CRISPR-Based Therapeutic Genome Editing: Strategies and *In Vivo* Delivery by AAV Vectors

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The development of clustered regularly interspaced short-palindromic repeat (CRISPR)-based bio-technologies has revolutionized the life sciences and introduced new therapeutic modalities with the potential to treat a wide range of diseases. Here, we describe CRISPR-based strategies to improve human health, with an emphasis on the delivery of CRISPR therapeutics directly into the human body using adeno-associated virus (AAV) vectors. We also discuss challenges facing broad deployment of CRISPR-based therapeutics and highlight areas where continued discovery and technological development can further advance these revolutionary new treatments.

Numerous human diseases arise from mutations that diminish or damage gene products. Gene therapy as a strategy to treat genetic diseases was formally proposed in 1972 (Friedmann and Roblin, 1972), introducing the concept that "genes can be medicine." In the ensuing decades, implementation of this medical concept was met with initial excitement, serious setbacks, resurgence of interest, and more recently, clinical successes (Dunbar et al., 2018; High and Roncarolo, 2019). Despite these successes, however, delivering a functional gene copy to replace a mutated one is not a perfect solution for many diseases. For example, an exogenous gene copy lacks many regulatory elements that are important for endogenous gene expression and function. In addition, for gain-of-function pathogenic mutations, simply supplying a wild-type copy of the gene is ineffective. These and other limitations can be theoretically addressed by directly "editing" a mutated gene, thereby restoring gene function in its natural context.

Indeed, many of our cells carry out something like this thousands of times a day, using a variety of DNA repair mechanisms that guard genome integrity in response to DNA damage. Similar to DNA damage, a targeted double-stranded break (DSB) can also trigger these cellular repair mechanisms, mainly, nonhomologous end-joining (NHEJ) and homology-directed repair (HDR), which can potentially introduce DNA sequence changes during repair of the DSB. HDR, which is a templated process, allows for the introduction of specific DNA changes, a phenomenon that has been leveraged to achieve insertion of new DNA sequences (Thomas and Capecchi, 1987). HDR-mediated insertion, however, requires the presence of a correct template (which is missing in most natural cases of genetic mutations) and is generally less efficient than NHEJ.

More efficient methods for gene editing arose from the observation that a targeted DSB generated by an endonuclease can dramatically stimulate HDR in eukaryotic cells (Rouet et al., 1994; Smih et al., 1995). This observation spurred a quest for programmable and efficient endonucleases (Urnov, 2018), leading to the development of meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENS), and clustered regularly interspaced short-palindromic repeat (CRISPR)-associated (Cas) proteins (Gaj et al., 2013; Hsu et al., 2014), the latter of which has been most widely adopted in research. Fundamental to the popularity of Cas nucleases is that they are guided by a short RNA sequence that recognizes the target DNA sequence through Watson-Crick base pairing (Brouns et al., 2008; Jinek et al., 2012; Garneau et al., 2010; Gasiunas et al., 2012), whereas the other enzymes rely on protein-DNA interactions to achieve target specificity. Following the demonstration of CRISPR-Cas9-mediated genome editing (Cong et al., 2013; Mali et al., 2013), CRISPR-based technology has rapidly advanced, benefitting greatly from a collective endeavor to characterize, improve, expand, and share the CRISPR-based molecular toolbox (Doudna, 2020; Doudna and Charpentier, 2014; Zhang, 2019). In addition to accelerating basic research, CRISPR-based technology also holds enormous potential as a therapeutic, offering an approach to permanently correct disease-causing mutations.

Deploying CRISPR-based therapeutics directly into the human body holds great promise for treating numerous diseases. Although the CRISPR-based toolbox enables diverse operations ranging from DNA and RNA editing to gene expression modulation, delivery remains a bottleneck for therapy development. Currently, adeno-associated virus (AAV) vector is the leading platform for *in vivo* gene therapy delivery (Wang et al., 2019).
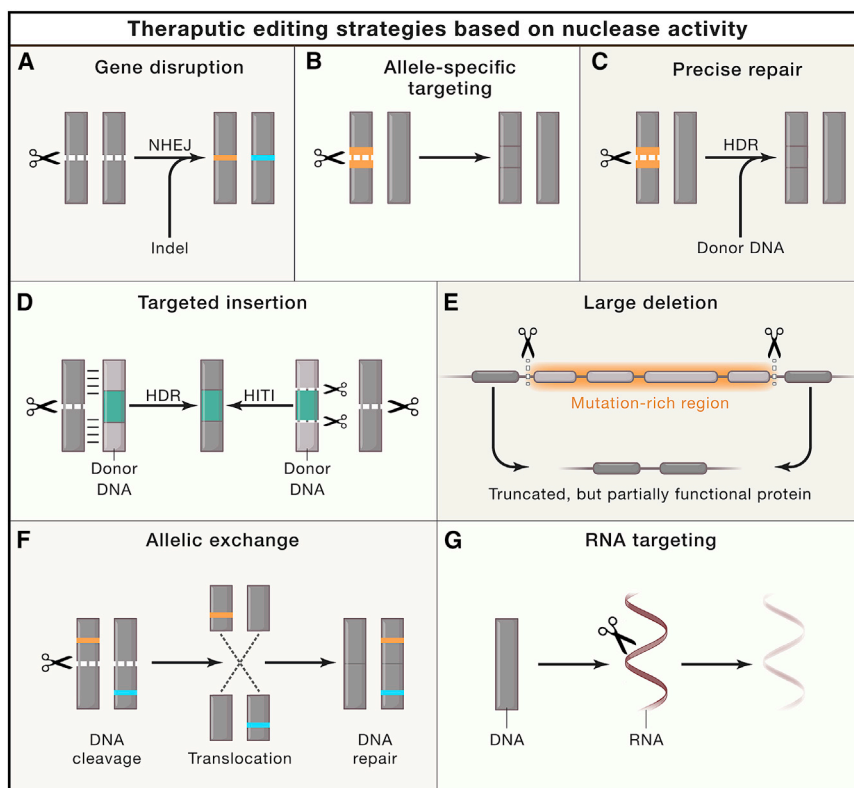
Theraputic editing strategies based on nuclease activity

A Gene disruption

B Allele-specific targeting

C Precise repair

D Targeted insertion

E Large deletion

Mutation-rich region

Truncated, but partially functional protein

F Allelic exchange

DNA cleavage   Translocation   DNA repair

G RNA targeting

DNA   RNA

Figure 1. Therapeutic Editing Strategies Based on Nuclease Activity
(A) Gene disruption introduces indel mutations (red and orange bars, indicating possibly different mutations) into a gene (orange and blue lines), silencing gene function.
(B) Targeting can be specific to the mutant allele (orange rectangle), sparing the normal allele.
(C) HDR mediates precise mutation repair in the presence of a donor template.
(D) Targeted insertion of a therapeutic gene (green bar) can be achieved by either HDR or HITI.
(E) Two cuts can delete a large gene fragment harboring a mutation, resulting in the production of truncated but partially functional protein.
(F) Allelic exchange converts a compound heterozygous genotype to heterozygous through translocation between two homologous chromosomes (dashed black lines). The targeting site is designed to be intronic so that the indel mutations caused by DNA repair (thin dark gray lines) do not compromise gene expression.
(G) RNA targeting achieves gene silencing by degrading RNA instead of DNA.
Indel, insertion or deletion; NHEJ, non-homologous end joining; HDR, homology-directed repair; HITI, homology-independent targeted integration.

AAV is safe, capable of delivering its single-stranded DNA (ssDNA) vector genome to various tissues and cell types, and only mildly immunogenic within a wide range of dosing regimens. Although the vector genome largely remains episomal inside host cells, it is stabilized through concatemerization and circularization to mediate long-term transgene expression in post-mitotic cells, leading to durable therapeutic efficacy. The successes of AAV vectors in delivering gene therapies to disease animal models and patients propelled their adoption for *in vivo* delivery of CRISPR-based therapeutics.

In this review, we focus on recent advances in CRISPR-based therapeutic strategies and *in vivo* delivery of CRISPR machinery using AAV vectors. We also discuss the limitations of using AAV vectors to deliver CRISPR-based therapeutics. Although the lessons learned from AAV gene therapy are generally applicable to delivering CRISPR-based tools, many challenges are unique to this new class of cargoes and call for distinct solutions from both the CRISPR and AAV fields to fully unleash the power of CRISPR-based therapeutic gene editing.

Human Health Applications
Editing Strategies Based on Nuclease Activity
Gene disruption by NHEJ. Typically, DSBs introduced with CRISPR are repaired via NHEJ, an efficient and prevalent DNA repair mechanism in human cells. NHEJ ligates two broken DNA ends together at the break site (Chapman et al., 2012). This process of targeted cleavage and repair can take place repeatedly until an insertion or deletion (indel) occurs that pre-

vents further recognition of the target site by the nuclease. An indel mutation in a protein-coding gene can cause frameshifting or exon skipping, thereby disrupting gene function (Figure 1A). This seemingly disruptive gene editing approach has several therapeutic potentials. For example, the *PCSK9* gene encodes an enzyme that binds to the cell surface receptor for low-density lipoprotein (LDLR) and triggers the lysosomal degradation of LDLR. When PCSK9 is diminished by gene editing, LDLR can return to the cell surface and continue to remove LDL, thereby lowering cholesterol levels (Ding et al., 2014; Ran et al., 2015). Similarly, loss of CCR5—a co-receptor exploited by HIV to infect T cells—confers HIV resistance on edited T cells (Xu et al., 2019b; Xu et al., 2017). Another application of gene disruption is silencing dominant negative mutations. Through careful design of the guide RNA, the mutant allele can be differentially disrupted while the normal one is preserved (Bakondi et al., 2016; György et al., 2019; Rabai et al., 2019) (Figure 1B).

Predictable editing with a single cut. Although the indel mutations generated by Cas9-initiated NHEJ repair are heterogenous, the mutation spectrum is not random but reproducible and dependent on the target site and sequence context (van Overbeek et al., 2016). A data-trained machine-learning model was developed to predict the types and frequencies of Cas9-mediated small indels in human and mouse cells with high accuracy (Shen et al., 2018). This model identifies human pathogenic variants that, following Cas9 cleavage, can be corrected by the predicted predominant indels. This template-free editing method was used to correct frameshift mutations and microduplication mutations in human cells (Iyer et al., 2019; Shen et al., 2018). Another class of predictable editing is targeting a splicing signal to induce exon skipping (Amoasii et al., 2017; Long et al., 2018).

Compared with a 2-cut approach (see below), using one single-guide RNA (sgRNA) to destroy an exonic splicing enhancer, a splicing acceptor site, or a splicing donor site offers a simplified therapeutic design.

Precise mutation repair by HDR. Although precise correction of DNA mutations by HDR is an intuitive therapeutic approach (Figure 1C), this DNA repair mechanism is inefficient in human cells, especially in post-mitotic cells like myofibers and neurons (Chapman et al., 2012). Furthermore, it requires the co-delivery of a repair donor template that carries homology arms matching the targeted locus, which complicates the therapy. Nevertheless, this is a powerful therapeutic strategy, especially in cases where a small number of corrected cells can improve symptoms or in cases where regenerative tissues are targeted. For example, hereditary tyrosinemia type I (HT-1) is a metabolic disease caused by mutations in the *FAH* gene, which is expressed in actively dividing hepatocytes. In a mouse model of HT-1, delivery of Cas9, sgRNA, and a donor template to the liver corrected a disease-causing *Fah* mutation in some hepatocytes, which then had a growth advantage compared to unedited cells, ultimately driving rescue of the disease phenotype (Yin et al., 2014, 2016).

Targeted gene insertion. Another application of HDR-based gene editing is targeted insertion of exogenous DNA sequence into the genome (Figure 1D). This is best exemplified by integrating a chimeric antigen receptor (CAR) gene into isolated T cells; after being infused back into the patient, these armed T cells are capable of recognizing and killing tumor cells specified by the CAR (Bailey and Maus, 2019). Using CRISPR to achieve targeted integration of the CAR by HDR can achieve concomitant disruption of the T-cell receptor α constant (*TRAC*) locus, resulting in engineered CAR-T cells that may be more amenable to allogeneic infusion (Eyquem et al., 2017). In addition to HDR-based approaches, homology-independent targeted integration (HITI) was developed to utilize the more robust NHEJ repair pathway for gene integration, which the authors reported achieved efficient rescue of a disease phenotype in mice (Suzuki et al., 2016). Notably, a single AAV vector design can incorporate elements allowing for both HDR-mediated gene insertion and HITI to achieve efficient genetic correction (Ohmori et al., 2017).

Large-scale DNA editing. CRISPR is inherently capable of multiplex editing, and this natural feature has been leveraged to achieve deletion of large sections of DNA. By introducing two guide RNAs targeting separate sites, fragments as large as several megabase pairs (Mbp) can be excised from the genome (Figure 1E). This approach can remove deleterious mutations while maintaining the open reading frame by deleting one or more exons. This strategy is particularly well suited for Duchene muscular dystrophy (DMD), which is caused by several different mutations, all of which can be treated with this strategy because truncated dystrophin protein with internal deletions is partially functional (England et al., 1990). In a DMD mouse model carrying a nonsense mutation in the dystrophin gene in exon 23, AAV delivery to the muscle of Cas9 and a pair of sgRNAs flanking exon 23 mediated deletion of this exon. Splicing between exon 22 and exon 24 preserved the normal reading frame, generating truncated but functional dystrophin that rescued the disease pheno-

type (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). Another example of this therapeutic strategy is removal of the IVS26 mutation in the *CEP290* gene, which causes Leber congenital amaurosis type 10 (LCA10) (Maeder et al., 2019). This point mutation is in intron 26 and causes aberrant splicing. Using a similar AAV-based approach for eye delivery in a humanized mouse model carrying the IVS26 mutation, a pair of sgRNAs was used to guide Cas9 to excise a segment of intron 26 that contains the mutation, restoring normal splicing between exon 26 and exon 27. The editing efficiency met the targeted therapeutic threshold and was translatable to nonhuman primates (Maeder et al., 2019). Based on these preclinical results, a phase 1/2 clinical trial for the treatment of LCA10 is currently open (ClinicalTrials.gov; identifier: NCT03872479), and it is expected to be the first *in vivo* clinical application of CRISPR-based gene editing. At an even larger scale, recessive compound heterozygous mutations—different mutations damaging the same gene—can be corrected by allelic exchange mediated by DNA cuts at two homologous chromosomes and inter-homologue translocation (Wang et al., 2018) (Figure 1F). Although compound heterozygous mutations are prevalent in patients, application of allelic exchange in a broad range of diseases will require enhancing the efficiency of allelic exchange.

RNA targeting. Following the discovery and development of RNA-targeting Cas enzymes for use in human cells (Abudayyeh et al., 2016, 2017; Konermann et al., 2018; Shmakov et al., 2015; Smargon et al., 2017; Yan et al., 2018), a number of human health applications for these novel proteins have been pursued (Figure 1G). Compared with editing at the level of the genome, gene knockdown at the RNA level is a potentially reversible therapeutic approach. In addition, RNA targeting enzymes, like Cas13, offer more flexible target selection than Cas9 and more specific target degradation than RNA interference (RNAi) (Abudayyeh et al., 2017). Repeated intratumoral delivery of Cas13a and guide RNA targeting a mutant *KRAS* transcript in the form of ribonucleoprotein (RNP) was shown to slow tumor growth in a xenograft mouse model (Zhao et al., 2018). To achieve long-lasting *in vivo* therapeutic effects by targeting RNA, AAV vectors are an advantageous delivery platform for Cas13-based tools because they allow for durable expression of the editing system.

Cas-Effector Fusion Platforms

CRISPR interference and activation (CRISPRi/a). In addition to its use for gene editing, Cas9 has been repurposed as an RNA-guided DNA binding domain by introducing mutations in the RuvC and HNH nuclease domains (Bikard et al., 2013; Qi et al., 2013). In mammalian cells, robust gene repression was achieved by fusing a nuclease-deactivated Cas9 (dCas9) to a repressor domain like the Krüppel-associated box (KRAB) domain and targeting it to the transcription start site (Gilbert et al., 2014; Gilbert et al., 2013) (Figure 2A). AAV delivery of CRISPRi targeting *Pcsk9* in mouse liver effectively silenced transcription and lowered serum PCSK9 and cholesterol levels (Thakore et al., 2018). Replacing the KRAB domain with a gene activating domain, like VP64, converts a gene repressor to a gene activator (Cheng et al., 2013; Gilbert et al., 2013; Konermann et al., 2013; Maeder et al., 2013; Perez-Pinera et al., 2013) (Figure 2A). CRISPRa is useful for treatment of haploinsufficiency, in which a mutant allele renders the remaining normal gene copy inadequate to
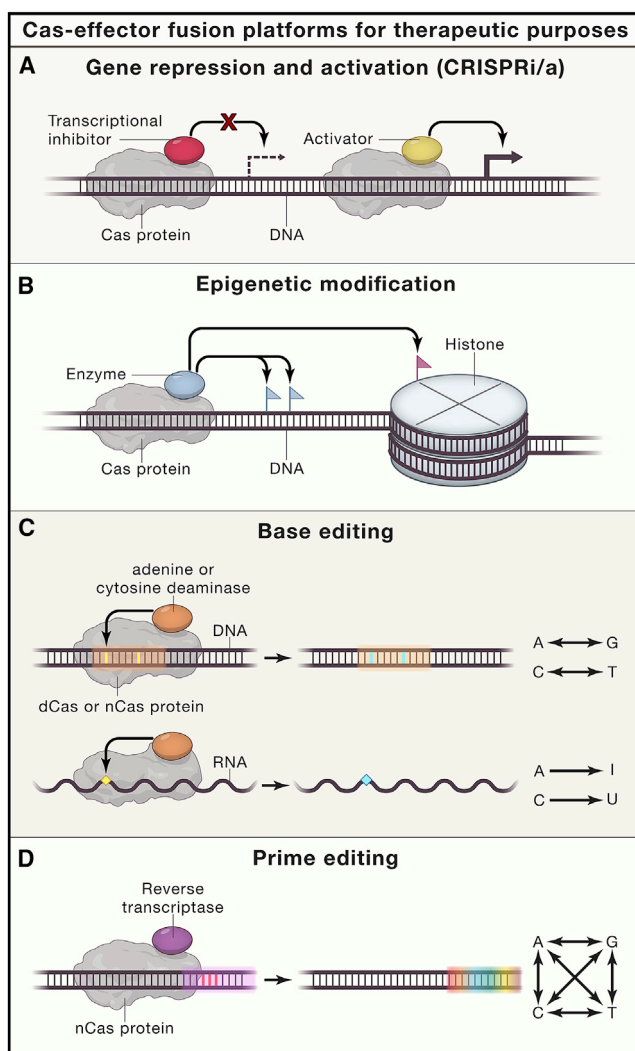
Cas-effector fusion platforms for therapeutic purposes

A   **Gene repression and activation (CRISPRi/a)**

B   **Epigenetic modification**

C   **Base editing**

D   **Prime editing**

Figure 2. Cas-Effector Fusion Platforms for Therapeutic Purposes
(A) Cas protein (gray shape) fused to a transcriptional inhibitor (red oval) or activator (yellow oval) can repress or boost transcription, respectively.
(B) Cas proteins fused with enzymes that can modify epigenetic marks of DNA (blue flags) or histones (red flag) can be used to change gene expression status.
(C) A DNA base editor consists of a dCas or nCas protein, and an adenine or cytosine deaminase (orange oval) that converts A to G or C to T, respectively (yellow bars to blue bars). Note that the editing window (orange shade) contains the desired target (yellow bar pointed by an arrow) and potentially bystander editing targets (the other yellow bar). An RNA base editor can mediate A to I or C to U base change (yellow diamond to blue diamond).
(D) Prime editor comprises an nCas protein fused with a reverse transcriptase (purple oval). Note that the editing window (purple shade) starts from 3 base pairs upstream of the protospacer adjacent motif (PAM) (red bars) of SpCas9, and the editing outcome can be diverse (rainbow shade), including all single base changes and small indels (not shown).

produce enough gene product. CRISPRa enhances transcription of the normal allele to compensate for the damaged allele, thereby restoring normal gene function. This therapeutic approach was demonstrated in a study involving two haploinsufficient mouse models of obesity caused by heterozygous mutations in the *Sim1* and *Mcr4* genes (Matharu et al., 2019). AAV

delivery of dCas9-VP64 and an sgRNA targeting the promoter region of *Sim1* or *Mcr4* to hypothalamus upregulated gene expression to or beyond normal levels and reversed the obesity phenotype. In a more recent report, a similar AAV-mediated CRISPRa approach restored *Scn1a* gene expression in the brain of a *Scn1a* haploinsufficient Dravet syndrome mouse model, which attenuated seizures (Colasante et al., 2019). Although CRISPRa can operate on large genes that are not amenable to viral vector delivery, this approach potentially risks toxicity associated with gene overexpression.

Editing the epigenome. Epigenetic markers, such as DNA methylation and histone modification, are crucial to proper gene expression and have been implicated in human disease (Jaenisch and Bird, 2003). Epigenetic marks are particularly important for brain function, and their dysregulation underlies many neurological disorders (Landgrave-Gómez et al., 2015). These marks are deposited and erased by many enzymes, some of which have been fused with dCas9 to generate target-specific epigenetic writers and erasers of therapeutic value (Liu and Jaenisch, 2019) (Figure 2B). For example, fragile X syndrome (FXS) is caused by CGG trinucleotide repeat expansion in the 5′ untranslated region (UTR) of the *FMR1* gene. This mutation leads to a series of epigenetic changes that silence *FMR1*, such as DNA hypermethylation of the CGG repeats, decreased activating histone modifications (H3K27Ac, HK4me3), and increased inhibitory histone modifications (H3K9me3). A CRISPR-based epigenetic tool comprised of dCas9 fused with the catalytic domain of a DNA demethylation enzyme TET1 (dCas9-TET1) was shown to demethylate the CGG repeats in human cells (Liu et al., 2018). This epigenetic editing converted the mutated *FMR1* promoter region to an active state, leading to gene reactivation and functional restoration in various cell types including FXS neurons. Although epigenomic editing was maintained after the edited neurons were transplanted into the mouse brain (Liu et al., 2018), the *in vivo* therapeutic efficacy and durability of this approach remain to be evaluated.

Base editing. Point mutations represent more than half of the known pathogenic human genetic variants. Therefore, the development of programmable DNA and RNA base editors greatly expanded the CRISPR-based toolbox for therapeutic applications (Rees and Liu, 2018) (Figure 2C). Base editors have been shown to correct disease-causing point mutations in human cells, mammalian embryos, and mouse disease models (Molla and Yang, 2019). In particular, base editing can correct nonsense mutations that account for ~11% of pathogenic mutations, which has been demonstrated in mouse models of DMD (Ryu et al., 2018), phenylketonuria (Villiger et al., 2018), HT-1 (Song et al., 2020), and Niemann-Pick disease type C (Levy et al., 2020). In addition, base editing was used to silence *Pcsk9* gene function by introducing a nonsense mutation into the gene (Chadwick et al., 2017) and to correct a *SOD1* missense mutation in a mouse model of amyotrophic lateral sclerosis (Lim et al., 2020). Compared with HDR-based gene correction, base editing functions in both dividing and post-mitotic cells, and it generally exhibits higher on-target editing efficiency and lower indel rate (Roccio et al., 2015; Yeh et al., 2018). However, bystander editing within a window of several base pairs may cause undesired changes, which has prompted efforts to refine

the editing window and increase editing precision (Thuronyi et al., 2019). RNA base editors were developed by fusing dCas13 with the deaminase domain of ADAR2 (ADAR$_{dd}$) (REPAIR) or an evolved cytosine deaminase (RESCUE) (Abudayyeh et al., 2019; Cox et al., 2017). REPAIR and RESCUE provide a programmable, reversible, and multiplexable approach to correct genetic mutations at the RNA level. Because the target base is defined by a mismatch between the guide RNA and substrate RNA, bystander editing is less likely to occur. A better understanding of the interplay between RNA editing machinery and endogenous RNA metabolism pathways like splicing and nonsense-mediated decay will facilitate its therapeutic utilization in vivo.

Prime editing. Current base editors are limited to four transition mutations (a purine to another purine, or a pyrimidine or another pyrimidine), leaving eight transversion mutations (a purine to a pyrimidine, or vice versa) and indel mutations uncorrectable by base editing. Recently, prime editing was developed to expand the scope of donor-free precise DNA editing (Anzalone et al., 2019) (Figure 2D). The prime editor (PE) consists of Cas9 nickase mutant (nCas9) fused with an engineered reverse transcriptase. PE is guided by a prime editing guide RNA (pegRNA) that serves three functions. First, pegRNA dictates target DNA specificity. Second, following nCas9 nicking at the target site, pegRNA hybridizes with the single-stranded DNA to initiate reverse transcription. Third, pegRNA encodes the desired editing information that can be reverse transcribed and incorporated into the target site. Overall, prime editing is a versatile genome editing tool that can correct not only all 12 types of point mutations, but also small indel mutations. The therapeutic potential of prime editing was demonstrated in the initial report, where a diverse range of disease-causing mutations were corrected in various cell types (Anzalone et al., 2019). Future work will likely improve prime editing efficiency and precision and assess genome-wide off-target editing.

Delivery Using AAV Vectors
Ex Vivo and In Vivo Approaches
The delivery of genome editing therapeutics can be broadly categorized into ex vivo and in vivo approaches. Both approaches have been extensively practiced in the broader gene and cell therapy field and have achieved clinical successes (Dunbar et al., 2018; High and Roncarolo, 2019). The lessons on gene delivery are broadly translatable to delivery of CRISPR-based therapeutics. In ex vivo delivery, genome editing reagents are introduced into isolated human cells to achieve the desired genetic modification. After expansion, the genetically modified cells are infused into patients to confer a therapeutic effect. In vivo delivery aims to introduce genome editing reagents into patients systemically or locally to directly manipulate cells in the body.

Ex vivo delivery has several advantages. First, multiple robust methods have been established to introduce nucleic acid and protein into cell culture, such as transduction by lentiviral vectors, transfection with DNA or RNA, or electroporation with ribonucleoproteins (RNPs). Furthermore, infection by AAV vectors with a ssDNA vector genome can provide donor template for HDR-mediated strategies (Bak et al., 2017). Second, before

the edited cells are infused into patients, they can be scrutinized to ensure gene editing efficiency and accuracy. Third, delivery of CRISPR-based genome editing reagents into cell culture is not limited by host immune responses. For these reasons, ex vivo cell therapy has rapidly advanced to a number of clinical trials involving various cell types and targeting multiple diseases (Table 1), such as disrupting CCR5 in T-cells for HIV infection, engineering immune cells to combat cancer, and editing the BCL11A gene in hematopoietic stem cells for treating β-hemoglobinopathies (Porteus, 2019). However, the human cell types amenable to isolation and ex vivo manipulation are limited, and most cell types, especially highly differentiated and post-mitotic cells, are only functional and manipulatable in vivo. Therefore, in vivo genome editing arguably has the potential to modify more cell types relevant to a broader range of human diseases.

Key to the success of in vivo genome editing is safe and effective delivery of genome editing reagents to the target tissue and cell types. AAV vectors have been widely used for in vivo gene therapy delivery (Wang et al., 2019). Using AAV vectors for in vivo delivery of CRISPR-based genome editing therapeutics has been reported in numerous studies involving disease models and wild-type animals (Lau and Suh, 2017). Along with these continuing successes in proof-of-concept animal studies, the first human application has gained regulatory approval. A phase 1/2 clinical trial employing AAV-CRISPR delivery directly to the eye to correct a CEP290 mutation is currently open for LCA10 patient enrollment (ClinicalTrials.gov; identifier: NCT03872479). Although the success of AAV vectors in gene therapy has paved the way for delivering genome editing therapeutics, the nature of CRISPR-based reagents presents unique challenges. Here, we focus on two issues associated with using AAV vectors for CRISPR delivery, namely the packaging size limit of AAV vectors and undesired editing outcomes.

Fitting Big CRISPR into Small AAV
Smaller Cas proteins. AAV is a small non-enveloped virus with a diameter of ~25 nm. The icosahedral protein capsid packs an ssDNA genome of ~4.7 kb flanked by two inverted terminal repeats (ITRs). Most of the viral genome sequence can be replaced with a transgene cassette generating recombinant AAV (rAAV), and only the two ITRs totaling ~0.3 kb are required during rAAV production. Despite decades of research, the vector genome seems to be limited to ~5.0 kb (Dong et al., 1996), leaving a maximal length of ~4.7 kb for the transgene cassette. Cas9 from Streptococcus pyogenes (SpyCas9), which has a permissive PAM and is widely used, is 4.2 kb and therefore necessitates the use of short gene regulatory elements including promoter and polyadenylation signal totaling less than 0.5 kb. This excludes some promoters that have desired expression strength and specificity. Furthermore, other genome editing components must be carried on a separate AAV vector, such as sgRNA expression cassette(s) and donor template for HDR or gene insertion. A dual-vector delivery scheme can achieve genome editing only when both vectors are taken up by the same cell, potentially limiting editing efficiency. Nevertheless, this approach is being pursued for many applications, some of which are discussed below.

An early approach to overcome this challenge was to identify smaller orthologs of Cas9 (Ran et al., 2015). Additional smaller

Table 1. Ongoing Interventional Clinical Trials Involving CRISPR-Based Gene Editing

Condition	Editing Strategy	Target Gene	Delivery Mode	Phase	Sponsor	ClinicalTrials.gov Identifier
Beta-thalassemia	ND	ND	ex vivo (HSCs)	NA	Shanghai Bioray Laboratory Inc.	NCT04211480
	ND	ND	ex vivo (HSCs)	Phase 1/2	Shanghai Bioray Laboratory Inc.	NCT04205435
	Gene knock-out	BCL11A	ex vivo (CD34+ HSPCs)	Phase 1/2	Vertex Pharmaceuticals Inc.	NCT03655678
Sickle cell disease	Gene knock-out	BCL11A	ex vivo (CD34+ HSPCs)	Phase 1/2	Vertex Pharmaceuticals Inc.	NCT03745287
Advanced stage EBV-associated malignancies	Gene knock-out	PD-1	ex vivo (CTL)	Phase 1/2	The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School	NCT03044743
B cell malignancy	Gene knock-out & insertion of CAR using lentiviral vector	TCR, B2M	ex vivo (T cells)	Phase 1/2	Chinese PLA General Hospital	NCT03166878
	ND	ND	ex vivo (T cells)	Phase 1/2	Chinese PLA General Hospital	NCT03398967
	Gene knock-out and insertion	TRAC, B2M, CAR	ex vivo (T cells)	Phase 1/2	CRISPR Therapeutics	NCT04035434
	Gene knock-out & insertion of CAR using lentiviral vector	HPK1	ex vivo (T cells)	Phase 1	Xijing Hospital	NCT04037566
Esophageal cancer	Gene knock-out	PD-1	ex vivo (T cells)	N/A	Hangzhou Cancer Hospital	NCT03081715
Metastatic non-small cell lung cancer	Gene knock-out	PD-1	ex vivo (T cells)	Phase 1	Sichuan University	NCT02793856
Multiple myeloma, Melanoma, Synovial sarcoma, Myxoid/round Cell Liposarcoma	Gene knock-out & insertion of NY-ESO-1 using lentiviral vector	TCR, PD-1	ex vivo (T cells)	Phase 1	University of Pennsylvania	NCT03399448
Multiple solid tumors	Gene knock-out & insertion of CAR using unspecified method	TCR, PD-1	ex vivo (T cells)	Phase 1	Chinese PLA General Hospital	NCT03545815
	Gene knock-out & insertion of CAR using unspecified method	PD-1	ex vivo (T cells)	Phase 1	Chinese PLA General Hospital	NCT03747965
Multiple myeloma	Gene knock-out and insertion of CAR	TRAC, B2M, CAR	ex vivo (T cells)	Phase 1	CRISPR Therapeutics	NCT04244656

(*Continued on next page*)

Table 1. Continued

Condition	Editing Strategy	Target Gene	Delivery Mode	Phase	Sponsor	ClinicalTrials.gov Identifier
T cell malignancy	Gene knock-out & insertion of *CAR* using unspecified method	*CD7*	*ex vivo* (T cells)	Phase 1	Baylor College of Medicine	NCT03690011
HIV infection	Gene knock-out	*CCR5*	*ex vivo* (CD34+ HSPCs)	N/A	Affiliated Hospital to Academy of Military Medical Sciences	NCT03164135
HPV-related cervical intraepithelial neoplasia	Gene knock-out	*HPV E6/E7*	*in vivo* (plasmid in gel)	Phase 1	First Affiliated Hospital, Sun Yat-Sen University	NCT03057912
LCA10	Gene correction	*CEP290*	*in vivo* (rAAV)	Phase 1/2	Allergan	NCT03872479

EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; HPV, human papillomavirus; LCA10, Leber's congenital amaurosis 10; ND, not disclosed; CAR, chimeric antigen receptor; HSC, hematopoietic stem cell; HSPC, hematopoietic stem and progenitor cell; CTL, cytotoxic T lymphocyte; rAAV, recombinant adeno-associated virus; NA, not applicable. Data are from www.clinicaltrials.gov (accessed on February 6, 2020).

Cas proteins have since been discovered or engineered and experimentally validated as effective genome editing tools in mammalian cells (Kim et al., 2017; Konermann et al., 2018; Liu et al., 2019; Strecker et al., 2019; Teng et al., 2018) (Table 2). These Cas proteins are more compatible with AAV delivery, enabling additional vector design options like expanded promoter choices and a streamlined delivery scheme. For example, Cas9 from *Staphylococcus aureus* (SauCas9) has a gene size of 3.2 kb, allowing a single AAV vector to express SauCas9 together with one or two sgRNAs (Ran et al., 2015). Recently, an "all-in-one" AAV8 vector was reported to not only express SauCas9 and two sgRNAs but also carry a self-linearizing repair template. Together, this vector was shown to correct an *Fah* mutation in mice to treat HT-1 (Krooss et al., 2020).

Generating full-length protein using dual AAV vectors. The above-mentioned Cas-effector fusion platforms, especially base editors and prime editors, require delivering even larger cargoes that exceed the packaging limit of AAV (Table 2). In general, delivering over-sized transgenes has been a longstanding hurdle in the AAV gene therapy field, and several approaches have been developed to address this challenge (Patel et al., 2019; Tornabene and Trapani, 2020). The principle is to split the large transgene into two or more segments, each packaged into an individual AAV vector. Delivering all AAV vectors to the same cell leads to the reconstitution of the full-length gene product (Figure 3). This can be achieved at any level along the flow of genetic information. At the DNA level, AAV ITR, a partial transgene sequence, or an optimized recombinogenic sequence present in both vector genomes, can promote inter-vector DNA recombination (Duan et al., 2000; Ghosh et al., 2008; Lai et al., 2005; Nakai et al., 2000; Sun et al., 2000) (Figure 3A). The recombined vector genome undergoes transgene expression, during which purposefully designed splicing signals mediate pre-mRNA splicing to remove the overlapping sequence, generating a mature transcript that is translated into the desired protein. This strategy was employed to deliver a base editor using dual AAV vectors into a DMD mouse model (Ryu et al., 2018). At the RNA level, two transcripts derived from separate AAV vector genomes can partially hybridize to promote *trans*-splicing mediated by the splicing donor and acceptor present in the two transcripts, respectively (Pergolizzi et al., 2003; Song et al., 2009) (Figure 3B). The fully spliced mature transcript leads to full-length protein production.

Reconstitution at the protein level by split inteins has been increasingly investigated as another approach to express large proteins using AAV vectors (Chew et al., 2016; Fine et al., 2015; Li et al., 2008; Truong et al., 2015; Villiger et al., 2018). An intein is a protein segment that can excise itself and ligate the nearby protein segments with a peptide bond, similar to an intron in pre-mRNA splicing (Shah and Muir, 2014). Split inteins are a pair of natural polypeptides that, when present at the termini of two other proteins, mediate protein *trans*-splicing (PTS) (Aranko et al., 2014) (Figure 3C). In contrast to pre-mRNA splicing that requires complex cellular machineries and has a certain promiscuity, split inteins are self-sufficient to mediate precise PTS. Therefore, a split intein-mediated protein reconstitution strategy is potentially robust and precise in various cell types, which is an important feature for therapeutics.

Table 2. Gene Sizes of Selected CRISPR-Based Tools in Comparison with AAV Vector Genome Size

	Tool	Function	Gene Size (kb)	Ref.
Within AAV packaging limit	SpCas9	Nuclease	4.2	Cong et al., 2013
	SaCas9	Nuclease	3.2	Ran et al., 2015
	PspCas13b	Ribonuclease	3.4	Cox et al., 2017
	RfxCas13d	Ribonuclease	2.9	Konermann et al., 2018
	dPspCas13b(tr)-ADARdd	RNA editing	4.1	Cox et al., 2017
	dSaCas9-KRAB	CRISPRi	3.6	Thakore et al., 2018
	FnCas12a	Nuclease	3.9	Zetsche et al., 2015
	BhCas12b	Nuclease	3.3	Strecker et al., 2019
Beyond AAV packaging limit	BE4	Cytosine base editing	5.6	Komor et al., 2017
	ABE7.10	Adenine base editing	5.3	Gaudelli et al., 2017
	dSpCas9-TET1	Editing DNA methylation	6.4	Liu et al., 2016
	PE2	Prime editing	6.4	Anzalone et al., 2019

AAV Vector Component	Function	Size (kb)
ITR x 2	Vector genome rescue and replication during packaging; Packaging signal	0.3 total
Transgene cassette	Transgene expression	<4.7

ITR, inverted terminal repeat; BE, base editor; ABE, adenine base editor; PE, prime editor.

In a recent report, base editors were systematically studied for split intein reconstitution and AAV vector delivery to mice (Levy et al., 2020). The optimized base editors achieved genome editing in multiple tissues including the brain, liver, retina, heart, and skeletal muscle, with editing efficiencies ranging from 9%–59%. As expected, the choices of split intein, split site, and base editor design are important parameters. In addition, AAV capsid, dose, and route of administration also have a profound impact on the final editing efficiency, highlighting the synergy between CRISPR-based reagents and AAV-mediated delivery to fully unleash the therapeutic potential. Notably, base editing constructs delivered by intein-mediated PTS were more efficient than reconstitution at the AAV vector DNA level in a side-by-side comparison, likely due to the multiple cellular processes in the latter approach that together compromise efficiency (Levy et al., 2020).

Control of Undesired Genome Editing Outcomes

Inducible expression. Although AAV delivery can mediate durable transgene expression in post-mitotic cells, for many genome editing applications, this is not necessary, because the mutation is permanently corrected after being edited. Furthermore, long-term expression of an active genome editing system poses a safety concern, because it has been shown to increase off-target cleavage (Zuris et al., 2015). Therefore, transient expression of CRISPR-based genome editing machineries is preferred in many therapeutic settings. To achieve this, non-viral delivery methods can be used to deliver mRNA or proteins, which will eventually be degraded in cells (We refer the reader to recent reviews for details about progress in this area [Chen et al., 2019; Wan et al., 2019; Xu et al., 2019a]). While these methods hold great potential for *in vivo* applications like those targeting the liver (Finn et al., 2018; Yin et al., 2016), their delivery efficiency

and range of targetable tissues and cell types beyond liver are currently limited compared to AAV vectors. A straightforward way to make AAV vectors compatible with transient delivery is to incorporate a molecular apparatus for inducible expression. Many approaches for this purpose have been reported to function in cell culture, such as using a drug-inducible promoter for gene expression, protein activation by light, drug-inducible assembly, or reconstitution from a split construct, and protein inactivation by a drug-inducible destabilizing domain (Davis et al., 2015; Dow et al., 2015; Kumar et al., 2018; Nguyen et al., 2016; Nihongaki et al., 2015; Rose et al., 2017; Tak et al., 2017; Truong et al., 2015; Wright et al., 2015; Zetsche et al., 2015). The translational value of these methods for *in vivo* applications remains to be investigated. Ideally, an AAV-compatible, inducible genome editing system would be a molecular circuit that comprises a simple transgene design to fit in AAV vectors and a safe trigger for activation or inactivation. Such a system was explored (Ye et al., 1999), but it remains to be translated to clinical applications.

Self-cleavage of AAV-CRISPR. An interesting idea to achieve transient function of AAV-CRISPR is to engineer an intrinsic destabilization mechanism into the vector itself by taking advantage of the DNA cleavage activity of the delivered CRISPR machinery. Along with the desired therapeutic genome editing event, DNA cleavage of the vector genome occurs simultaneously (Li et al., 2018; Li et al., 2019). This intriguing approach has been shown to limit SauCas9 expression in mice without significantly compromising on-target editing. However, the reduction in SauCas9 protein levels was not complete, leaving some possibility of off-target cleavage by the remaining vector expression (Li et al., 2018). Moreover, the cleaved AAV vector DNA was found to integrate into the on-target genomic site
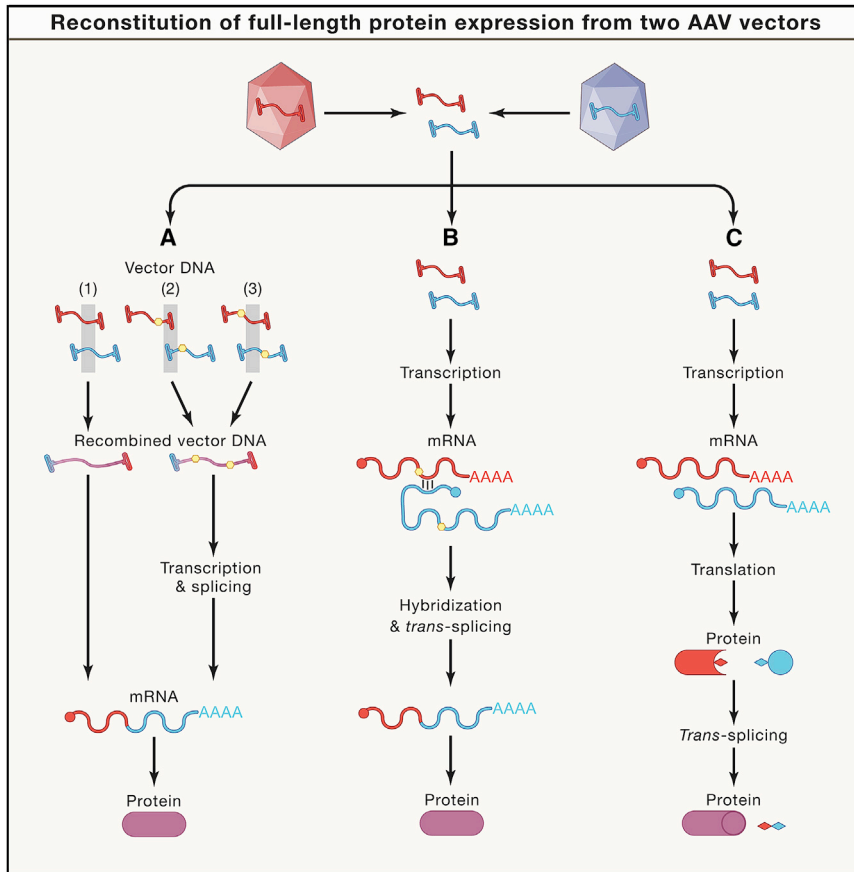
Figure 3. Reconstitution of Full-Length Protein Expression from Two AAV Vectors

(A) Two AAV vector genomes can undergo recombination mediated by an overlapping sequence (1), inverted terminal repeat (ITR) (2), or a recombinogenic sequence (3) to form a recombined vector DNA that encodes the full-length protein. The transcript derived from the recombined vector DNA may undergo splicing by engineered splicing signals (yellow hexagons).

(B) The two mRNAs transcribed from individual vector genomes can undergo *trans*-splicing to form a chimeric mRNA encoding the full-length protein, which is promoted by the hybridization domains (short black bars).

(C) The two half proteins expressed from the two AAV vectors can undergo protein *trans*-splicing mediated by split inteins (diamonds) to form a full-length protein.

teins are co-delivered by AAV vectors. Acr protein expression is subjected to tissue-specific miRNA-mediated suppression, which in turn allows for Cas protein expression and genome editing in that tissue.

AAV vector DNA integration at on-target DSBs. It was shown that AAV vector DNA can integrate at pre-existing chromosomal breaks induced by the endonuclease I-SceI or irradiation in human cells (Miller et al., 2004). Similarly, when AAV was used to deliver CRISPR to generate DSBs in model animals *in vivo*, AAV vector DNA integration at the on-target cleavage site occurred with frequencies approaching or exceeding those of indels in some studies (Hanlon et al., 2019; Jarrett et al., 2017; Maeder et al., 2019; McCullough et al., 2019; Nelson et al., 2019; Yoon et al., 2018). In general, such integration events are undesired, and their biological consequences remain to be evaluated in the context of specific applications, where parameters including the AAV vector design, on-target and off-target genomic loci, and editing goals will all need to be taken into consideration. Furthermore, it is worth exploring whether AAV vector integration is a therapeutically relevant editing outcome in certain applications, such as to mediate permanent gene insertion in dividing cells (Wang et al., 2020). It has been suggested that AAV ITRs facilitate vector integration at DSBs (Miller et al., 2004), and the ITR has been found in most integrated sequences (Jarrett et al., 2017). Therefore, a better understanding of the mechanistic role of ITR in mediating AAV vector integration at DSBs may provide not only insight into this genome editing outcome but also clues to harness this phenomenon for meaningful applications.

Challenges and Future Perspectives
The advancement of AAV-CRISPR therapeutics faces the same set of obstacles facing the development of AAV-based gene therapy in general, such as pre-existing immunity against AAV capsids and vector-induced immune responses, delivery

(Li et al., 2019), and the potential impact of this on-target but undesired editing on safety remains to be evaluated.

Spatial control of genome editing. In addition to temporal control, avoiding genome editing in unintended tissues and cell types will also improve the clinical safety profile of CRISPR-based therapeutics. Although different AAV capsids provide a spectrum of tissue tropism, they often differ only in the tropism strength but not absolute specificity. Tissue-specific promoters are widely used in AAV vector design for spatial control of transgene expression. As mentioned earlier, the large gene sizes of Cas proteins require compact promoters that usually do not exhibit high specificity. A potentially viable approach to exclude Cas protein expression in certain tissue types is to include binding sites for an endogenous microRNA (miRNA) in the 3′ UTR of the Cas protein gene cassette (Brown et al., 2006; Geisler et al., 2011; Qiao et al., 2011; Xiao et al., 2019; Xie et al., 2011). Delivery to tissues that highly express the miRNA will silence Cas protein expression, whereas other tissues lacking the miRNA will express the Cas protein for genome editing. In addition, silencing Cas protein expression in antigen-presenting cells may help mitigate immune responses against Cas9 (Xiao et al., 2019). Utilizing this de-targeting concept and combining it with natural inhibitors of Cas proteins, known as anti-CRISPRs (Acr), can lead to cell- and tissue-specific targeted genome editing (Hoffmann et al., 2019; Lee et al., 2019). In this elegant system, Cas and Acr pro-

efficiency and specificity, and AAV vector manufacturing. These topics were discussed in depth in recent reviews (Colella et al., 2017; Domenger and Grimm, 2019; Rabinowitz et al., 2019; Verdera et al., 2020; Wang et al., 2019). In addition, clinical application of AAV-CRISPR presents unique challenges that require distinct solutions. For example, pre-existing humoral and cellular immunity against commonly used Cas9 orthologs has been reported in general human populations due to widespread infections of the bacteria from which these proteins are derived (Charlesworth et al., 2019; Simhadri et al., 2018; Wagner et al., 2019). It remains to be evaluated whether delivering vectored Cas9 proteins directly into the human body in the presence of pre-existing anti-Cas9 immunity will compromise safety or therapeutic efficacy (Crudele and Chamberlain, 2018). Potential routes to addressing the issue of immunogenicity includes use of immune-orthogonal Cas9 orthologs (Moreno et al., 2019) and further exploration of the natural diversity of CRISPR systems to identify new enzymes that may be less immunogenic in humans. Furthermore, various protein engineering approaches can improve clinically relevant features, such as mapping and editing epitopes for a better immunological profile.

Off-target editing needs to be carefully assessed for any genome editing therapy. A number of methods to characterize off-targets of CRISPR-based reagents have been developed (Akcakaya et al., 2018; Cameron et al., 2017; Crosetto et al., 2013; Kim et al., 2015; Tsai et al., 2017; Tsai et al., 2015; Wienert et al., 2019; Yan et al., 2017; Zuo et al., 2019). Although these methods provide sensitive measurement of off-targets at nucleic acid sequence level, the potential biological consequences stemming from any off-target event largely have to be evaluated empirically. Because off-targets heavily depend on the species-specific genomic sequence and the *in vivo* delivery method, using animal models that are humanized at the cellular or tissue level can provide valuable pre-clinical information. To improve specificity, various protein engineering approaches have yielded high-fidelity versions of Cas and Cas-effector fusion proteins (Casini et al., 2018; Chen et al., 2017; Hu et al., 2018; Kleinstiver et al., 2016; Kleinstiver et al., 2019; Lee et al., 2018; Slaymaker et al., 2016; Vakulskas et al., 2018). The other components of CRISPR-based therapeutics, such as sgRNA and donor template, have also been subjected to modifications to enhance editing precision and efficiency (Cromwell et al., 2018; Fu et al., 2014; Kocak et al., 2019; Mir et al., 2018; Yin et al., 2018).

In addition to off-target editing, on-target editing can also lead to undesired editing events, such as large deletions extending to nearby genes (Adikusuma et al., 2018; Kosicki et al., 2018), chromosome rearrangements including inversions and translocations (Frock et al., 2015; Maeder et al., 2019), and AAV vector genome integration (Hanlon et al., 2019; Nelson et al., 2019). Profiling these events usually requires a set of techniques different from the ones used to monitor off-target cleavage (Frock et al., 2015). It is worth emphasizing that a therapeutic genome editing strategy synergizes the action of the editor and multiple cellular processes triggered by the editor, such as DNA repair pathways in the case of utilizing the nuclease activity of Cas9. Therefore, a better understanding of these relevant cellular processes will provide valuable insights into genome editing outcomes and safety (Yeh et al., 2019).

Disease animal models are critical in translational research and therapy development. Because *in vivo* delivery is a major bottleneck in AAV-CRISPR therapeutics, directly testing in disease animal models is the gold standard to evaluate therapeutic efficacy. For AAV gene replacement therapy that aims to deliver a functional gene copy to correct recessive diseases, animal models created by various gene knockout strategies are usually sufficient. In contrast, many genome editing strategies, such as base editing and prime editing, often target a particular mutation. Therefore, animal models harboring deliberately engineered mutations are required to provide a suitable testing platform. For example, several DMD mouse models with representative dystrophin gene mutations were purposefully generated to demonstrate the effectiveness of various genome editing strategies (Amoasii et al., 2017). Although the advent of CRISPR-based genome editing technology has greatly facilitated the generation of genetically modified animals, the lack of suitable animals with patient-relevant mutations remains a major challenge. Developing easy, robust, and rapid animal modeling approaches will likely expedite the development of precise genome editing therapeutics.

Conclusions

CRISPR and AAV are just two examples illustrating how natural systems can be harnessed for revolutionary biotechnologies, highlighting the exciting opportunities that can emerge from a deeper understanding of and appreciation for natural diversity. However, adaptation of microbial machineries to meet human needs is not guaranteed to succeed. This adaptation process involves multidisciplinary expertise spanning various research fields like microbiology, biochemistry, molecular biology, protein engineering, structural biology, and bioinformatics. For CRISPR-based technology, the simplicity of its design and utilization should not disguise the importance of openly sharing valuable resources ranging from plasmids and sgRNA libraries to transgenic animals and bioinformatic tools, which has encouraged cross-validation and speedy adoption of the technology for novel applications and will continue to foster rapid scientific and technological advances.

In general, delivery is among the most urgent obstacles hindering *in vivo* gene therapy, including genome editing therapeutic approaches. AAV vectors are currently the most effective gene delivery vehicle and the only delivery vehicle approved to introduce CRISPR-based genome editing therapeutics directly into the human body. It will be exciting to follow the ongoing AAV-CRISPR clinical trial and glean new insight into the strengths and weaknesses of this approach that can then inform further development and refinement of the system. Such refinements are expected to come both from the efforts to modify CRISPR-based reagents to fit the intrinsic features of AAV vectors and from ongoing research in AAV biology and vectorology. Merging the findings from these two fields will yield a broader range of delivery platforms that can better serve emerging molecular therapeutics.

DECLARATION OF INTERESTS

D.W. is an inventor on issued patents and patent applications relating to AAV- and CRISPR-based technologies. F.Z. is an inventor on issued patents and patent applications relating to CRISPR-based technologies. F.Z. is a co-founder and scientific advisor of Editas Medicine, Beam Therapeutics, Pairwise Plants, Arbor Biotechnologies, and Sherlock Biosciences. G.G. is an inventor on issued patents and patent applications relating to AAV- and CRISPR-based technologies. G.G. is a co-founder of Voyager Therapeutics and Aspa Therapeutics.

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