Viral Vector Delivery Techniques for CRISPR-Based Homology-Directed Repair Gene Therapy with a Focus on Cystic Fibrosis

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ABSTRACT

CRISPR-Cas9 is a revolutionary technology used to edit or alter an organism's DNA sequence and gene functions. CRISPR-Cas9 technology is implemented using gene therapy vectors to effectively deliver gene editing reagents while protecting the genetic material and bypassing extracellular and cellular barriers [1]. Among current gene therapy vector technologies, viral vectors have demonstrated highly specific targeted delivery and high transduction efficiency. Viral vectors are modeled after pathogenic virus systems and integrate therapeutic genes into an organism's genome [2]. Due to their diverse types and unique advantages and disadvantages, thorough deliberation must be applied to select an optimal classification to maintain efficacy and safety of CRISPR-Cas9 gene therapy techniques. Viral vectors can be used to effectively deliver gene therapies to repair mutations employing 2 techniques: Homology-Directed Repair (HDR) and prime editing [3, 4]. Single mutations can cause certain genetic diseases, such as cystic fibrosis, which is most commonly caused by a deletion of the 508th amino acid residue (phenylalanine) of the cystic fibrosis transmembrane conductance regulator (CFTR) protein [5, 6]. HDR repairs double-stranded breaks following the gene editing process, while prime editing, a newer technology, edits and repairs DNA without creating double-stranded breaks. These techniques are both effective options for employing CRISPR-Cas9 to repair the CFTR mutation [7, 8]. This review explores the range of available viral vector technologies used for gene delivery and compares prime editing with HDR with an emphasis on the application of repairing the CFTR deletion mutation that causes cystic fibrosis.

Introduction

Genome editing, or gene editing, is a revolutionary technique that allows for precise targeting and addition, deletion, or other alteration of specific sequences within the genome. Gene editing can be used for a wide range of purposes, ranging from altering physical traits to eliminating genetic disease risk. Although it was first invented in the late 1900s, the popularity of gene editing has increased greatly over the last decade because of the new genome-editing tool CRISPR, or Clustered Regularly Interspaced Short Palindromic Repeats, which allows for cheaper, faster, and more accurate editing [1].

Gene-editing therapy techniques are being used successfully in many single-gene disorders, such as hemophilia, sickle cell disease, cystic fibrosis, and more complex diseases such as HIV, cardiovascular disease, various cancers, and mental illnesses. Although CRISPR is far more advanced than its predecessor, it has limitations when applied to therapeutic gene editing in humans [2]. One technical limitation is the possibility of off-target editing, i.e. editing at the wrong location in the genome, and its impact on human health is inconclusive [9]. Mosaicism is another limitation that occurs when a cell divides before the gene editing process has been completed. In such cases, a daughter cell may carry either the edited gene or the original mutated gene. In addition, targeting may be mono-allelic or bi-allelic, and it is often necessary to mutate both copies of a gene. Mosaicism and incomplete targeting are very common

in gene editing as CRISPR techniques often require cells that are actively dividing [10]. However, although both cause instability in genome functionality and pose a major concern for biomedical and clinical applications, multiple DNA repair pathways have been utilized in concert with CRISPR methods while minimizing these limitations [11]. These include double-stranded break-mediated homology-directed repair (HDR) and prime editing, the latter of which only requires single-strand breaks but also has limitations, which are described herein. These pathways determine the efficacy of such gene editing techniques in clinical settings as they must be implemented to repair genetic damage deliberately caused by editing systems. Therefore, consideration must be applied to the implications and efficiencies of both systems. In addition, viral vectors are a highly efficient for introducing the necessary DNA into this system. Nonetheless, specific categories of these vectors are optimal only in particular circumstances. To ensure the safety and precision of gene editing systems, a complete understanding of these nuances is imperative. CRISPR-Cas9 gene editing, viral vectors, and the HDR and prime editing pathways are discussed in detail herein with particular focus on applications to the repair of CFTR deletion mutation on cystic fibrosis transmembrane conductance regulator (CFTR), which causes cystic fibrosis.

Cystic Fibrosis (CF)

Insertion, deletion, and substitution mutations can cause a myriad of genetic diseases, cystic fibrosis (CF) being one of the most common [12]. CF is a genetic disorder that affects a number of different organs and causes obstructive lung disease with chronic bacterial infection, pancreatic enzyme insufficiency, and high salt content in sweat. CF is most commonly caused by a mutation affecting amino acid residue 508 of the cystic fibrosis transmembrane conductance regulator (CFTR) protein [12]. The mutation in question is a deletion of three nucleotides, causing the removal of the amino acid phenylalanine. This causes a mutant protein to be created that fails to fold properly, which results in defective function as a channel for chloride ions. Thus, with the deletion of AA508, there is an absence or shortage of chloride ion channels, causing secretory epithelial cells to overproduce mucus and obstruct passageways in the lungs. Additionally, CF causes defects in secretory epithelial cells that transport water, electrolytes, and other solutes across cell membranes.. CF is an autosomal recessive disorder. Individuals with CF inherit two copies of mutated CFTR, while those with one copy of the mutated gene are asymptomatic carriers that can pass the disease on to their offspring. This can be a life-threatening illness resulting in lung infections and extreme coughing. However, the CRISPR viral vector toolbox can be used to repair the deletion mutation that causes cystic fibrosis. Since CF can result in organ damage and long-term detrimental effects from subsequent infections, gene therapy to repair this mutation must be performed before severe symptoms begin, so it must be proven safe for young children. Additionally, there are numerous different mutations—nearly 2,000—that can result in CF [13], making other therapeutic strategies for the disease less efficient than gene therapy [14]. Because of this, CRISPR-Cas9 gene therapy remains one of the most lucrative strategies in possible CF treatment. Specifically, HDR and prime editing are potential tools for editing of the CFTR gene.

The CRISPR-Cas9 Gene Editing Tool

CRISPR is a gene-editing technology composed of bacterial DNA sequences that use captured DNA from foreign viruses as a defense against pathogens. It consists of repeating genetic sequences that are interrupted with spacer sequences and acts as a system to maintain genetic memory of viral invaders. The CRISPR system uses viral DNA to generate DNA sequences called CRISPR arrays, which are then used to produce guide RNA segments targeting specific viral DNA sequences. The Cas9 (CRISPR-associated protein 9) enzyme then cuts the viral DNA and disables the virus. In order to use the system without relying on bacteria, the CRISPR-Cas9 system has been adapted for in vitro use. The bacterial process is replicated using Cas9 and a guide RNA sequence that attaches to a target DNA sequence. Once the guide RNA detects the target sequence, Cas9 is used to cut the DNA, and the cell's biological

mechanisms can be used to make precise edits in the target gene. The CRISPR-Cas9 editing process is shown in Figure 1.

Single genes can be critical for the cell's biological function. Therefore, minuscule mutations or errors can cause debilitating genetic diseases. For example, a point deletion mutation causing a loss of a *PMP22* gene copy can cause hereditary neuropathy, and a point insertion mutation resulting in nucleotide repeats on chromosome 19 can cause myotonic dystrophy. Additionally, cystic fibrosis is most commonly caused by the deletion of amino acid residue 508 in CFTR on chromosome 7. CRISPR technology can remedy these mutations by modifying the mutated genes, leaving all other genes unaffected and restoring biological function [1, 2].

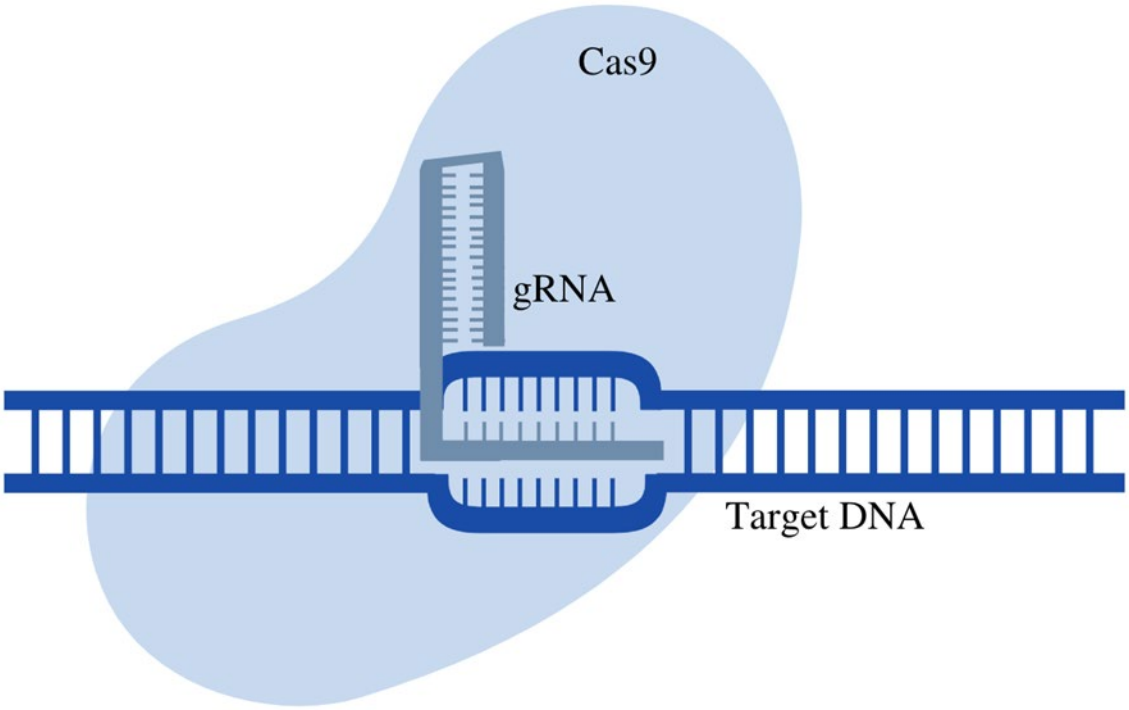


Figure 1. The target DNA sequence is edited by the guide RNA using the CRISPR-Cas9 system

Cas9 in Human Cells

In 2013, the CRISPR-Cas9 system was adapted for genome editing in eukaryotic cells, including human cells [15]. This system was programmed to target multiple locations on the same chromosome [4]. The CRISPR prokaryotic immune system of the *Streptococcus pyogenes* type II CRISPR locus was engineered for gene editing purposes. This system consisted of a trans-activating CRISPR RNA (tracrRNA) and a precursor CRISPR expression RNA (pre-crRNA) to create double-stranded breaks and cleave DNA. The *S. pyogenes* Cas9 (SpCas9) system was used alongside nuclear localization signals to facilitate nuclear transport. This system was successful in performing genome editing in both human and mouse cells, thus creating new therapeutic opportunities [2].

Prime Editing, an Advancement in the Cas9 Toolbox

Since 2013, the Cas9 toolbox has been expanded to deliver genetic material and repair mutations without requiring donor DNA templates or creating double-stranded breaks (DSBs) [16]. Prime editing is a technology that provides

precise gene editing and a wider variety of base changes than the traditional CRISPR/Cas9 system. Prime editing is accomplished using a Prime Editing RNA (pegRNA) as the guide RNA composed of a Single Guide RNA (sgRNA) complexed with a reverse transcriptase , a primer binding sequence, and a template with the necessary RNA sequence. The pegRNA guides the system to the target site to be edited and identifies the edit to be made. Instead of the traditional Cas9 enzyme, prime editing utilizes Cas9 nickase to simply nick the DNA without creating DSBs. The resulting "nicks" result in reduced harm to the DNA than DSBs as they only impact one strand of the double-stranded nucleic acid, which is preferred in clinical settings. Three iterations of prime editing have been created to refine and increase the gene-editing ability of CRISPR Cas-9 [17]. Prime editor 1 was able to repair most simple mutations, and prime editor 2 ensured steady thermal inactivation and binding improvements. The most recent iterations, prime editor 3 and prime editor 3b, allow for the repair of mismatched DNA sequences that may occur when the prime editor is repairing a deletion, substitution, or transversion.

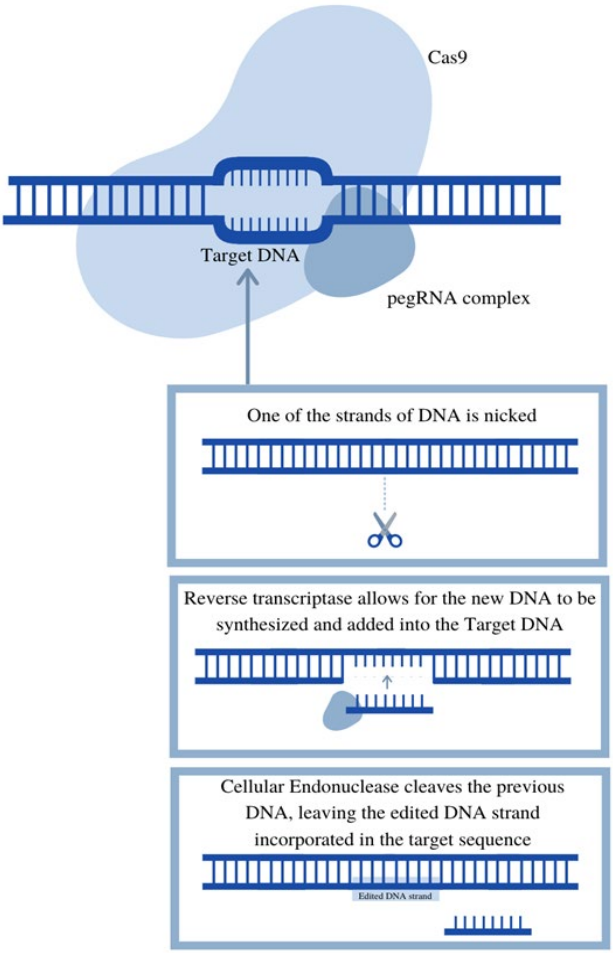


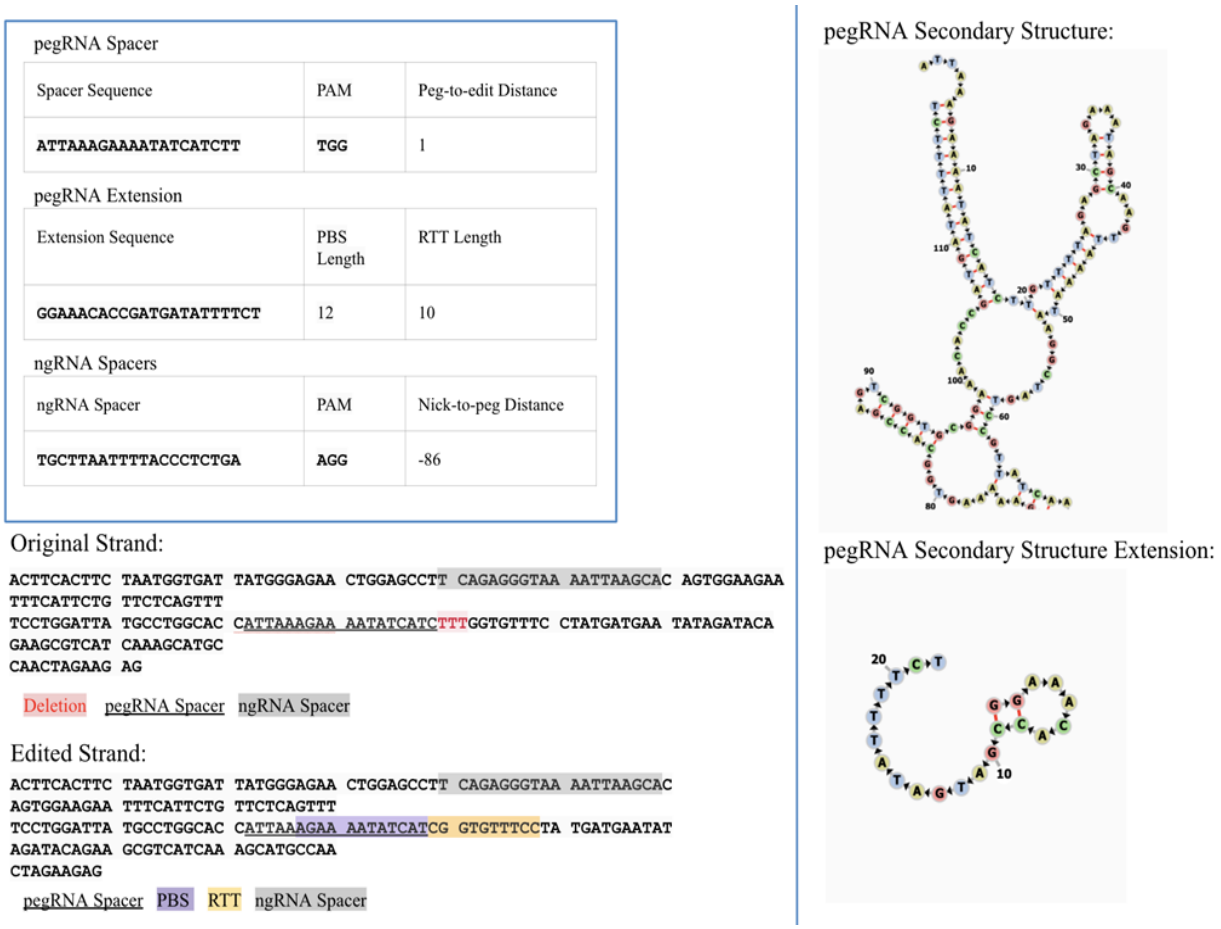
Figure 2. The prime editing process is composed of nicking, reverse transcription and synthesis of DNA, and incorporation of the edited strand.

The prime editing process [Figure 2] begins when the pegRNA binds to the target sequence, causing the Cas9 nickase to nick one of the two DNA strands. The RNA template of the prime editor is reverse transcribed, and this edited sequence is inserted into the single-stranded nick in the target DNA. Cellular endonuclease, an enzyme that cleaves nucleotides, is then used to degrade the previous DNA sequence that was nicked. After this process, the other

DNA strand is edited by a similar process using the first edited strand as a template. However, the newest iterations of prime editors, prime editor 3 and 3b, can use a second guide RNA to edit the second strand.

Applications of Prime Editing to CF

Prime editing is a viable solution for repairing the CFTR AA508 deletion. Figure 8 shows the necessary pegRNA spacer and extension and the ngRNA spacer necessary for this edit. The mutated strand is repaired using the necessary pegRNA structure. With an application to CF, prime editing has been implemented to repair the CFTR-F508 and CFTR-R785* mutations to restore CFTR function in intestinal organoids modeled after CF patients. The prime editing template for this mutation is illustrated in Figure 3. Prime editing was used in order to create the mutated organoids containing the CFTR-R785* or the CFTR-F508 mutation. In both scenarios, after electroporation, application of an electric current to introduce the needed DNA into the cell, it was concluded that CFTR functionality had been restored. Additionally, it was proven that this prime editing did not result in genome-wide off-target effects, contributing to the understanding of the safety of this method [18]. Although prime editing exhibited low efficiency, repairing the function of CFTR by this method was proven possible, indicating the potential for future studies to improve efficiency and accuracy.



pegRNA Spacer

Spacer Sequence	PAM	Peg-to-edit Distance
ATTAAAGAAAATATCATCTT	TGG	1

pegRNA Extension

Extension Sequence	PBS Length	RTT Length
GGAAACACCGATGATATTTTCT	12	10

ngRNA Spacers

ngRNA Spacer	PAM	Nick-to-peg Distance
TGCTTAATTTTACCCTCTGA	AGG	-86

Original Strand:

ACTTCACTTC TAATGGTGAT TATGGGAGAA CTGGAGCCTT CAGAGGGTAA AATTAAGCAC AGTGGAAGAA
TTTCATTCTG TTCTCAGTTT
TCCTGGATTA TGCCTGGCAC CATTAAAGAA AATATCATCTTTGGTGTTTC CTATGATGAA TATAGATACA
GAAGCGTCAT CAAAGCATGC
CAACTAGAAG AG

Deletion pegRNA Spacer ngRNA Spacer

Edited Strand:

ACTTCACTTC TAATGGTGAT TATGGGAGAA CTGGAGCCTT CAGAGGGTAA AATTAAGCAC
AGTGGAAGAA TTTCATTCTG TTCTCAGTTT
TCCTGGATTA TGCCTGGCAC CATTAAAGAA AATATCATCG GTGTTTCCTA TGATGAATAT
AGATACAGAA GCGTCATCAA AGCATGCCAA
CTAGAAGAG

pegRNA Spacer PBS RTT ngRNA Spacer

pegRNA Secondary Structure:

pegRNA Secondary Structure Extension:

Figure 3. The prime editing template for repairing the CF mutation including the pegRNA spacer, pegRNA extension, and ngRNA extensions. Sequences were derived using PrimeDesign (https://drugthatgene.pinellolab.partners.org/)

DNA Repair Pathways in Gene Editing

Homology Directed Repair (HDR) is a naturally initiated nucleic acid repair mechanism that is used to repair DNA lesions [19]. DNA lesions are sites of either structural or base pairing-related damage to DNA. Lesions can form when both strands of the DNA backbone are broken. These are referred to as double-strand breaks (DSBs), and often occur as a natural phenomenon. For example, when DNA breaks occur during meiosis, cells deliberately initiate DSBs to facilitate the exchange of genetic information between homologous chromosomes. However, DNA lesions that are caused by deleterious intracellular and external factors are potentially lethal to cells [20]. Intracellular factors such as free radicals, which are unstable molecules containing oxygen that are capable of reacting with other molecules, can cause DSBs and cell death. Another intracellular factor is nuclease, an enzyme capable of cleaving bonds between nucleotides and nucleic acids resulting in the formation of DSBs. Many external factors can cause DSBs, including but not limited to ultraviolet radiation and ionizing radiation. Although there are many causes of DSBs and their occurrence is often unpredictable, it is critical to repair DNA damage from DSBs in order to retain genomic stability. Unrepaired DSBs can cause chromosomal translocations that are correlated with many developmental defects as well as neurological and immunological dysfunctions.

HDR is a universally applicable mechanism for repairing DSBs. Apart from repairing DSBs that originate from intracellular and external factors, HDR is also used to repair DSBs that are engineered by CRISPR Cas-9 genome modification techniques. Genome engineering techniques such as CRISPR deliberately introduce DSBs at precise target sites by directing nucleases to modify genomes. In many cases, donor DNA is introduced and copied into the DSB as HDR is induced to repair these DSBs. Including HDR, there are 3 main DNA repair mechanisms derived from eukaryotic cells:

- HDR is the second most common repair pathway for DSBs in eukaryotes. HDR heavily relies on homologous repair templates at the DSB site. This pathway is induced in late S and G2 phases of the cell cycle where sister chromatids (homologous templates) are present. The HDR pathway results in repair of DSBs, meaning that there are no insertions and deletions at the breakage site. Although HDR is regarded as less efficient than other methods, specifically non-homologous end joining, it also presents a higher accuracy in repair. Hence, HDR is often a preferable choice for precise gene editing and efficient repair when using CRISPR, especially when it is induced.
- Non-homologous end joining (NHEJ) is the predominant rapid cellular repair pathway and is present throughout the cell life cycle in most eukaryotic cells [21]. The NHEJ repair mechanism works by rejoining the blunt ends of DNA, and has a higher capacity for repair as it doesn't require a homologous repair template. However, this leads to a higher error rate. NHEJ is prone to creating insertions/deletions (indels) at the breakage site. These indels can cause frameshift mutations in the genetic sequence and result in dysfunctional or semi-functional proteins. Due to high efficiency in generation of indel mutations, NHEJ is often the obvious choice for CRISPR-Cas9 genetic knockout initiatives. However, it is a time-consuming and error-prone process to target the indels accurately given that there is little control over the exact location or length of indels created using this method.
- Microhomology-mediated end joining (MMEJ) is a less commonly occurring repair mechanism in cells [22]. This pathway is known to be active only in the S (DNA synthesis) and G2 (cell growth) phases of the cell cycle. Unlike the NHEJ pathway, MMEJ depends on regions of homology at the repair site. However, MMEJ remains prone to error and results in the deletion of various lengths of both ends at DSBs. This deletion causes chromosomal disruptions, including translocations and rearrangements. The role of MMEJ is not entirely known and is currently being investigated.

Of these mechanisms, HDR provides the most accurate repair of DSBs because of its requirement for higher sequence homology between the damaged DNA donor DNA strands. This requirement is paramount to prevent the introduction of specific mutations at damaged DSB sites.

Applications of HDR Pathways in Gene Editing for CF

There are different HDR pathways used to repair DSBs that are categorized as either conservative or non-conservative [23]. The conservative HDR pathways are less error-prone because they require a homologous template for DSB repair. They include classical double strand repair (DSBR), synthesis dependent strand annealing (SDSA), and break induced repair (BIR). The non-conservative pathway is a single-strand annealing pathway (SSA) that relies on flanking repeat sequences rather than a homologous template from the sister chromatid and is therefore error-prone. Gene editing methods such as Zinc finger nucleases (ZFNs), TAL effector nucleases (TALENs), and CRISPR can direct nucleases encoded on plasmids to induce specific DSBs.

The HDR pathway is optimal for gene editing tools because of its accuracy and success rate compared to NHEJ. However, the HDR pathway naturally occurs in 1 out of 10^6 repair events. Therefore, it is difficult to exploit this pathway for experimental gene therapy [24]. However, sequence replacement happens at a higher rate at specific sites when DSBs are nuclease-induced. With the current technological advancements in the realm of genome editing, HDR can be induced in cells that are resistant to gene replacement techniques. Therefore, HDR is a preferable choice for repairing genetic mutations.

Designing an HDR template is a vital step to creating the required amount of homology around the gene-editing target sequence. Homology arms that begin at the induced DSBs have been proven to be the most successful in ensuring close proximity between the target site and the DSBs. However, once CRISPR induces DSBs, the Cas9 enzyme will continue to cleave the target, resulting in several iterations of cutting and repairing as long as the genomic RNA (gRNA) target site and the protospacer adjacent motif (PAM) sequence is intact. This repeated editing can pose problems if the intent is to introduce a very specific mutation or sequence. However, repeated editing can be prevented by mutating either the PAM sequence or the gRNA sequence after the first DSB is induced and repaired. As the most predominant DSB repair mechanism, NHEJ can be inhibited to promote HDR.

Figure 4. An HDR template for the repair of the AA508 deletion mutation on CFTR. Sequence was derived using Benchling (benchling.com).

A single type II deletion mutation of AA508 is known to cause 70% of the mutant CFTR genes [25]. CRISPR-Cas9 can be utilized to repair this mutation by restoring the function of the CFTR chloride ion conductance channel. In addition, CRISPR-Cas9 has been utilized for repairing the CFTR locus through homologous recombination (HR)—a type of HDR—in intestinal stem cells of CF patients [26]. The HDR template is depicted in Figure 4. Through repair of the mutated F508 gene in two CF patients, function was restored in the organoid system [26]. HDR was also shown to be optimized when Cas9 protein is introduced into cells as a ribonucleoprotein (RNP), a complex with sgRNA, through adeno-associated viral vectors [27], which can stimulate HR. These components are discussed below. The RNP complex also generally results in fewer instances of off-target cutting, emphasizing the importance of this

method, combined with efficient donor delivery by AAV [27]. Therefore, CRISPR-Cas9 is a viable tool to induce guided HDR for gene therapy applications with the promise to advance CF clinical research and therapy

Viral and Non-Viral Delivery

To deliver gene editing therapeutics to the desired target sequence for techniques such as prime editing, gene therapy vectors are used as delivery mechanisms to protect the genes being transported, target the appropriate target cells, and perform delivery in a safe and effective manner. Vectors used for clinical gene therapy are classified as either viral or non-viral. Viral vectors are modeled after pathogenic viruses, which introduce their genetic material into a host cell and can cause detrimental effects in the host organism. However, viral vectors are modified to be non-pathogenic, and instead introduce therapeutic genetic material for a specific effect in the organism. As these vectors are modeled after highly efficient pathogenic viruses capable of quickly commandeering a host cell's mechanisms, viral vectors are used more widely in clinical trials than non-viral vectors [28]. Non-viral vectors have lower production costs and more favorable safety profiles [29]. However, these methods have lower transduction efficiencies and less accurate targeted delivery [30]. Lower transduction efficiency decreases the overall efficiency of modifying genes, and less specific targeted delivery leads to potential off-target effects when non-viral vectors are used.

Widely used non-viral technologies include plasmids and solid lipid nanoparticles. Plasmids are circular DNA molecules outside of the chromosome that can be engineered to contain designer DNA sequences, making them a useful tool for gene editing. Their independent replication, small size, and large quantity relative to host chromosomes make them amenable to the implementation of recombinant DNA. However, despite their convenience, they are associated with increased off-target effects because of their stability and persistence in host cells [30]. Solid lipid nanoparticles (SLNs) have the capability to deliver large amounts of nucleotides to cells and provide protection from nuclease degradation. SLNs can be produced in large quantities and remain stable in an organism, and are an alternative to other less affordable and stable colloidal carriers. They consist of a lipid core that solubilizes lipophilic molecules, an emulsifier, and a solvent. However, SLNs have limited DNA loading capacity and lower transduction efficiency than viral vectors [28, 29, 30].

Viral Vectors

Viral vectors can integrate genetic material into a cell's chromosomes, have high delivery efficiency, and can be used for applications such as stable protein overexpression [28, 30] (Figure 5). In addition, they are useful for targeting cells that are difficult for other delivery methods to target, specifically non-dividing cells. Vectors that have been selected for clinical research are genetically stable in order to preserve efficacy and target specificity. Viral vectors that are used in vivo, or within an organism, are optimized for low antigenicity in order to avoid activating the body's immune response [30]. They are also replication defective because the therapeutic gene replaces viral genes that are essential for replication [31]. Although viral vectors may pose risks, such as being inserted into the incorrect genomic location and causing mutations, animal testing and other precautions that are taken before human trials, and optimization for low antigenicity and genetic stability can reduce risk of such issues [30]. A broad spectrum of viral vector types is utilized for gene therapy, including both DNA and RNA viruses. Common viral vectors and their advantages and disadvantages are summarized in Tables 1. and 2.

Avoiding an immune response remains a vital consideration in regards to viral vector gene therapy. Although certain types of viral vectors reduce the risk of such a response, viral vectors retain many structural components of their pathogenic counterparts. In fact, the immune system is often unable to distinguish between the pathogenic virus and an unharmful viral vector. As the vector enters a host cell, it releases its genome which can then trigger an innate or adaptive immune response if it is mistaken for a pathogen. Cytokine-mediated toxicity and cytokine storm are possible risks of provoking such a reaction [32], resulting from severe cytokine production from an innate immune

response to repeated high doses of adenovirus-based vectors in immunocompromised individuals. To avoid such complications, various strategies may be employed, such as increasing transduction efficiency, blocking costimulation of lymphocytes, or depletion of T and B cells with antibodies [33]. Furthermore, viral vectors such as adeno-associated viruses may be employed, which are associated with a decreased likelihood of eliciting a substantial immune response.

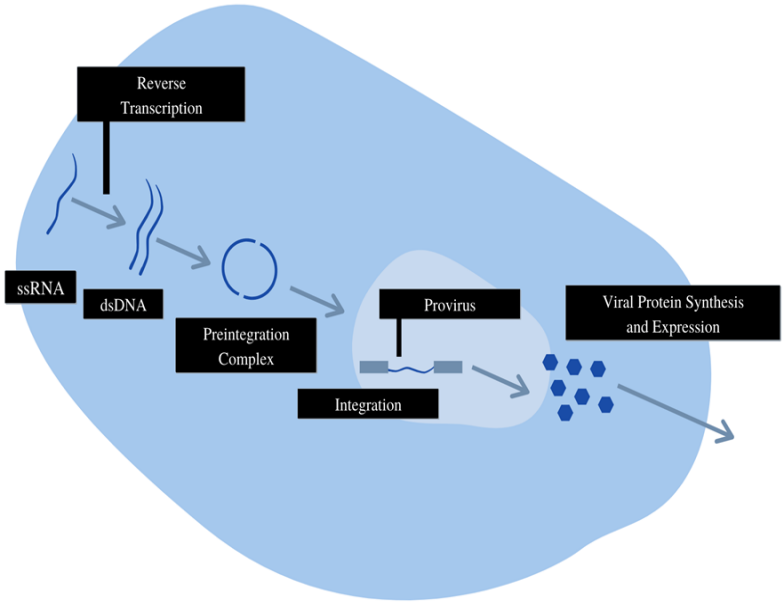


Figure 5. The process of integration and expression in viral delivery

Retroviral Vectors

Retroviruses are RNA viruses that convert their own RNA genome into double-stranded DNA to be inserted into a target cell genome. Retroviral vectors provide effective genome editing in human somatic cells. The retrovirus contains three open reading frames to be translated to proteins, including group-specific antigen (gag), envelope (env), and polymerase (pol) [34] (Figure 6). The gag polyprotein encodes the structural proteins of the virus, env codes for coat proteins that allow macromolecule transfer, and pol codes for enzymes such as reverse transcriptase. The reverse transcriptase enzyme is pivotal in reverse transcription, the process of deriving a DNA sequence from the RNA retrovirus genome. The next step involves the pre-integration complex, a nucleoprotein that forms to allow the transcribed viral DNA sequence to enter the nucleus without disrupting the nuclear membrane. Integrase, another enzyme that pol encodes, is responsible for incorporating this DNA sequence into the host cell's chromosomes. After the incorporation of the proviral DNA sequence into the chromosome, the cell will replicate the viral DNA during cell division.

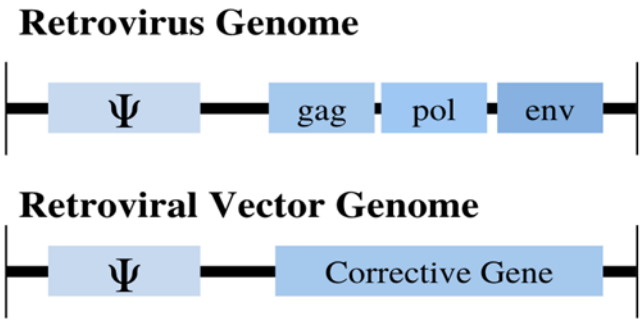


Figure 6. Retrovirus and retroviral genomes

In order to transform the pathogenic retrovirus into a viral vector for genome editing, a packaging cell is introduced that "packages" the retroviral vector for delivery. Packaging cell lines have proteins that are necessary for synthesizing capsids and for the maturation of virions to optimize them for reinfection. The retroviral vector contains the edited gene sequence in place of the gag, env, and pol polyproteins, ensuring safety and proper incorporation in the target host cell.

Despite the ability of retroviral vectors to efficiently enter mammalian cell types, disadvantages include insertional mutagenesis, which may disrupt the functions of functional genes and cause issues, such as uncontrolled cell division [35]. However, technologies such as zinc finger nucleases can be used to direct the retroviral vector to the correct sequence and avoid off-target editing.

Lentiviral Vectors

Lentiviruses are also RNA viruses and a subset of retroviruses. However, unlike retroviruses, which can only infect dividing cells, lentiviruses can be used for genome editing in both dividing and non-dividing cells with high efficiency, making them viable alternatives to retroviral vectors. Notable examples of lentiviruses are Human Immunodeficiency Virus (HIV) and Simian Immunodeficiency Virus (SIV), the latter of which impacts non-human primates. The unique and high-performing qualities of lentiviral vectors make them useful for remedying deletion mutations and introducing new genes. In clinical trials involving mice, lentiviral vectors produced positive therapeutic results for hemophilia and diabetes. Another potential application of lentiviral vectors is gene introduction into T-cells to provide heightened immunity [16].

Adenoviral Vectors

Adenoviruses are DNA viruses, where, unlike retroviruses, their DNA is not incorporated into a target cell's genome and instead separately stays in the nucleus of the cell [31]. When the cell replicates, the viral DNA is not incorporated in the daughter cell genomes. In gene therapy, these characteristics require that the vector be re-administered as cell division occurs and the population increases. Adenoviruses have three exterior structural proteins; fiber, hexon, and penton base. The hexon protein is a coat protein that plays a role in capsid structure by forming 240 homotrimers (proteins that consist of 3 polypeptides) to encapsulate the virus. In order for the virus to enter a host cell, the fiber protein's knob receptor must bind to the cell receptor. Cellular integrin αV, which facilitates the attachment of cells to the extracellular matrix, then interacts as a co-receptor for the penton base protein. A co-receptor is a cell surface protein that binds a ligand molecule along with other signaling molecules. Binding to this molecule causes cell signaling that results in the engulfment of the virion into the target cell in an endosome, an organelle in the cytoplasm that brings materials into the cell. After the virus enters the target cell, chemical changes ensue because of the acidification of the endosome, including viral capsid disassociation, leading to the virion being released into the cell's

cytoplasm. Subsequently, the virion is transported through the nuclear pore complex, where the adenovirus structure disassembles. The viral DNA is then released into the nucleus and associates with histone proteins in the chromosomes, allowing the viral DNA to be associated with the host cell DNA without direct integration into it.

There are three generations of adenoviral vectors (AdV) with different capabilities and transformation processes [36]. First-generation AdVs have the E1 and E3 regulatory genes removed, prohibiting the virus from replicating or being produced anywhere except in mammalian cells. First-generation vectors quickly trigger an immune response during in vivo expression. Second generation AdVs have the E1, E2, E3, and E4 regulatory genes removed, resulting in increased DNA packaging capacity and reduced immunogenicity. Third generation AdVs are also known as gutless AdVs because all viral sequences are removed. These have extremely high DNA packaging capacity. However, these gutless vectors are technically difficult to produce. Adenoviral vectors are highly efficient for targeting cancer. In fact, the first clinically approved gene therapy product, Gendicine, is an adenoviral vector for the treatment of cancer.

Adeno-Associated Viral Vectors

Adeno-associated viruses (AAV) are also DNA viruses but are nonpathogenic and do not elicit an immune response when used for clinical treatment [37]. AAV vectors have a low probability of insertional mutagenesis and result in stable expression of the transgene. Although they have low DNA carrying capacity and are complex to produce, they are useful for gene delivery in non-dividing cells. The AAV genome consists of inverted terminal repeats (ITRs) on each end, single-stranded DNA, and the rep and cap open reading frames. The rep open reading frame encodes the genes necessary for the AAV life cycle, and the cap open reading frame encodes structural capsid proteins. The ITR sequences are necessary for the virus to be incorporated into host cell DNA and for the virus to multiply. They are symmetric and consist of 145 base pairs each. The AAV has a naked icosahedral structure that makes virus purification efficient.

To develop AAV vectors for gene therapy, the rep and cap open reading frames are removed to minimize the possibility of viral DNA integration into a random location in the host cell's genome [38]. AAV vectors have been implemented successfully in clinical trials of cystic fibrosis and hemophilia B treatments.

Table 1. Comparison of Viral Vectors [28-30, 38, 39]

Method	**Adenovirus**	**Lentivirus**	**Retrovirus**	**AAV**
Genome Type: DNA or RNA	DNA	RNA	RNA	DNA
Gene Expression	Transient	Transient or Stable	Stable	Transient

Integration into Target Cell Genome?	No	Yes	Yes	No
Immune Response in Target Cell	High	Low	Moderate	Very Low
Transduction Efficiency	High	Moderate	Moderate	Moderate
Infects Dividing Cells	Yes	Yes	Yes	Yes
Infects Non-Dividing Cells	Yes	Yes	No	Yes

Table 2. Advantages and Disadvantages of Viral Vectors

Adenoviral vectors	
Advantages	Disadvantages
Highly efficient gene transfer: ● Relatively large therapeutic genes. ● Very high expression of genes due to the delivery of a large number of genome copies per target cell High fidelity of gene transfer: ● Vector genomes are genetically stable.	Transient Expressions and Transfer: ● Adenoviruses are non-integrating. Therefore, expression is short-lived (1–2 months in non-dividing cells) and even shorter in dividing cells.
Extensive clinical testing	Vectors are lethal when given in high doses
Effective insertional mutagenesis	Vectors trigger immune responses and trigger inflammation
Low immune response in *vivo*	Transgene delivery and expression is

	significantly reduced in individuals with a pre-existing immunity against adenoviruses
Adeno Associated Vectors (AAV)	
Advantages	Disadvantages
Highly efficient gene transfer: ● Very high expression of genes due to the delivery of a large number of genome copies per target cell High fidelity of gene transfer: ● Vector genomes are genetically stable ● Integrate with target cell genomes ● Stable gene expression	Small transgenes In some settings when vectors do not integrate, transfer and expression are not stable
Generally stable	
Lentiviral vectors	
Advantages	Disadvantages
Stable and efficient gene transfer: ● High transduction rates up to 90% of hematopoietic stem cells (HSCs) ● Extensive clinical experience ● High transgene expressions	The imitations of precision transgene insertion cause uncertainty and safety concerns Insertion mutations may lead to the activation of protooncogenes (cancer-causing genes), increasing the risk of cancer.
The absence of chromosomal rearrangements and intact integrations promote high fidelity gene transfer.	
Lentiviral vectors have high safety levels with minimal immunogenicity	
Lentiviral vectors induce transgene expression more rapidly than single-stranded AAV	
Retroviral Vectors	
Advantages	Disadvantages

High fidelity of gene transfer: ● Long-lasting gene expression ● Efficiently enters the target cell	Only infects dividing cells Low production yield

Next-Generation Viral Technologies

While retroviral, lentiviral, adenoviral, and adeno-associated vectors are useful tools for gene delivery, there are many potential improvements. Next-generation viral technologies include adeno-associated virus mutagenesis, nanoblades, and lentiviral vector-based insertional mutagenesis. These are reviewed below.

Adeno-Associated Viral Mutagenesis

AAV capsids are used to deliver reagents for gene editing and have many therapeutic applications. Therefore, next-generation methods are currently being explored to further improve this tool's accuracy and efficacy in genome editing. In the VP1 structural component of AAV, membrane associated accessory protein (MAAP) is a frameshifted open reading frame encoded protein that hinders AAV production, reducing the system's overall efficiency [40]. MAAP is associated with the cell membrane and impacts AAV production through competitive exclusion. Consequently, mutations to this protein are needed as an inhibitor of functional MAAP to eliminate competition with AAV. Therefore, AAV-containing MAAP mutations exhibit stronger tropism in vivo and are more useful for targeting specific tissues [40]. Implementing a machine-guided strategy to foresee potential mutations is a viable solution for this issue.

Nanoblades

Nanoblades are a CRISPR tool used in gene editing to cut DNA strands with precision. They are virus-like particles (VLPs) that are based on the murine leukemia virus. VLPs cannot replicate and do not have the same pathogenic effects as viruses [41]. Nanoblades consist of viral structural proteins embedded in membranes, exemplified by the Gag protein of the mouse leukemia virus, which is utilized in conjunction with SpCas9. A protease is included in this system in order to break down VLP proteins to release Cas9, a guide RNA to provide specificity to the target sequence, and viral envelope proteins, which protect the genetic material being delivered and facilitate transport of the vector.

Compared to traditional CRISPR-Cas9 gene-editing technologies, nanoblades have fewer off-target effects in the clinical setting and higher efficiency for gene delivery. Nanoblades are also easy to produce and can be used in a variety of situations, including injecting genetic material into a zygote. Recent literature explores potential applications of nanoblades, such as genome editing in human T, B, and $CD34^+$ cells [42] and transfer of Cas9-sgRNA ribonucleoproteins in both in vitro and in vivo delivery [43], and suggests promising prospects for future advancements in this research domain.

Nanoblades have also shown promise in targeting the CFTR gene in mouse colon organoids [44]. Gene knockout, or inactivation, was achieved with high accuracy, proving that nanoblades are an accurate approach to targeting this gene. Additionally, low toxicity was reported as a result of this gene editing, underscoring the promising potential of nanoblades in gene editing with applications to CF.

Conclusions

Both prime editing and HDR are viable systems for repairing the deletion mutation that causes cystic fibrosis. Prime editing avoids indels that may be caused by HDR and NHEJ because of the creation of single stranded DNA breaks instead of double stranded breaks. Prime editing is precise because of the use of a PAM sequence, and it allows for

the repair of all types of mutations. Prime editing's off-target effects are minimal compared to the traditional CRISPR/Cas9 system because of the 3 iterations of DNA binding that occur: between the DNA and the guide RNA, between the binding site and the DNA, and between the nicked DNA and pegRNA. However, prime editing is a fairly new technology that has not been implemented for large edits. While prime editing represents the more efficient and accurate method, more clinical research must be performed before its applicability to the repair of the CFTR mutation can be fully determined. The specificity of HDR owes to the presence of an HDR template, ensuring precise DSB repair. HDR is currently more accessible than prime editing and has been studied thoroughly for the repair of double stranded breaks after gene editing.

For gene delivery we have reviewed and compared retrovirus, lentivirus, adenovirus, and adeno-associated virus vectors. AAV vectors are by far the most specific and are likely to be the most effective in repairing the cystic fibrosis mutation. AAV vectors are nonpathogenic, and because the rep and cap open reading frames are removed, the viral DNA will not integrate into the host genome. AAV vectors have a low probability of insertional mutagenesis and are genetically stable. In addition, a large number of genome copies per cell leads to high levels of gene expression, optimizing gene delivery for cystic fibrosis gene therapy.

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