

# Chapter 2

## Combining Engineered Nucleases with Adeno-associated Viral Vectors for Therapeutic Gene Editing

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**Abstract** With the recent advent of several generations of targeted DNA nucleases, most recently CRISPR/Cas9, genome editing has become broadly accessible across the biomedical community. Importantly, the capacity of these nucleases to modify specific genomic loci associated with human disease could render new classes of genetic disease, including autosomal dominant or even idiopathic disease, accessible to gene therapy. In parallel, the emergence of adeno-associated virus (AAV) as a clinically important vector raises the possibility of integrating these two technologies towards the development of gene editing therapies. Though clear challenges exist, numerous proof-of-concept studies in preclinical models offer exciting promise for the future of gene therapy.

**Keywords** AAV • Gene therapy • Gene editing • CRISPR/Cas9 • Zinc-finger nuclease

### Abbreviations

AAV	Adeno-associated virus
CCR5	C-C chemokine receptor type 5
CRISPR	Clustered regularly interspaced short palindromic repeats

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crRNA	CRISPR targeting RNA
DMD	Duchenne's muscular dystrophy
Dmd	Dystrophin
Fah	Fumarylacetoacetate hydrolase
FIX	Coagulation factor IX
HBV	Hepatitis B virus
HDR	Homology-directed repair
HSC	Hematopoietic stem cell
NHEJ	Non-homologous end joining
OTC	Ornithine transcarbamylase
PCSK9	Proprotein convertase subtilisin/kexin type 9
SaCas9	<i>Staphylococcus aureus</i> Cas9
sgRNA	Single guide RNA
SpCas9	<i>Streptococcus pyogenes</i> Cas9
TALE	Transcription activator-like effector
TALEN	Transcription activator-like effector nuclease
tracrRNA	Trans-activating crRNA
ZF	Zinc finger
ZFN	Zinc-finger nuclease

## 2.1 Introduction

Gene therapy, the treatment of disease via the delivery of genetic material to cells, has enabled incurable diseases to now be considered as therapeutic targets, including both monogenic diseases with well-defined underlying genetic etiology as well as idiopathic diseases with candidate gene targets. Throughout most of its history, the major barrier to gene therapy has been delivery. A major advance has been the development of safe and effective delivery vectors, and the most prominent for *in vivo* gene therapy have been based on adeno-associated viruses (AAV). Natural AAVs offer reasonable infectivity, a lack of pathogenicity, numerous variants with different tissue tropisms, and negligible genomic integration. As a result, vectors based on AAV have begun to show increasing clinical promise, primarily in studies involving gene augmentation where additional copies of genes are delivered to either replace the functionality of null alleles in recessive diseases or to overexpress a potentially therapeutic factor. In particular, AAV has been successful in trials for monogenic recessive disorders including Leber's congenital amaurosis type 2 (LCA2) [1, 2], hemophilia B [3, 4], spinal muscular atrophy [5], and lipoprotein lipase deficiency [6, 7]. The last of these is the basis for a clinically approved gene therapy product in the European Union, and it is anticipated that a gene therapy for LCA2 may be approved in the US in 2017. In addition, early-stage clinical trials have demonstrated some positive signs in harnessing AAV to treat more complex disorders, such as overexpressing SERC2A in heart failure patients [8] and

expressing the vascular endothelial growth factor (VEGF) inhibitor sFLT-1 in patients with age-related macular degeneration [9].

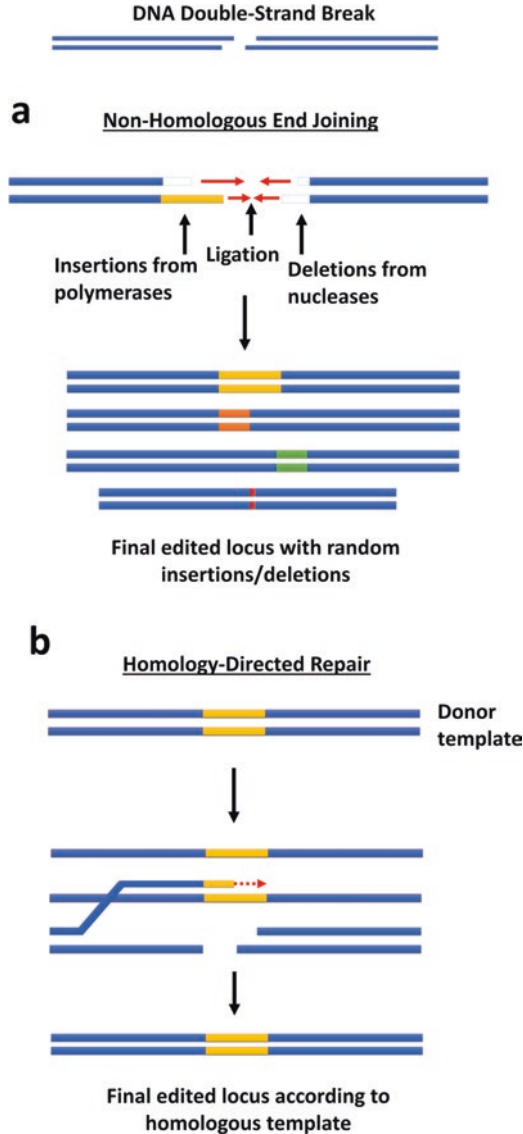
While gene therapy is thus gathering increasing momentum, particularly for monogenic diseases, a number of disorders are not amenable to gene augmentation therapy. For instance, autosomal dominant genetic diseases require the elimination or modification of the disease-causing allele. In addition, AAV has a limited carrying capacity of <5 kb [10], and mutated genes whose cDNAs exceed this threshold require alternate approaches. Furthermore, while non-integrating vectors like AAV might be safer than integrating vectors, they can be insufficient to treat disease requiring gene delivery in actively mitotic cells due to progressive dilution of the delivered extrachromosomal genetic cargo with each cell division [11].

Gene-editing technology in the form of targeted nucleases, with the capacity to directly and permanently edit and modify the cellular genome, can potentially address such challenges. For example, these nucleases may offer the capability to specifically eliminate dominant disease alleles, correct endogenous genes, or integrate exogenous genes at safe harbors, resulting in permanent changes that are heritable in mitotic cells. These approaches could be applied for direct *in vivo* therapy, and AAV's potential for high delivery efficiency coupled with the enhanced efficacy of AAV genomes as DNA donors for homology-directed repair also offers the capacity for *ex vivo* modification of cells for subsequent engraftment. Coupling the potential of gene-editing technology with the increasingly well-established, safe, and effective gene delivery capabilities of AAV may thus render new classes of genetic diseases accessible to gene therapy.

## 2.2 Therapeutic Gene Editing

Targeted gene editing has two primary goals—disrupting a sequence or introducing a precisely defined modification to a sequence—and both strategies begin with generating a DNA break at the locus of interest. For disruption, the non-homologous end joining (NHEJ) cellular repair mechanism directly rejoins the two ends and typically introduces small insertions or deletions (indels) at the cut site [12]. When placed near the 5' end of a coding sequence, such indels generally disrupt the reading frame and thereby effectively knock out the target gene. For precise modification, a DNA template containing both the desired modification and flanking regions of DNA homologous to the target area, known as homology arms, is co-delivered with the nuclease. The homology-directed repair (HDR) pathway can then splice the template in place of the damaged DNA within the region between the homology arms, thereby mediating specific gene modification (Fig. 2.1) [13, 14].

Both strategies require a means to generate targeted DNA strand breaks, and the first such engineerable tool was zinc-finger nucleases (ZFNs). An “alphabet” of individual zinc finger (ZF) DNA binding domains that bind to specific three-nucleotide targets was identified; these ZFs could then be modularly assembled to target new



**Fig. 2.1 Cellular mechanisms of DNA repair following DNA double-strand break.** When a double-strand DNA break occurs, one of two cellular mechanisms repairs the damage. **(a)** In non-homologous end joining, polymerases and nucleases clean up the damaged ends by adding or deleting small numbers of nucleotides until they can be rejoined by ligases. The final ligated product contains small insertions or deletions (indels) at the damage site, often resulting in a frameshift. **(b)** In homology-directed repair, the 3' overhang strand at a site of DNA damage can displace a strand in a separate DNA duplex with homology to that strand (a donor template). Polymerases extend the damaged end according to the homologous template DNA duplex, and the strand either returns to its original complementary strand, annealing to the other original damaged end, or the donor template strand and the previously damaged strand can undergo complete crossover and recombination. Either option results in a repaired strand

desired genetic loci and fused with *FokI* nuclease domains to yield custom nucleases [15, 16]. This advance opened the door not only to a broad range of important basic research applications but also to the potential capacity to treat disorders including HIV infection [17], hemophilia [18], sickle cell anemia [19], and others. For instance, the use of ZFNs to knock out the HIV co-receptor CCR5 within T cells, and thus render them resistant to HIV infection, is currently in clinical trials [17]. While established ZFNs are indeed quite effective enzymes, generating new nucleases is difficult since the target specificity of each individual ZF domain can depend on the context of its neighboring domains [20], requiring a high level of expertise, ZFN library selection methods, and thus a time-consuming process to generate specific ZFNs for new targets.

In 2009, the DNA binding domains of the transcription activator-like effector (TALE) class of bacterial transcription factors was found to consist of modular elements [21, 22]. Excitingly, these individual TALE domains were found to bind single nucleotides with strong specificity and, importantly, with minimal context dependence, unlike ZFNs. Thus, TALE DNA binding domains could be linked together with near-ideal modularity to target virtually any desired DNA sequence, and fusion to a *FokI* nuclease domain yielded TALE nucleases (TALENs) [23]. Simple assembly kits made the generation of new functional TALENs rapid and accessible to researchers. That said, the resulting TALEN constructs were very large and thus challenged the carrying capacity of delivery vectors like AAV, and the repetitive nature of the TALEN coding sequence led to concerns with recombination in the context of these ssDNA viral vectors.

While the simplicity of TALENs seemed unlikely to be surpassed, in 2012, it was demonstrated that the bacterial anti-viral adaptive immune mechanism known as the CRISPR/Cas9 system [24–26] could be re-engineered for targeted gene editing [27], a finding that was subsequently applied for genome editing in human cells [28–30]. Three components of the system from *Streptococcus pyogenes* are necessary and sufficient for enzymatic activity: the CRISPR-associated protein 9 (Cas9) nuclease; the CRISPR targeting RNA (crRNA) that is complementary to a target DNA sequence; and the trans-activating crRNA (tracrRNA) that hybridizes with a crRNA, enables it to bind to Cas9, and helps direct cleavage activity to the encoded locus. Fusing the two RNA components into a single guide RNA strand (sgRNA) further simplified the system such that virtually any desired target strand of DNA could be targeted and cleaved by simply changing the targeting RNA sequence, limited only by the requirement for a small adjacent sequence known as the protospacer-adjacent motif. With this discovery, DNA cleavage and editing no longer required even simple modular protein assembly but merely modification of ~20 nucleotides of the targeting sgRNA. The simplicity and efficacy of the resulting CRISPR/Cas9 system make effective gene editing broadly accessible.

With the potential to effectively modify virtually any locus, CRISPR/Cas9 gene editing offers promise for both *in vitro* and *in vivo* genome editing. Successful application of this work for therapeutic purposes, however, will hinge upon an effective and reliable method for delivering the CRISPR/Cas9 machinery to affected cells.

### 2.2.1 AAV

Adeno-associated virus (AAV) is the most clinically successful *in vivo* gene therapy vector to date. AAVs are a family of non-enveloped, single-stranded DNA viruses that naturally require the presence of a helper virus, such as an adenovirus, to replicate. The 4.7 kb AAV genome contains two short (~150 nucleotide) viral inverted terminal repeat sequences (ITRs) flanking two genes, *rep* and *cap*, encoding proteins for replication and capsid formation, respectively. Because these genes can function in trans, the virus can be engineered for assembly of virus particles with recombinant genomes containing only the desired genetic cargo flanked by the ITRs. AAV has numerous natural serotypes with somewhat different viral capsid sequences and tissue tropisms, indicating that differences in the viral capsid proteins can lead to differences in infectivity. As a non-integrating virus with a strong safety record, AAV has strong promise as a clinical gene delivery vehicle, and, as mentioned above, there are numerous examples of strong proofs of concept in clinical trials as well as one regulatory approval in the European Union [1–9].

In addition to delivering DNA sequences for direct expression, the single-stranded nature of the AAV genome can serve as an effective template that inherently stimulates the homologous recombination pathway to mediate gene targeting [31]. Specifically, viral delivery of a HDR construct with homology to a chromosomal locus, even without a nuclease component, can result in recombination into the target locus at a rate of up to 1% [32], a rate >1000-fold higher than conventional plasmid donors [33] or other viral vectors [34]. Successful AAV-mediated gene targeting has been achieved in neural stem cells [35], human pluripotent stem cells [36], and hematopoietic stem cells (HSCs) [37], among other cell types. In addition, as discussed below, combining AAV with a nuclease offers even stronger potential.

Despite its success, AAV has significant challenges as a gene therapy delivery system. Natural serotypes of AAV are inefficient at infecting many target cells and tissues, do not have the capacity for targeted delivery to specific cells, and can be neutralized by antibodies prevalent within the human population due to prior natural AAV exposure. Additionally, the packaging capacity of AAV is approximately the size of its wild-type genome (4.7 kb), and cargos that are greater than 10% beyond this size are not possible to package [10]. Finally, while a non-integrating virus offers the potential for lower genotoxicity and thus greater safety than an integrating one, the lack of a specific mechanism for vector integration means that the cargo will be diluted over time in mitotic target cells, and treating diseases with a cargo that must persist for efficacy is thus more difficult.

Different approaches have been taken to address these challenges. For example, directed evolution—or the generation of large AAV variant libraries and iterative selection *in vitro* or *in vivo* for enhanced gene delivery properties—has generated novel AAV variants with greatly improved delivery efficiencies for a range of applications and targets [38]. These include enhanced delivery to lung epithelium in human organotypic culture tissue [39] and a pig model of cystic fibrosis [40],

enhanced retrograde transport for targeting specific neuronal populations *in vivo* [41], greatly improved biodistribution to target tissues such as outer retinal photoreceptors upon simple administration to the vitreous [42], and other applications. In addition, a large cargo can be delivered in AAV by packaging fragments of a gene in two separate vectors, and the full product can then be reconstituted *in vivo* via trans-splicing or homologous recombination of the two separate vectors [43], though with a significant decrease in overall efficiency. At any rate, the potential for highly efficient natural and, in particular, engineered AAV delivery to therapeutically relevant targets makes it a strong choice for gene therapy, including for therapeutic applications of CRISPR/Cas9.

### 2.2.2 Nucleases and AAV for Therapeutic Gene Editing

One major focus of gene editing has been *ex vivo* engineering of cellular therapies, in which a specific patient's cells are harvested, edited, and re-engrafted. Compared to a direct *in vivo* therapy, more *in vitro* delivery options are available. As a prominent example, CD4<sup>+</sup> T cells harvested from HIV-infected patients were edited to disrupt the *CCR5* locus and thereby confer resistance to HIV infection, followed by reintroduction into patients. This approach has been implemented with both ZFNs and TALENs, and the ZFN-based approach—in which the nuclease was delivered with an adenoviral vector—is currently in clinical trials in which the engineered cells were shown to persist following administration [17]. In addition to *CCR5* disruption, this *CCR5* locus has been edited within HSCs via AAV donor template delivery and the ZFNs transiently expressing through mRNA electroporation [44].

In addition to *CCR5* disruption for HIV [45, 46], therapeutic treatment of  $\beta$ -globinopathies [47] such as sickle-cell disease and  $\beta$ -thalassemia [48, 49] has been explored. *Ex vivo* cell therapy thus has strong potential to address an unmet medical need, though efforts are currently focused predominantly on the hematopoietic system since its cells can readily be harvested and cultured. *In vivo* delivery will be needed to address most other tissue targets.

For *in vivo* editing, AAV's packaging capacity posed initial challenges for CRISPR/Cas9 delivery, as the combined size of the initially best-characterized *Streptococcus pyogenes* Cas9 (SpCas9), the sgRNA, and promoters for each was simply too large to fit into a single AAV vector. However, two primary approaches for utilizing AAV as a CRISPR/Cas9 delivery vector have since emerged. Since the initial discovery and characterization of SpCas9, thousands of CRISPR/Cas9 proteins have been identified [50], many of which are significantly smaller than SpCas9. The best-characterized alternative Cas9 protein, derived from *Staphylococcus aureus* (SaCas9), is nearly 1 kb shorter than SpCas9 and can thus be accommodated along with its sgRNA in AAV [51]. Other non-Cas9 CRISPR proteins, such as Cpf1 [52], offer new binding and cleavage characteristics in addition to being more compact. With these smaller CRISPR/Cas9 proteins, the entire system can fit comfortably in a single AAV vector, though there are still inflexible limitations on the



maximal size of promoters. As an alternative, some studies have packaged SpCas9 and the sgRNA in separate vectors for co-administration [53]. This approach is particularly useful for HDR-modification applications, where, for example, one vector could be used to deliver the nuclease and sgRNA and a second vector the HDR template.

Both the use of smaller Cas9s and the dual vector approach have been successfully implemented *in vivo* for an increasing number of applications, both to disrupt endogenous gene expression as well as to precisely correct disease alleles. In early 2015, SaCas9 and its sgRNA were combined in a single AAV8 vector to disrupt and thereby knock out expression of a cholesterol regulatory gene, proprotein convertase subtilisin/knexin type 9 (PCSK9), in the adult mouse liver [51]. The result was reduced circulating cholesterol levels.

Later in 2015, another group demonstrated the ability to correct the ornithine transcarbamylase (OTC) locus, a gene responsible for a potentially life-threatening metabolic disease, in the liver using two vectors [54]. One AAV8 vector contained SaCas9, and the second harbored the sgRNA along with the HDR repair template. Co-delivery successfully corrected a mutation in ~10% of the cells within neonatal mouse liver, leading to significantly greater survival of affected mice.

Two-vector systems have also been successfully used for targeted gene disruption. In 2016, three research groups used either SaCas9 or SpCas9 in a two-vector system to disrupt an exon within the dystrophin gene that harbored a disease-causing mutation within a mouse model of Duchenne's muscular dystrophy (DMD) [53, 55, 56]. Loss of dystrophin expression in the corresponding human monogenic recessive disorder leads to progressive muscle degeneration. To restore expression of this essential protein, they targeted loci within the splice sites flanking the mutation-containing exon 23, and the resulting successful elimination of this non-essential exon from the mRNA led to a functional protein product. AAV was administered via several routes—including direct intramuscular injection, intravenous injection, retro-orbital injection, and neonatal intraperitoneal injection—which resulted in varying levels of functional dystrophin production in muscle tissue. While the fraction of muscle cells corrected was low, as in the OTC liver study, it was sufficient in these models of DMD to restore significant levels of muscle function.

A comparison of each of these gene editing strategies using AAV in combination with engineered nucleases is provided in Table 2.1.

### 2.2.3 Challenges

While gene editing therapy offers considerable promise, numerous challenges still must be overcome. First, there is a risk of engineered nucleases cutting unintended sites with imperfect but very close homology to the nuclease target site. Such off-target editing is well known to occur within *in vitro* contexts [57], and this risk can be further amplified by viral delivery methods such as AAV that can lead to persistent Cas9/sgRNA expression in non-dividing cells for durations far longer than



**Table 2.1** Therapeutic applications of gene editing via AAV combined with engineered nucleases

Disease	Target gene	Target cell/tissue	Nuclease used	AAV serotype used	Modification type	Reference	Note
HIV infection	CCR5	CD4+ T cells	ZFN	rAAV6	Disruption	[44]	Donor template only in AAV; nuclease mRNA
Hemophilia B	FIX	Hepatocytes	ZFN	rAAV8	Correction	[67]	
Cardiovascular/cholesterol homeostasis	PCSK9	Liver/hepatocytes	SaCas9	rAAV8	Disruption	[51]	
DMD	Dmd	Muscle	SpCas9	rAAV9	Exon excision	[53]	
		Muscle	SaCas9	rAAV9	Exon excision	[55]	
		Muscle	SaCas9	rAAV8	Exon excision	[56]	
Metabolic disease	OTC	Liver/hepatocytes	SaCas9	rAAV8	Correction	[54]	
Hepatitis B infection	HBV polymerase	HepAD38 cells	ZFN	rAAV2	Disruption	[68]	
Tyrosinemia	Fah	Hepatocytes/liver	SaCas9	rAAV8	Correction	[69]	Donor template only in AAV; nuclease mRNA

needed for the genome editing—an undesirable condition since the likelihood of undesired off-target cutting increases with nuclease residence time [58, 59]. This can be mitigated *ex vivo* through delivery of mRNA encoding the nuclease or even recombinant nuclease proteins [44], but these methods do not translate well to *in vivo* contexts.

Numerous approaches have thus been developed to reduce such off-target effects. One strategy is Cas9 protein engineering. For example, a mutant form of Cas9 capable of only nicking one strand of DNA, rather than cleaving both, was combined with two sgRNAs targeting opposite strands near the desired locus. The resulting paired nicks yielded double-stranded breaks that could be harnessed to generate indels or achieve HDR, but single nicks (such as at an off-target site that matches one sgRNA but not the other) instead lead to high-fidelity repair through the base excision repair pathway. The result is reduced off-target editing [60]. In another approach, rational modifications were introduced into Cas9 to reduce non-specific DNA contacts and thereby decrease binding affinity to non-specific targets without substantially affecting on-target editing rates [61, 62].

A third approach, based on the correlation between residence time and off-target activity, has been controlling the activity of Cas9 after delivery to minimize the total duration of its activity. One approach introduces inteins into the structure of Cas9 that only splice themselves out and generate active Cas9 in the presence of a small molecule ligand. By providing the small molecule for only a short duration, the activity window for editing can be reduced, thereby limiting off-target editing [63]. Another approach is the use of self-inactivating Cas9 vectors, where sgRNA target sites are engineered into the delivered viral genome itself to target the Cas9 expression cassette for destruction at the same time as targeting the desired genomic locus. The result is reduced residence time and off-target editing [64, 65].

While it would clearly be preferable to use a system with reduced off-target cutting, assessing the actual clinical risks of off-target modifications is challenging. *In vitro* assays that detect off-target cutting can be highly sensitive, such that only a subset of at-risk sites are actually cut *in vivo*. Furthermore, off-target modifications can be highly variable in location and sequence, and understanding how sequence changes translate to functional risk of an adverse event is very difficult. Future work may focus increasingly on functional assays of off-target cutting impact, such as cell transformation.

Persistent expression also raises the risk of an immune response to the expression of a bacterial protein in a human cell, which can result in immune elimination of therapeutically corrected cells. For example, expression of AAV-delivered SpCas9 in a mouse has elicited clear immune recognition, though the subsequent cellular damage in this animal model was minimal [66]. Methods of effective transient delivery, such as self-inactivating vectors, may reduce immune responses by limiting the time of exposure.

Efficacy *in vivo* is an additional challenge. While successes in the highly accessible liver bode well for future work, low editing rates in other tissues, while therapeutically sufficient for the strong work in the DMD model, raise concerns for diseases that may require greater levels of correction or for larger animals (or

humans) that tend to be more difficult targets for gene delivery than mouse models. Delivery therefore remains a major challenge, particularly *in vivo*, and improved delivery systems, including novel AAV variants engineered by directed evolution or rational design, are therefore needed for human gene therapy. Improving delivery efficiency to target tissues will increase the efficacy of AAV-mediated genome editing, and improving vector selectivity or targeting to these tissues can enhance the safety profile by reducing potential side effects in tissues unaffected by the disease.

## 2.3 Conclusion

The era of gene editing has transformed virtually every area of biology, and clinical gene therapy is among the most exciting. The advent of CRISPR/Cas9 has enabled readily engineerable, accessible, and effective gene editing, and this technology is positioned to combine with AAV vectors to assist with *in vitro* editing and to make *in vivo* clinical gene-editing therapy a reality. Addressing additional challenges in the field—including Cas9 target fidelity, Cas9 immunogenicity, and AAVs engineered for optimal gene delivery in the clinic—will enable next generation gene- and genome-editing therapeutics.

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