Delivery and Specificity of CRISPR/Cas9 Genome Editing Technologies for Human Gene Therapy

Jennifer L. Gori, Patrick D. Hsu, Morgan L. Maeder, Shen Shen, G. Grant Welstead, and David Bumcrot*

Editas Medicine, Cambridge, Massachusetts.

Genome editing using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated 9 (Cas9) technology is revolutionizing the study of gene function and likely will give rise to an entire new class of therapeutics for a wide range of diseases. Achieving this goal requires not only characterization of the technology for efficacy and specificity but also optimization of its delivery to the target cells for each disease indication. In this review we survey the various methods by which the CRISPR/Cas9 components have been delivered to cells and highlight some of the more clinically relevant approaches. Additionally, we discuss the methods available for assessing the specificity of Cas9 editing; an important safety consideration for development of the technology.

INTRODUCTION

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) bacterial immunity systems[1–9] and adaptation of RNA-guided CRISPR/Cas9 for genome editing[10–11] have had a profound impact on a wide array of research efforts, including the identification and validation of disease targets[12,13] as well as the development of disease models.[14,15] The ease and flexibility of the system has also generated tremendous excitement about its clinical potential. With steady improvement in functionality, delivery, and specificity, it is possible that the CRISPR/Cas9 system will enable the development of necessary treatment options for genetic diseases that affect a variety of organs and tissues. Rapid advances in the CRISPR/Cas9 technology as well as delivery modalities for gene therapy applications are removing barriers to the clinical translation of this technology, as exemplified by the recent characterization of the smaller *Staphylococcus aureus* Cas9 homolog[16] that can be packaged into adeno-associated viral vectors (AAV). However, to address the expanded scope of genetic disease targets amenable to gene editing with the versatile CRISPR/Cas9 system, additional work needs to be done to resolve outstanding delivery and technological complexities.

First and foremost, CRISPR/Cas9 must be rigorously tested in primary human cells and, when available, clinically relevant animal models to show that the technology is both safe and effective. Here, the key factors that will influence the pace and path of CRISPR/Cas9 advancement to the clinic are discussed. Specific topics include a review of the available *ex vivo* and *in vivo* delivery methods for CRISPR/Cas9 components (i.e., Cas9, guide (g)RNAs, donor repair templates) and a review of the currently available technologies for evaluating off-target gene modification, which is one of several elements that must be addressed to validate the safety of the CRISPR/Cas9 platform for clinical application.

DELIVERY OF CRISPR/CAS9 GENOME EDITING COMPONENTS

Both viral and nonviral delivery approaches with historical precedence in gene therapy are currently being evaluated for CRISPR/Cas9 delivery. Unlike traditional gene therapy, in which sustained transgene expression is generally required, genome

*Correspondence: Dr. David Bumcrot, Editas Medicine, Inc., 300, 3rd Street, Cambridge, MA 02142. E-mail: david.bumcrot@editasmed.com

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editing can be achieved through transient expression of Cas9. This permits consideration of a range of delivery options for therapeutic application. Viral vectors that are potential delivery vehicles for CRISPR/Cas9 components (with or without donor repair template sequences) include self-inactivating lentivirus, adenovirus, and AAV. Cargoes for nonviral delivery systems include plasmid DNA, Cas9 mRNA, *in vitro* transcribed or synthesized gRNA, Cas9/gRNA ribonucleoprotein complexes,[17] and donor nucleic acid templates. Nonviral delivery of nucleic acids and proteins can be achieved through several methods, including electroporation, lipid-based transfection, or induced osmocytosis.[18] Features of multiple promising delivery approaches are discussed below.

Lentiviral delivery

Self-inactivating lentiviral vectors (LVs) are useful tools for gene therapy given their ability to efficiently transduce both dividing and nondividing cells.[19] The HIV-1-based lentivirus vector is a replication-incompetent enveloped retrovirus that contains two copies of the ~10 kb single-stranded, positive-sense RNA genome. Segregation of the viral genes encoding structural and enzymatic proteins among different plasmids and elimination of certain accessory genes from the RNA genome render the lentivirus vector unable to replicate in transduced cells.[20] In addition, the packaging signal is restricted to the transfer vector that encodes the transgene expression cassette (e.g., the Cas9 and gRNAs), thereby preventing packaging of LV structural and enzymatic encoding genes.[21] To produce virus, the transfer vector containing the transgene is co-transfected with separate plasmids that encode the viral proteins required for packaging the transgene expression cassette, and an envelope protein (usually the vesicular stomatitis virus glycoprotein-G [VSV-G]) that expands tropism of the virus.[21]

After years of preclinical testing in animal models, integrating recombinant lentiviruses are being used to genetically modify hematopoietic stem/progenitor cells (HSPCs) *ex vivo* to treat X-linked adrenoleukodystophy, metachromatic leukodystrophy, and Wiskott–Aldrich syndrome.[22–24] Lentivirus vectors are also being successfully applied in the clinic for the production of cancer-specific chimeric antigen receptor-expressing T lymphocytes to treat leukemia[25] and glioblastoma.[26] Feasibility of the lentiviral approach for CRISPR/Cas9 is supported by studies demonstrating successful delivery to mammalian cells both *ex vivo* and *in vivo*. The diverse applications of lentivirus vectors for delivery of Cas9/gRNA components include modeling cancer in mice,[27–30] development of selection based gene disruption assays to conduct complex gain and loss of function genetic screens,[13,31–33] and eradication of latent viral infections such as HIV,[34] hepatitis B virus,[35–37] and herpesvirus.[38] In contrast to the effective use of integrating lentivirus vectors for gene replacement therapy, integrating vectors may not be suitable for delivery of Cas9, as the safety profile of sustained Cas9 expression (e.g., off-target cleavage and potential effects of Cas9 expression on primary cell homeostasis, functionality, and immunity) is not yet well understood.

Integrase-deficient lentivirus vectors (IDLVs) are able to transduce primary human cells but cannot integrate genetic cargo into the host cell genome. Given the packaging capacity of lentivirus vectors (~10 kb),[39] IDLVs are a useful tool for the delivery of Cas9, gRNAs, and donor repair templates for homology-directed repair (HDR)-based genome editing strategies. IDLVs have been used to deliver zinc finger nucleases (ZFNs),[40,41] TAL effector nucleases (TALENs),[42] meganucleases,[43] and donor repair templates for site-specific modification of primary target cells *ex vivo* and *in vivo*. While the transient expression of IDLV-delivered Cas9 in rapidly dividing cells could represent a safety advantage, in quiescent and slowly dividing cells, including long-term HSPCs, neurons, and hepatocytes, Cas9 expression may persist after IDLV delivery leading to undesired effects such as higher off-target cleavage.

AAV delivery

Recombinant AAV (rAAV) vectors are promising gene delivery vectors for a spectrum of genetic disorders because of their nonpathogenicity, low levels of immune stimulation, capability to transduce both dividing and nondividing cells, non-integrating nature, and diverse tissue-targeting profiles.[44] AAV is a nonenveloped parvovirus with a protein capsid and relatively small single-stranded DNA genome of 4.7 kb. More than 200 naturally occurring AAV serotypes have been discovered and the AAV toolkit is expanding with re-engineered capsids exhibiting low reactivity to neutralizing antibodies and novel tissue specificity *in vivo*.[45–49] The past decade witnessed the advancement of recombinant AAV vectors into clinical trials for inherited and acquired genetic diseases, including Leber Congenital Amaurosis Type 2, Hemophilia B, and Canavan's disease.[50] In 2012, the first AAV-based gene therapy product was approved by the European Medicines Agency to treat Lipoprotein Lipase Deficiency.[51,52] These results demonstrating the safety and efficacy of AAV vectors suggest that

they may be promising candidates for *in vivo* delivery of CRISPR/Cas9 components.

One challenge of using AAV to deliver *Streptococcus pyogenes* Cas9 is the packaging limit of AAV vectors. Wild-type AAV capsids package 4.7 kb of single-stranded DNA, including two inverted terminal repeats that comprise approximately 300 bp. This leaves 4.4 kb for packaging of CRISPR/Cas9 components. However, the coding sequence for *S. pyogenes* Cas9 is 4.14 kb, leaving little room for a promoter, polyadenylation signal, or gRNA coding sequence. While Senis et al. were able to package *S. pyogenes* Cas9 plus a gRNA gene into AAV8 and achieve modification of the miR122 target in mouse liver,[53] oversized AAV vectors generally yield inconsistent results.[54–57] An alternative approach is to deliver Cas9 and gRNA coding sequences in two separate AAV vectors. Swiech et al. achieved robust multiplex gene modification at three genomic loci following administration into the mouse brain with this approach.[58]

A novel approach to fit *S. pyogenes* Cas9 into AAV vectors is to design split AAV/CRISPR/Cas9 cassettes that reconstitute to form a functional protein after delivery. Based on the crystal structure, 11 potential split sites on *S. pyogenes* Cas9 have been identified that allow for division of the protein into N- and C-terminal fragments.[59] In this study, rapamycin induced reassembly of the two fragments into a functional nuclease. Novel AAV variants with genomes greater than 4.7 kb are also being studied. Human bocavirus virus-1 (HBoV1) is an autonomous parvovirus with a genome 5.5 kb in length. A chimeric AAV2/HboV1 vector was shown to accommodate a transgene up to 5.5 kb and transduce human airway epithelium 70-fold more efficiently than AAV2.[60]

More recently, a CRISPR/Cas9 homolog from *S. aureus* emerged for research and potential therapeutic applications.[16,61] The coding sequence of *S. aureus* Cas9 is shorter than that of *S. pyogenes* Cas9 by 942 bp, and thus could be packaged into the 4.7 kb AAV genome together with a gRNA gene expression cassette. Ran et al. achieved >40% genome modification efficiency of the *Pcsk9* gene in murine liver within one week following intravenous administration of an "all-in-one" AAV8 vector. Inflammation and abnormal pathology were not observed in murine liver after gene modification, and serum alanine aminotransferase and bilirubin elevation were not detected. However, systematic and thorough studies of the immunological effects of AAV vectors encoding CRISPR/Cas9 components are necessary to facilitate optimization of AAV for therapeutic application.

Nonviral delivery

Nonviral methods that introduce CRISPR/Cas9 components into primary cells are currently under investigation.[17,62] A variety of nonviral options exist for both *in vivo* and *ex vivo* delivery of the CRISPR/Cas9 system, including electroporation, hydrodynamic delivery, and lipid-based nanoparticles. Depending on the modality used, the components (e.g., Cas9 and the gRNAs can be formulated as DNA, RNA, or a complex of protein (Cas9) and [gRNA]), referred to as ribonucleoprotein (RNP). The majority of these methods support delivery of the CRISPR machinery for transient expression that is sufficient for gene editing, potentially providing a safety advantage over viral delivery methods.

Electroporation. Originally described over 30 years ago, the application of an electrical current to cells in order to delivery DNA, or electroporation, is now a commonly used research method. Electroporation effectively delivers DNA, RNA, and protein to cells and has been used more recently for delivery of mRNA in a clinical setting, thus establishing the utility of this delivery method for clinically based genome editing.[63] For delivery of the CRISPR components, electroporation has been utilized to deliver plasmid DNA encoding Cas9 and gRNAs to cancer cell lines, pluripotent stem cells, and primary hematopoietic cells such as $CD4^+$ T-cells and $CD34^+$ stem cells.[15,64–67] Electroporation has also been successfully used to deliver RNA to fertilized mouse embryos in order to generate genetically modified rodents.[68,69]

Hydrodynamic delivery. In preclinical models, a method that has been successfully used for *in vivo* gene delivery is hydrodynamic delivery in which a large volume of a DNA-containing solution is rapidly delivered via intravenous injection, resulting in uptake of DNA by liver cells. Recently, hydrodynamic delivery was successfully used to deliver plasmids encoding Cas9 and target-specific gRNAs to achieve editing in a mouse model of[70] and to model cancer in the mouse liver.[71] Although these experiments provide a proof-of-concept validation for gene editing of cells in an adult animal, this approach is not directly applicable for clinical use. Nevertheless, hydrodynamic delivery of CRISPR/Cas9 components is useful for the rapid generation of mouse models for liver cancer and other diseases.

Lipid-mediated transfection. One of the most common methods for nucleic acid delivery to cells in a research context makes use of lipid nanoparticles or complexes. This approach has entered clinical

testing with siRNA therapeutics.[72,73] Although the spectrum of lipid-based formulations is broad, they typically involve encapsulation or complexing of nucleic acid cargo through interactions between the negatively charged phosphate backbone and the positively charged lipid head groups. Cellular uptake is generally mediated by endocytosis and macropinocytosis mechanisms. Researchers have routinely made use of lipid particles to deliver CRISPR/Cas components to cancer cell lines,[10,67] and a recent article demonstrated *in vivo* delivery of gRNAs to Cas9 transgenic mice using a lipid-like delivery formulation.[27] It has also been shown that a complex of Cas9 protein and gRNA (RNP) could be delivered by lipid-based transfection both *in vitro* and *in vivo*.[17] The highly negative charge of the gRNA allows the RNP complex to be encapsulated by the cationic lipids. The RNP-containing lipid complex is capable of delivering its payload to the cells of the inner ear of the mouse after injection into the cochlea of P2 neonates. Encouragingly, it was possible to achieve nearly 20% Cas9 genome editing in the hair cells of the inner ear using this method. Although additional experiments need to be performed in order to confirm the clinical feasibility of this approach, delivery of RNP by lipid particles could be a desirable way to introduce CRISPR/Cas9 components to certain target cells *in vivo*.

CRISPR/Cas9 SPECIFICITY

By generating DNA double-stranded breaks (DSBs) that are repaired by endogenous cellular repair mechanisms, Cas9-mediated gene editing creates permanent changes in the genomes of the treated cells. While this allows for precision in human gene therapy by correcting disease-causing mutations, the specificity of Cas9 targeting, or any permanent genomic modification, needs to be carefully considered. While specificity analyses can serve as surrogates for safety, potential CRISPR/Cas9-based therapeutics share many of the same safety concerns as gene augmentation approaches such as toxicity or immunogenicity of the transgene and delivery modality. For example, early gene therapy trials in which retroviruses were used to treat SCID lead to leukemic transformation after integration of the provirus into patient genome.[74] Insertional mutagenesis caused by integration in proximity to a protooncogene led to leukemic transformation in four of the nine gene-modified hematopoietic stem cell-transplanted patients, illustrating that restricting gene modification to more precise locations could potentially improve the safety of gene therapies.

The Cas9 enzyme utilizes a two-step process to identify target DNA cleavage sites. The initial step comprises a scanning mechanism wherein the Cas9 protein recognizes a short nucleotide sequence called the protospacer adjacent motif (PAM). After Cas9 recognition of this sequence, gRNA–DNA homology drives the cleavage event. Early efforts to characterize the specificity of Cas9 cleavage focused exclusively on the *S. pyogenes* Cas9 nuclease, initially demonstrating that some mismatches between the gRNA and target DNA could be tolerated, especially if they were at the PAM-distal, 5′ end of the guide sequence.[10] Using a combination of mismatch guide RNAs,[75,76] fluorescent reporter assays,[67] and *in vitro* selection,[77] multiple groups extensively analyzed the role of guide-target mismatches on Cas9-induced insertion/deletion mutations (indels). Collectively, these studies showed that the 5′-NGG *S. pyogenes* PAM is likely the most stringent determinant of targeting specificity, but that *S. pyogenes* Cas9 could cleave with lower efficiency at a 5′-NAG PAM.[76] Furthermore, while mismatches far away from the PAM were generally more tolerated and could preserve Cas9 activity, this effect varied widely among target sites. Finally, reducing the enzymatic concentration of Cas9 and gRNA in the cell could reduce off-target modification rates, although at the cost of also reducing on-target efficiency.[76,77] Overall, while sequence homology between the DNA target site and the guide sequence within the gRNA appeared to be a major determinant of Cas9 targeting fidelity, there are no simple rules yet defined that govern Cas9 targeting.[78]

Over the last two years, efforts in Cas9 protein engineering and guide RNA optimization have led to strategies that can greatly improve their off-target activity. By converting Cas9 into a nickase enzyme (which cleaves only one of the two DNA strands) and requiring two Cas9 nickase-gRNA complexes to mimic a DSB by generating cooperative nicks on opposite strands of DNA, activity at certain off-target sites could be greatly reduced.[67,79] Truncating the guide sequence from 20nt to 17nt or 18nt also appears to improve targeting fidelity, presumably by increasing the sensitivity of the guide to mismatches.[80] Taking inspiration from ZFNs and TALENs, Cas9 can also be converted into a catalytically inactive, "dead" Cas9 (dCas9) and fused to the catalytic domain of the FokI endonuclease.[81,82] Similar to Cas9 double nicking, dCas9-FokI dimers then generate DSBs by reconstituting a functional FokI endonuclease at the target site of interest. While all of these methods greatly improve Cas9 cleavage specificity, they

also possess various drawbacks such as a reduced number of potential on-target sites, the need to deliver multiple guides, increasing the protein size, or typically lower on-target efficiencies. Further developments such as directed evolution of Cas9, characterization of novel orthologues,[16,65,83] or chemical modifications on the gRNA may serve as future grounds for improvement.

Given a particular gRNA, it has been difficult to characterize the subsequent level or nature of Cas9 off-target mutagenesis genome-wide. Work to date using targeted deep sequencing has been fundamentally limited by the sequence homology assumptions inherent in the computational prediction of secondary targets. Whole-genome sequencing of Cas9-edited clones can be prohibitively expensive for identifying low frequency yet potentially deleterious off-target events.[84,85] Finally, while ChIP-seq of dCas9 can identify many off-target binding sites in the genome, the vast majority of these binding sites do not appear to be actually cleaved.[86] To address these challenges, multiple groups have recently reported the development of unbiased approaches to enrich or detect Cas9 off-target events throughout the genome (Table 1). Each of these methods has unique advantages and drawbacks, highlighting the need to comprehensively evaluate the specificity of Cas9-guide RNA combinations in clinically relevant cell types and dosages across a variety of unbiased off-target detection technologies.

GUIDE-seq

GUIDE-seq is a recently developed method that relies on incorporation of an end-protected, double-stranded oligodeoxynucleotide (dsODN) into nuclease-generated breaks, presumably via the nonhomologous end-joining repair pathway.[87] Integration events are then identified through deep sequencing of a library constructed by ligating adaptors to sheared genomic DNA and PCR-amplifying with a dsODN-specific primer. While this method also relies on capture of foreign DNA to mark the sites of DSBs, incorporation of the dsODN appears to be significantly more efficient than IDLV capture (described below) and can identify sites cleaved at frequencies of less than 0.1%. Tsai et al.[87] demonstrate the broad functionality of this method by using it to analyze the off-target profile of 10 different gRNAs in 2 cell types, and show that it can also be used to detect translocations, not only between nuclease cleavage sites, but also between cleavage sites and breakage hot spots in the genome. One drawback to this method is that it relies on efficient delivery of the dsODN into cells, and it therefore remains to be seen how it could be applied *in vivo*, or to cells that are resistant to transient transfection or require viral transduction.

IDLV capture

IDLV capture relies on trapping of the viral vector into nuclease-generated DSBs, and subsequent identification of these sites by linear amplification-mediated PCR (LAM-PCR).[88] Originally described as a method for identifying off-target cleavage sites of ZFNs,[89] it has been more recently applied to both TALENs and CRISPR/Cas9.[90,91] Wang et al. employ this method to characterize the off-target activities of six novel gRNAs targeted to the *WAS* and *TAT* genes, as well as one previously characterized gRNA targeted to *VEGFA*.[91] Deep sequencing to quantify indel frequency revealed that this method is capable of identifying off-target sites that are cleaved at a frequency greater than 1%, likely because of the background integration rate of IDLVs, although the authors suggest that sensitivity can be increased by pooling a larger number of IDLV-containing colonies.

High-throughput genome-wide translocation sequencing

The previously published high-throughput genome-wide translocation sequencing (HTGTS) method[92] has been recently optimized and applied to the identification of CRISPR/Cas9-induced off-targets.[93] This method relies on the fact that, at variable frequencies, all DSBs have the potential to lead to chromosomal translocations, and the translocation junctions can be used to identify the sites of DSBs in the cell. Amplifying an endogenous "bait" sequence, which can be the on-target site, a known off-target site, or the target site of an additional nuclease that is co-introduced into the cells, allows for capture of "prey" sequences (genomic regions that have been joined to the bait sequence via translocation) by LAM-PCR. Frock et al. use this method to characterize the specificity of four novel gRNAs targeted to the *RAG1* gene, as well as two previously published *EMX1* and *VEGFA* gRNAs.[93] The authors note that because of the effects of 3D proximity on translocation frequency, HTGTS is much more likely to identify off-target sites on the same chromosome as the bait sequence, a bias that could be mitigated by performing the assay using a panel of bait sites from each chromosome.

BLESS

BLESS (direct *in situ* breaks labeling, enrichment of streptavidin, and next-generation sequencing) is an *in situ* assay that captures DSBs by

Table 1. *Comparison of genome-wide methods for assessing Cas9-induced DNA double-stranded breaks*

Method	*Major features of technology*	*Method of DSB capture*	*Time scale of DSB capture*	In vivo *applicability*	*Sensitivity*
Genome-wide, unbiased identification of DSBs enabled by sequencing (GUIDE-seq)[87]	Simple and straightforward protocol Requires phosphorothioate protection of bait oligonucleotide Cells need to be efficiently transfected or nucleofected with bait oligo May not work for non-DSB lesions such as DNA nicks	Oligo integration into DNA break site Bait: double-stranded DNA oligonucleotide (dsODN)	Oligo integration events accumulate post-Cas9 expression until genomic DNA extraction	Not as currently configured, unless the dsODN is delivered via nonviral particles	Reported 0.1%
Integrase-deficient lentiviral vectors for off-target detection (IDLV capture)[91]	Simple and straightforward protocol Cells containing viral integrant can be enriched via antibiotic selection Useful for cell types easily transduced by lentivirus IDLV retains a background nonspecific integration rate, likely leading to lower sensitivity than other methods	Viral genome tagging of DNA DSB Bait: Integrase-deficient lentiviral vector (IDLV)	IDLV integration events accumulate post-Cas9 expression/IDLV infection until genomic DNA extraction	Yes, assuming high transduction levels and sufficient target IDLV capture relative to background integration rates	Reported 1%
High-throughput, genome-wide translocation sequencing (HTGTS)[92]	No exogenous bait needs to be introduced Translocation events preferentially occur between DSBs on the same chromosome, potentially biasing relative off-target frequencies	Translocation-based sequencing, using Cas9-induced DSBs as bait for translocation events between on- and off-target cleavage sites	Translocations accumulate post-Cas9 expression until genomic DNA extraction	Yes, assuming sufficient cell number to capture low-frequency translocation events	Not reported
Direct *in situ* breaks labeling, enrichment on streptavidin, and next-generation sequencing (BLESS)[16]	No exogenous bait needs to be introduced Direct measure of DNA DSBs present in the assayed population of cells Sensitive to time of cell fixation, as DNA DSBs at on- and off-target sites may occur with different kinetics Nuclease-induced DSBs can be computationally separated from naturally occurring, endogenous DSBs	Biochemical ligation of sequencing adapters Requires cell fixation and nuclear deproteination to free genomic DNA ends for adapter capture	DNA breaks are captured at time of cell fixation post-Cas9 exposure (time between introduction of nuclease and fixation of cells may be optimized for maximum break detection)	Yes, assuming sufficient cell number for sensitive adapter ligation	Not reported
In vitro nuclease-digested whole-genome sequencing (Digenome-seq)[95]	Requires whole-genome sequencing at high coverage for four different sets of genomic DNA per Digenome-seq assay Does not account for cellular factors that may influence off-target cleavage (e.g., chromatin context, transcription status) because genome is digested *in vitro*	Computational identification of Cas9-induced cut sites via alignment of 5′ end of sequencing reads	Mutations accumulate in cells post-Cas9 expression until genomic DNA extraction in nuclease-treated cells	Yes, assuming sufficient cell number and using nuclease-treated and mock-treated tissues as the two input DNAs	Reported 0.1%

DSBs, double-stranded breaks.

chemically fixing the cells, purifying the chromatin, and ligating biotinylated DNA linkers to all available DNA break sites.[94] PCR amplification of the ligated linkers enables specific enrichment for transient DSB sites present at the time of cell fixation, which can then be quantified via deep sequencing. Unlike methods such as GUIDE-seq and IDLV capture, which should be able to continuously capture DSBs between the time of transfection and genomic DNA extraction, BLESS is more sensitive to experimental timing as nuclease-induced breaks will be quickly repaired by the NHEJ pathway. Additionally, it is likely sensitive to Cas9-specific kinetics at on- and off-target sites, as well as any ligation bias because of local sequence or chromatin composition near the break site. This method has been applied to map cleavage events induced by the I-SceI endonuclease, as well as by Cas9 nucleases derived from *S. pyogenes* and *S. aureus.*[16] Encouragingly, the frequency of BLESS-enriched sites exhibited a strong correlation to their corresponding indel levels, demonstrating that it can accurately detect the presence, and also rank order, of genomic off-target sites.

Digenome-seq

Digenome-seq is a method that assays whole-genome cleavage activity of Cas9 by a mixed approach involving cellular Cas9 nuclease-mediated genome editing followed by a cell-free or *in vitro* Cas9 nuclease-mediated digestion of isolated genomic DNA from the treated and untreated control cells.[95] The *in vitro* digested genomes are then subjected to whole-genome sequencing at high coverage to identify the sites of Cas9 nuclease activity.

Identification of the sites from whole-genome sequencing data is enabled by counting the number of WGS reads with 5′ positions that either begin or terminate at each position in the genome. At the high coverage used in digenome sequencing, sites of *bona fide* Cas9 activity have large clusters of forward and reverse reads that originate or terminate at the same position. Examining these regions and comparing the read counts in treated and untreated samples, all of which were digested *in vitro*, allows for a measurement of cellular gene editing.

Kim et al. use this method to interrogate *HBB*- and *VEGFA*-targeting gRNAs in HAP1 and K562 cells, reporting a detection sensitivity of indel frequencies as low as 0.1%.[95] Although Digenome-seq requires high sequencing depth and multiple WGS libraries, the ability to digest the genome to completion *in vitro* may be a potential advantage because it identifies a large set of potential off-targets that can then be checked *in vivo* by targeted deep sequencing.

CONCLUSIONS

The CRISPR/Cas9 system is a powerful genome editing technology with the potential to create a variety of novel therapeutics for a range of diseases, many of which are currently untreatable. The first application of therapeutic genome editing has entered clinical testing,[63] While it remains to be seen which disease indications are most suited for CRISPR/Cas9-mediated genome editing, it is clear that optimization of delivery and assessment of specificity are critical for the safe and effective clinical translation of this technology. Traditional gene therapy programs are also paving the way for the use of integrating and nonintegrating viral delivery vectors, and these approaches will likely be applicable to CRISPR/Cas9 therapeutic editing. Nonviral delivery methods, such as electroporation, may be among the first strategies used for *ex vivo* editing in the cell therapy setting. Lipid-based formulations, such as those used for siRNA delivery, could also prove useful for local *in vivo* editing. Regardless of the delivery approach, measuring and understanding the relationship between efficacy and specificity will be an important aspect of ongoing CRISPR/Cas9 clinical development.

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AUTHOR DISCLOSURE

J.L.G., P.D.H, M.L.M., S.S., G.G.W., and D.B. are employees of Editas Medicine, which is developing therapeutics based on CRISPR/Cas9 genome editing technology.

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