequence, where all but the 12th and 13th amino acids are highly conserved. By selecting a combination of repeat segments with the appropriate variable regions (Repeat Variable Diresidues, or RVDs), specific DNA binding domains can be engineered.

TALENs utilize the same non-specific DNA cleavage domain from the *Fok*I endonuclease to confer cleavage activity. As a result, this strategy also requires two TALENs to work together as a pair, binding non-palindromic sequences on complementary strands of DNA. Proper positioning of the DNA binding and cleavage domains around the cute site allows the *Fok*I endonuclease domains to dimerize and produce a site-specific DSB (Figure 4B) [48,49].

Compared with ZFNs, TALENs can cleave a broader, more comprehensive range of DNA sequences. In addition, they tend to be more accurate, reducing the potential for off-target cleavage events. Furthermore, as a result of the fast ligation-based automatable solid-phase high-throughput (FLASH) system reported in 2012, large-scale assembly of TALENs has also become a more efficient and cost-effective alternative [50].

5.3. CRISPR/Cas9

In 2012, the use of a novel genome-editing tool was described in human cell culture [51]. In bacteria and archaea, clustered regularly interspaced short palindromic repeats (CRISPR) work together with Cas genes to form a prokaryotic adaptive immune system that protects against foreign genetic elements such as plasmids or phages. Upon detecting viral DNA, for instance, this system converts segments of the foreign DNA into CRISPR RNAs (crRNA); the crRNA then combines with a trans-activating crRNA (tracrRNA). The crRNA–tracrRNA

combination then guides a Cas9 DNA nuclease to a specific location within the viral DNA, called the protospacer, where a DSB is induced.

Investigators discovered that by designing a new crRNA and combining it with the tracrRNA, a "single-guide RNA" (sgRNA) could be produced that would direct the Cas9 nuclease activity to any desired location. Studies have shown that delivery of two components, the Cas9 nuclease and a corresponding sgRNA (containing both the crRNA and tracrRNA), were sufficient to elicit cleavage in a desired gene [52–55]. Hence, by retargeting the crRNA portion of the sgRNA, a site-specific genome-editing tool could be developed (Figure 4C).

Unlike ZFN and TALEN strategies, the nuclease cleavage domain in the CRISPR/Cas9 system is not fused to the DNA binding domain: instead, these are delivered to the cell in two separate components (Figure 4C). As a result of this design, only a single DNA binding domain has to be created. As this single protein-binding domain is significantly shorter than those required for TALEN or ZFN designs, CRISPR/Cas9 components are significantly easier and more cost effective to synthesize, making this technology more widely available to the research community at large. Despite lower costs and greater accessibility, the functional activity of CRISPR/Cas systems appears to be equal to or greater than their ZFN and TALEN counterparts.

As the CRISPR/Cas9 system is a relatively new genome engineering technology, it will be important for the field to thoroughly study any potential shortcomings. For instance, since a relatively short DNA binding domain and cleavage site are utilized, the risk of low specificity and potential off-target recognition may be greater [56].

5.4. Dimeric CRISPR RNA-guided *Fok*I nucleases

In an effort to reduce the risk of unwanted off-target mutations associated with monomeric CRISPR/Cas9 nucleases, a modified dimeric version has now been developed [57]. Where the monomeric Cas9 nuclease is recruited by one sgRNA of only ~100 nucleotides in length (with 17–20 nucleotides of complementarity to the target), dimerization offers an attractive strategy for improving the binding specificity of the Cas9 system (Figure 4D).

In this approach, a wild-type *Fok*I nuclease domain is fused to a catalytically inactive Cas9 (dCas9) protein. Two such *Fok*I-dCas9 fusions are recruited by two corresponding guide RNAs, where both are required to bind their respective target sites in order for *Fok*I dimerization and DSB induction to occur (Figure 4D). An appropriately designed spacer and protospacer adjacent motif (PAM) are also critical for driving efficient cleavage. Overall, this RNA-guided *Fok*I nuclease (RFN) strategy has been shown to elicit robust genome editing efficiencies while reducing known off-target mutations to undetectable levels [57].

5.5. Meganucleases

Meganucleases are another form of endonuclease utilized for genome editing approaches. They are unique in that their DNA recognition and cleavage functions are naturally combined in a single domain. There are five classes available, where I-SceI, I-CreI, and I-DmoI are perhaps the most widely used. Consisting of a large recognition site of 12 to 40 base pairs, meganu-

cleases also offer high specificity and precision; however, historically, they were only capable of tolerating minor variations in their recognition site sequence, decreasing the probability of an available meganuclease for each desired application. In recent years, investigators have begun customizing meganucleases to expand their targeting repertoire. Two main approaches have been taken: modifying the specificity of existing meganucleases, and/or developing chimeric meganucleases with new recognition sites. In the latter approach, by fusing the DNA-binding domains of two different meganucleases, functional heterodimers can be designed for optimal efficacy and specificity (Figure 4E).

6. The best vehicle for the job

In genome editing, the identification of components capable of eliciting HDR is only half the battle. Efficiently delivering those components to the target cell can be an equally important hurdle to overcome. Unlike gene and transcript replacement therapies, where the goal is to achieve stable, long-term expression of a supplemental protein, gene editing has the advantage of requiring only short-term expression of the foreign components in the cell. Once these components have been expressed, induced a DSB, and triggered HDR, their presence is no longer necessary. In fact, in the attempt to minimize unwanted off-target cleavage activity and prolonged DSB induction, the ideal nuclease-delivery vehicle should only be transiently expressed.

Additionally, without the need for integration to promote stable expression, non-integrating delivery vehicles are also preferable, to minimize the risk of insertional mutagenesis. Delivery vehicles must also be capable of transducing target cells efficiently, to facilitate gene correction in enough cells to overcome the initial defect. To summarize, since transient expression of the nuclease is sufficient for stable modification of the genome, the ideal nuclease delivery vehicle should be:

- Short-lived,
- Non-integrating, and
- Able to enter target cells efficiently.

A variety of vectors have been utilized to deliver genome-editing reagents to the cell. Initial *in vivo* studies have included AAV viral vectors as well as integrase-defective lentiviruses. Using AAV-encoded ZFNs, for instance, Li and colleagues demonstrated direct *in vivo* gene correction using HDR in a murine model of FIX deficiency [36]. Despite these successes, early *in vivo* strategies have not fulfilled two of the critical components for nuclease delivery vehicles: transience and lack of potential integration. In addition, with respect to translation of these approaches to CF airway disease, none of these strategies reported targeting cells in the lung.

Due to its short half-life and inability to integrate into the genome, modified mRNA is gaining interest as an ideal vector for site-specific nuclease delivery: one that would address two of the main outstanding issues previously discussed. Transient expression of mRNA-encoded

nucleases would also minimize the long-term threat of off-target events associated with the use of stably expressing, and possibly integrating viral vectors.

7. Gene correction in the lung

In a recent study, nuclease-encoding chemically modified mRNA (nec-mRNA) was described as a novel vehicle for delivering genome-editing components directly to the lung [37]. Using a murine model of SP-B deficiency, nec-mRNA-encoded ZFNs were able to demonstrate the first report of life-prolonging gene correction specifically within lung tissue.

In the transgenic mouse model of SP-B deficiency, SP-B cDNA is under the control of a Tetracycline-inducible promoter [58]. Administration of doxycycline allows SP-B to be expressed at wild-type levels. If doxycycline is removed, SP-B expression drops and mice begin to exhibit phenotypic changes similar to those seen in humans with the disease: thickened alveolar walls, heavy cellular infiltration, increased macrophages and neutrophils, interstitial edema, congestion, augmented cytokines in the lavage, a significant drop in lung function, and acute onset of respiratory distress leading to death within days [59,60].

In order to demonstrate the value of nec-mRNA for lung-based genome-editing applications, this report utilized HDR to insert a constitutive CAG promoter immediately upstream of the SP-B cDNA. The resulting doxycycline-independent expression was able to significantly prolong the life of treated mice [37].

While this study was not performed in a humanized mouse model, the approach was able to demonstrate that extra-chromosomal nec-mRNA is capable of transducing airway epithelial cells, expressing genome-editing reagents, and achieving HDR rates sufficient for therapeutic levels of protein expression. Main findings from the study include:

i. mRNA modification schemes can be customized to optimize expression and minimize immunity.

ii. Intratracheal delivery of nec-mRNA is able to target airway epithelial cells.

iii. Complexing nec-mRNA to chitosan-coated nanoparticles can increase transduction efficiency in the lung.

iv. nec-mRNA expression is transient in comparison to AAV-encoded ZFNs.

v. nec-mRNA-mediated ZFN delivery can facilitate HDR rates comparable to AAV-encoded ZFNs.

8. Hurdles to success: Limitations to gene correction for cystic fibrosis

The lung has evolved with natural defense mechanisms against foreign pathogens. As such, a number of intracellular and extracellular barriers must be overcome in order to target new

technologies to the lung [61]. In addition to this, the lungs of CF patients are even more difficult to target, owing to the increased airway mucus (sputum) lining the lungs. Especially in more progressed CF patients, thickened mucus linings have proven prohibitive in several gene replacement therapy approaches. Hida and colleagues reported that expectorated sputum from CF patients effectively traps and slows the diffusion of both Ad and AAV viral vectors [62]. For instance, where the sputum penetration of muco-inert nanoparticles is reduced by only 40-fold compared with pure water, that of Ad and AAV particles is slowed by 3,000-fold and 12,000-fold, respectively. Poor penetration of mucus layers may be a major component preventing effective viral gene replacement therapy or the use of viral vectors to deliver genome-editing components for CF.

Limits to stable expression of functional CFTR also play an important role. For gene replacement therapy approaches, this may include immune responses against the vector capsid, the inability to re-administer, as well as turnover of CFTR-expressing lung cells. For gene correction approaches, transient expression of nucleases does not destabilize the downstream effects of HDR. However, lung cell turnover continues to be an issue, making the possibility of re-administration important.

The levels of CFTR expression required to halt the progression of CF lung disease remain largely unknown, as well as the cell types most suited as targets. While airway epithelial cells are generally considered to be the ideal target, airway histology and entry receptor expression patterns may impact the ability of this cell type to be transduced. The absence of adenoviral entry receptors on the apical surface of airway epithelium, for instance, is one of the major reasons that adenoviral gene therapy vectors are no longer pursued. Furthermore, turnover of these terminally differentiated cells will eventually require therapeutics to be redelivered to new target cells.

In addition to the most relevant cell type, another question remains: is it preferable to obtain low levels of CFTR in a high percentage of cells, or high levels of CFTR in only ~10% of cells? Gene replacement and correction approaches are more likely to attain the latter, although it remains unknown whether this will be sufficient to show therapeutic effect. One recent study has shown that restoration of normal mucus transport rates in cultured CF human airway epithelial cells required at least 25% of surface epithelial cells to be targeted by CFTR gene replacement therapy [63]. Whether this figure will translate to clinical benefit in an *in vivo* setting remains to be seen.

9. Supplemental strategies and future directions

Due to the limitations of targeting the lung, it remains unclear whether novel replacement and correction approaches will find success for Cystic Fibrosis lung disease. Efforts to overcome these barriers remain the subject of further investigation.

As one recent study demonstrated, combining nec-mRNA with chitosan-coated poly(lactide-co-glycolide) nanoparticles may be one viable method for overcoming the CF sputum barrier to lung cell targeting [37,64]. Recent progress in the development of mucus penetrating

nanoparticles (MPP) may provide an opportunity to further overcome this barrier [65]. Drug-loaded MPPs with non-adhesive coatings have been shown to penetrate mucus layers at rates nearly as fast as pure water. These developments may allow the penetration of delivery vehicles to airway epithelium, without reducing the protective function of the mucus itself. Furthermore, adjuvant regimens of N-acetylcysteine (NAC) with or without recombinant human DNase (rhDNase) were used to increase diffusivity of nanocomplexed, non-viral gene delivery vectors through sputum layers [66]. This strategy was able to increase gene expression by ~12-fold, making it another potential avenue for improving targeting in the lungs of CF patients. Complexes of pDNA or mRNA with GL67:DOPE:DMPE-PEG5000 (GL67) liposomes have also been described as a potential avenue for augmenting non-viral respiratory gene transfer [67]. Overall, developments in nanoparticle technology combined with advancements in aerosol-delivery devices may hold promise for the field.

Route of administration may be an important consideration as well, especially given the tendency for inhaled therapeutics to be entrapped in the mucus layer. Intratracheal high-pressure spraying approaches have been effective in targeting airway epithelial cells in pre-clinical models [30,31,37], although efficiency is likely to decrease in the face of CF sputum. Preliminary evidence supports the claim that intravenous routes of administration may also target airway cells efficiently, while avoiding the barriers to a direct airway approach.

The continued development of humanized animal models of CF, including mouse, pig, and ferret models, will further our ability to investigate novel therapeutic strategies [68,69]. An early mouse model, *CFTR*^{tm1UNC}, knocked out murine *CFTR* through a stop codon in exon 10; however, these mice showed a drastic drop in survival rates due to severe intestinal obstruction [70,71]. To overcome lethal intestinal defects, the mice were then 'gut-corrected' with a human *CFTR* construct driven by an intestinal-specific FABP promoter [72]. Studies in the FABP-hCFTR/*Cftr*^{tm1UNC} gut-corrected model have demonstrated that the human CFTR protein is indeed functional in mice. Using this or other models as a foundation, it may be possible to introduce a transgenic construct containing a mutated human *CFTR* driven by a lung-specific promoter. Creating humanized mice expressing the *CFTR*-ΔF508 mutation, for instance, may offer an excellent tool for studying gene correction using nucleases and repair templates designed for direct translation to the clinic.

In addition to the development of novel animal models, the identification of human lung stem cell populations has offered new hope for overcoming the issue of lung cell turnover [73]. If genome-editing vehicles could be efficiently targeted to lung stem cell populations, such as bronchioalveolar stem cells (BASCs) [74], Clara cells [75], or alveolar type II (ATII) progenitors [76], HDR in these self-renewing populations could support indefinite CFTR production.

Engineering strategies to minimize the risk of off-target cleavage and donor integration will also continue to be an important area of development. Along these lines, it will be critical to more thoroughly define standardized parameters for measuring off-target effects. State-of-the-art techniques that can be used for measuring outcome parameters will also aid in assessing overall efficacy. Combining efforts to overcome these barriers to lung targeting, cell turnover, proper animal models, and off-target effects will enable the field to make continued progress toward a novel gene correction strategy for the treatment of Cystic Fibrosis.

10. Conclusion

Over two decades since the cloning of the CFTR gene, numerous strategies have been investigated to identify clinically relevant genetic variants, target cells of the airway, and overcome deleterious mutations. Rather than masking symptoms of the disease, novel therapies strive to address the underlying genetic cause of the Cystic Fibrosis phenotype. Agents have approached this goal with varying strategies, including attempts to overcome the patient's functional CFTR defects, supplement their cells with a functional copy of the protein, or directly repair genomic mutations at their source. Innovations in viral and non-viral delivery vehicles and methods for overcoming barriers to lung targeting have allowed for promising progress in recent years. Coupled with novel genome-editing reagents, such as ZFNs, TALENs, and the CRISPR/Cas9 system, the promise of a novel therapeutic approach is becoming an increasingly attainable goal within the field. Further advancement in minimizing off-target activity, increasing the efficiency of site-specific cleavage, and optimizing robust, transient, non-integrating nuclease delivery vehicles will bring us closer to achieving stable modification of the genome in the race toward *in vivo* gene correction of Cystic Fibrosis.

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