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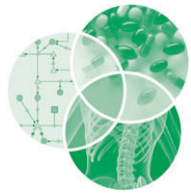

Therapeutic genome engineering via CRISPR-Cas systems

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Differences in genomes underlie most organismal diversity, and aberrations in genomes underlie many disease states. With the growing knowledge of the genetic and pathogenic basis of human disease, development of safe and efficient platforms for genome and epigenome engineering will transform our ability to therapeutically target human diseases and also potentially engineer disease resistance. In this regard, the recent advent of clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas) RNA-guided nuclease systems have transformed our ability to target nucleic acids. Here we review therapeutic genome engineering applications with a specific focus on the CRISPR-Cas toolsets. We summarize past and current work, and also outline key challenges and future directions. © 2017 Wiley Periodicals, Inc.

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INTRODUCTION

Gene therapy entails altering, replacing, or regulating the expression of affected genes to a degree that reverses a diseased phenotypic state. In principle, therapeutic interventions can be effected at two levels: *in vivo* or *ex vivo*. *In vivo* gene therapy involves local or systemic administration of a therapeutic vector, while *ex vivo* approaches involve isolating cells from the human body, such as CD34+ hematopoietic stem cells, application of gene therapy on the isolated cells, followed by re-transplantation of these cells back into the body (Figure 1). In this review, we discuss gene therapy in conjunction with gene transfer platforms that encompass both *in vivo* and *ex* vivo targeting modalities, with a focus on therapeutic interventions that directly perturb the genome of host cells.

In this regard, fundamental to enabling gene therapeutics are genome engineering tools that enable the ability to precisely edit or tune the regulation of genomic elements of interest. For instance, for targeted genome editing one harnesses programmable double-stranded breaks to effect precise edits in the genome. Creation of double-stranded breaks (DSBs) can be resolved by the cell via one of two processes: homologous recombination (HR) or nonhomologous end-joining (NHEJ). Whether a cell repairs the break through HR or NHEJ depends on several aspects, including the cell's cycle phase and whether a homologous donor is present or not. Specifically, one can effect HR by providing a synthetic homologous donor to introduce a desired sequence of DNA or to create specific point-mutations.[1] The alternative route for DSB repair is via NHEJ, which unlike HR is active throughout the cell cycle. In NHEJ, an indel (insertion or deletion) or substitution is created. Importantly, if this occurs in a coding exon, the translational reading frame of a gene can be disrupted, which can lead in turn to an inactive or truncated protein.[2]

Early genome engineering methods were based on programmable nucleases, such as zinc finger nucleases (ZFNs) and subsequently transcription activator-like effector nucleases (TALENs). ZFNs are comprised of zinc finger proteins (ZFPs), which are DNA binding domains frequently found in eukaryotic transcription factors,[3] that are fused to a DNA-cleavage domain (typically FokI). The ZFP region of ZFNs typically contains Cys2-His2 fingers which

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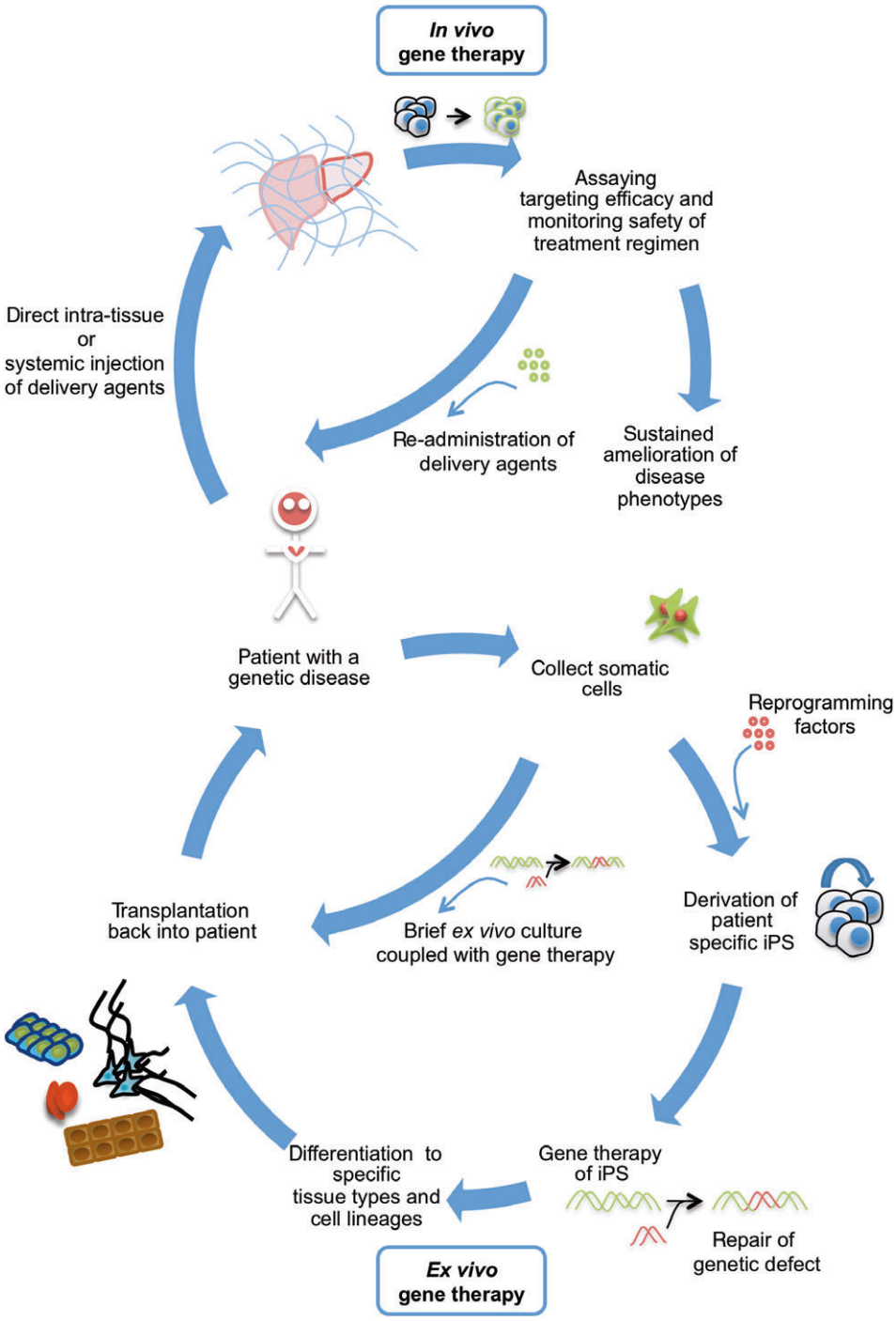

FIGURE 1 | Schematic of *in vivo* and *ex vivo* gene therapy modalities. *In vivo* gene therapy involves the direct intra-tissue or systemic injection of delivery agents, followed by assaying of targeting efficacy with close monitoring of safety and efficacy of treatment. In this regard, re-administration of delivery agents might be necessary to achieve therapeutic efficacy. A patient might also be treated via *ex vivo* gene therapy, where patient somatic cells are isolated and either (1) reprogrammed into patient-specific iPSCs, followed by gene therapy of these cells, and which are then differentiated into specific tissue types and cell lineages for transplantation; or (2) edited via *ex vivo* culture coupled with gene therapy (for instance in HSCs).

predominantly interact with nucleotide triplets, and combinations of ZFPs can thus be arranged to recognize a wide range of DNA sequences.[4] ZFNs induce DSBs in a targeted fashion via the DNA-cleavage domains tethered to this engineered sequence. An important advantage of utilizing ZFNs is their specific targeting, which is due to two independent binding events that must occur in order for FokI to dimerize before cleaving DNA.[5] On the other hand, ZFNs are difficult to engineer, requiring a high level of technical expertise.[4]

TALENs utilize proteins derived from transcription activator like effector (TALE) repeat domains from bacteria, such as *Xanthomonas*, which are comprised of highly conserved amino acid repeat domains of 33–35 residues in length, each targeting a single nucleotide, fused in turn to a FokI nuclease.[6] TALENs are easier to engineer than ZFNs and have been observed to similarly induce high efficiency genome editing with limited cytotoxicity.[7] TALENs relative to ZFNs are however significantly larger in size, and also have repetitive sequences which complicates their construction and incorporation into delivery systems.[8] Furthermore, they have a prokaryotic origin and can potentially elicit a negative immune response. Taken together, ZFNs and TALENs are robust genome engineering systems, but have certain drawbacks such as difficult programmability and/or limited multiplex genome targeting capabilities. A more detailed review on these platforms can be found in Ref 8.

More recently, the emergence of CRISPR-Cas systems has dramatically transformed our ability to target nucleic acids. In comparison to ZFNs and TALENS, CRISPR-Cas systems depend on simple Watson-Crick base-pairing between a RNA guide and a corresponding DNA target site making them remarkably simple to re-engineer. In addition, CRISPR-Cas systems can be utilized for multiplex targeting, which is especially useful when creating disease models or targeting complex diseases in which multiple loci are affected. Their ease of use, coupled with low cost, high efficiency, and broad versatility has resulted in the CRISPR-Cas systems rapidly becoming the genome engineering method of choice. In this review, we will focus on this system and provide a brief overview of CRISPR-Cas technologies for genome editing and regulation (Figure 2), and also outline the latest advances in CRISPR-Cas mediated *ex vivo* and *in vivo* genome manipulations. We will discuss appropriate delivery approaches for these, and also delineate the current challenges of existing platforms and outline potential applications and areas of further research and development.

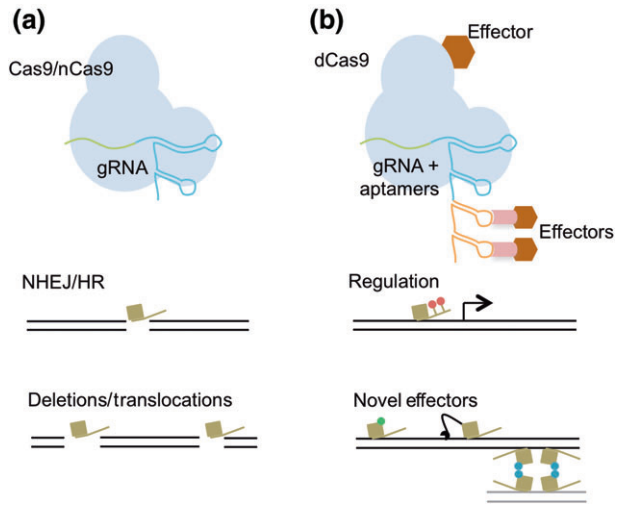

FIGURE 2 | The CRISPR-Cas9 genome-engineering toolset. (a) Wild-type Cas9 induces double-stranded DNA breaks, which the cell repairs through either nonhomologous end-joining (NHEJ) or homologous recombination (HR) pathways. A mutated version of Cas9, 'nickase' Cas9, nCas9, is created by mutating one of the two catalytic sites, typically the RuvC domain, which results in engineering of only single stranded breaks. Modifications by Cas9 and nCas9 can used to also engineer genomic deletions or translocations. b) One can also utilize dead-Cas9, dCas9, with both of its catalytic sites mutated, RuvC and HNH. dCas9 can in turn be tethered to effectors, such as activation or repression domains, to induce targeted genome regulation. In addition, other novel effectors can be utilized, such as fusion to the cytidine-deaminase enzyme for targeted 'base editing.'[9]

CRISPR-CAS SYSTEMS

CRISPR-Cas systems have evolved as adaptive immune defense systems in bacteria and archaea. These systems function in three phases: adaptation, expression, and interference, and typically utilize short RNA to direct degradation of foreign nucleic acids. During adaptation, short pieces of foreign DNA are acquired and integrated as 'spacer' elements into the CRISPR loci. During expression and interference, the CRISPR locus, which consists of acquired 'spacers' separated by repeat regions, is transcribed, which yields a pre-crRNA, which in turn is processed to generate crRNAs that guide effector nuclease complexes (*cas* proteins) to disrupt sequences that are homologous to the spacer.[10–12] In type II Cas9 systems, such as that of *Streptococcus pyogenes* Cas9 (SpCas9), pre-crRNAs are processed with the help of a trans-activating crRNA (tracrRNAs), to create a tracrRNA:crRNA:Cas9 complex that induces double-stranded breaks.[13] Notably the tracrRNA and crRNA can be fused to create a single guide RNA (gRNA). This consists of two functional regions: a variable spacer region which guides target

loci recognition, and a constant scaffold region which forms hairpin loops to facilitate binding to the Cas9. The variable spacer region is typically a short ~20 bp sequence that is complementary to the target loci, and must be flanked at the 3′ end by a conserved protospacer adjacent motif (PAM) sequence, for instance, NGG in SpCas9.[14] The PAM sequence is necessary for CRISPR-Cas targeting and also crucial for self vs. nonself discrimination.[15] By delivering crRNAs in addition to the Cas9-like effectors, one can thus readily enable programmable genome engineering.[16] Indeed the CRISPR-Cas9 system has now been shown to work in a range of eukaryotic systems,[16–18] and has also greatly facilitated our ability to engineer genomes in diverse cell types and organisms (Table 1). Some organisms that have been targeted *in vivo* include mice, rats,[19] rabbits,[20] and also nonhuman primates.[21]

Notably, one can also engineer nuclease-dead versions of Cas9 (dCas9) via point mutations in the endonuclease domains, HNH and RuvC. This engineered protein retains RNA-guided DNA binding activity but lacks endonuclease activity.[42,43] dCas9 coexpressed with a gRNA can be utilized for gene repression as it interferes with transcriptional elongation, RNA polymerase binding, or transcription factor binding.[44] Additionally, dCas9 can also be fused to effector domains and be programmed to enable diverse genome engineering functionalities such as gene repression (via KRAB fusions[43]), or gene activation, (such as via fusions of VP64, Rta, and P65 or combinations thereof),[45] and furthermore for targeted chromatin modulation by acetylation or methylation via fusions of methyltransferase (DNMT3A) and acetyltransferase (p300).[46,47] Another strategy is to fuse dCas9 to a multimeric tag such as 'Suntag,' which comprises of a repeating peptide array which can in turn recruit multiple copies of effector domains.[48] A primary drawback of utilizing dCas9 for permanent gene activation or repression is that it needs to be continuously expressed, whereas nuclease-Cas9 can enable irreversible changes in the genome, even with limited expression. Because of this, a patient would necessitate repeat treatments of dCas9. However, an advantage to utilizing dCas9 for genome activation over traditional methods is that one can activate/repress genes in a multiplexed manner, which could be beneficial for complex diseases that have multiple loci involved, or one could target genes that are otherwise difficult to edit. Additionally, since dCas9 lacks endonuclease activity, there is no permanent change to the genome[49] and thus off-target effects can also be avoided by using this system.

The CRISPR-Cas toolset has also been rapidly expanding with characterization of several new Cas9-like proteins. For instance, recently a class 2 type V CRISPR-Cas system, Cpf1, (CRISPR from *Prevotella* and *Francisella* 1), has been shown to effect robust *in vitro* genome editing in human cells.[50] Some differences between Cpf1 and other Cas9 proteins is that it does not require an additional trans-activating crRNA (tracrRNA),[51] and that it creates a 4–5 nt staggered dsDNA break distal to its T-rich PAM, which could thus expand the range of potential targetable genomic sites. A recent paper compared the specificity and efficiency of Cpf1 with Cas9. They first tested four Cpf1 orthologs, and found *Acidaminococcus sp. BV3L6* (AsCpf1) and *Lachnospiraceae bacterium N D2006* (LbCpf1) to be the most efficient. They then went on to compare the frequency of targeted mutations by LbCPf1, AsCpf1, and SpCas9 at ten chromosomal target sites, each containing two PAM sequences, one recognized by Cpf1 (5′-TTTN-3′) and the other recognized by SpCas9 (5′-NGG-3′). They discovered that Cpf1 is a less efficient endonuclease than SpCas9 in some human cell lines, but is highly specific.[52] Notably, mutant mice have also been generated by injecting crRNAs with Cpf1 (AsCpf1 and/or LbCpf1) mRNA into fertilized eggs and do not exhibit overt off-target effects.[53]

DELIVERY APPROACHES

To realize the versatility of CRISPR-Cas systems in genome engineering applications, appropriate delivery systems for the same will be critical. In fact, a key technological barrier to gene-based therapies has been the development of efficient and safe delivery systems. Towards this, both viral and nonviral methods have been utilized, with most vectors utilized in gene therapy clinical trials being engineered viruses, and below we discuss these technologies in the context of delivery of CRISPR-Cas for gene therapy (summarized in Table 2).

Viral Delivery Systems

Viral vectors, such as retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses (AAVs) have naturally evolved to transduce mammalian cells efficiently, and consequently have been a preferred format for gene delivery over the past few decades. These vector systems can in turn be broadly divided into two major classes, those whose genome integrates into the host chromatin, such as retroviruses and lentiviruses, and those that persist inside the host

TABLE 1 | Representative Table of *In Vivo* and *Ex Vivo* CRISPR-Cas9 Studies, via a Range of Viral and Nonviral Delivery Methods

Genes/Disease	Delivery System	Ortholog	Organism	Tissue Type	Reference	Ref. Num.
Representative *in vivo* and *ex vivo* CRISPR-Cas9 studies						
Tet1, Tet 2	Injected Embryos	SpCas9	Mice	–	Wang et al., Cell, 2013	22
P53, Pten	Hydrodynamic injection	SpCas9	Mice	Liver	Xue et al., Nature, 2014	23
Fah/Tyrosinemia Type I	Hydrodynamic injection	SpCas9	Mice	Liver	Yin et al., Nature Biotechnology, 2014	24
Pcsk9	Adenovirus	SpCas9	Mice	Liver	Ding et al., Circ Res., 2014	25
NeuN	AAV1/2	SpCas9	Mice	Brain	Platt et al., Cell 2014	26
DMD (Duchenne Muscular Dystrophy)	Injected Zygotes	SpCas9	Mice	Muscular Skeletal Tissue	Long et al., Science, 2014	27
X-linked androgen receptor	Injected Embryos	SpCas9 nickase	Mice	–	Shen et al., Nature Methods, 2014	28
Pten, NKx2-1	Lentiviruses	SpCas9	Mice	Lung	Sánchez-Rivera et al., Nature 2014	29
Kras, p53, Lkb1	AAV9	SpCas9	Mice	Lung	Platt et al., Cell, 2014	26
Tet2, Runx1, Dnmt3a, Ezh2, Nf1, Smc3, p53,Asxl1	Lentiviruses	SpCas9	mHSCs	Myeloid	Heckl et al., Nature Biotech, 2014	30
MeCP2/Rett syndrome, Dnmt3a, Dnmt1, Dnmt3b	AAV 1/2	SpCas9	Mice	Brain	Swiech et al., Nature Biotechnology, 2015	31
Pcsk9, ApoB	AAV9	SaCas9	Mice	Liver	Ran et al., Nature, 2015	32
Apc, Trp53	Hydrodynamic injections	inducible SpCas9	Mice	Intestine/ Thymus	Dow et al., Nature Biotech, 2015	33
Eomes	*in utero* Electroporation	SpCas9	Mice	Brain	Kalebic et al., EMBO Rep., 2016	34
DMD	AAV9	SaCas9	Mice	Muscular Skeletal Tissue	Tabebordbar et al., Science, 2016	35
DMD	AAV8	SaCas9	Mice	Skeletal and Cardiac Muscle	Nelson et al., Science, 2016	36
DMD	AAV9	SpCas9	Mice	Skeletal and Cardiac Muscle	Long et al., Science, 2016	37
OTC	AAV8	SaCas9	Mice	Liver	Yang et al., Nature Biotechnology, 2016	38
EGFP	Cationic Liposomes	SpCas9	Mice (Atoh1-GFP)	Ear	Zuris et al., Nature Biotechnology, 2015	39
Ptch1; Trp53,Pten,Nf1	Cationic Polymer	SpCas9	Mice	Brain	Zuckermann et al., Nature Communications, 2015	40
B2M and CCR5	Electroporation	SpCas9	CD34+ HSCs and CD4+ T cells	HSCs transplanted into NSG mice	Mandal et al., Cell Stem Cell, 2014	41

TABLE 2 | Methods of Gene Therapy Delivery

Mode of Delivery	Duration of Expression	Risk of Genomic Integration	Immunogenicity[1]
Retro-viruses	Long term	Yes (observed oncogenecity)	Low
Lenti-viruses	Long term	Yes (low oncogenecity)	Low
Adenoviruses	Medium term	Low	High
Adeno-associated viruses	Medium term	Low	Low
Mini-circle Plasmids	Short term	Low	Low
Conventional Plasmids	Transient	Low	Low
Proteins	Transient	No	Under evaluation
RNA	Transient	No	Low
Nanoparticles/biomaterials	Transient	Low	Under evaluation
Liposomes	Transient	Low	Under evaluation

Some key factors distinguishing current delivery methods are outlined: duration of expression, risk of genomic integration (which can lead to oncogenicity), and immunogenicity (which is especially important if repetitive gene therapy treatments are required).
Observed efficiency of genome targeting is also a function of innate properties of the target cells, in particular the mitotic state (Ref 38,58,113), and ability to efficiently deliver the genome engineering effectors (Ref 58,113).
[1] Immunogenicity is also a function of the delivery payload.

nucleus or cytoplasm, such as adenoviruses, AAVs, sindbis, and sendai viruses.[54] Each of these systems however come with certain tradeoffs, for instance, retroviral integration into the host chromosome can lead to increased frequency of random mutagenesis and oncogene activation,[55] but corresponding delivery systems are also best suited for long-term stable expression of their payloads. Thus nature of the application often governs choice of the delivery chassis and below we highlight some prominent viral systems relevant to CRISPR-Cas.

A preferred viral delivery system for gene therapy is AAVs. These have been widely utilized for gene therapy due to their overall safety, mild immune response, long-term transgene expression, high-infection efficiency, and are now already being used in clinical trials.[56] A primary drawback of AAVs, however, is that they have a limited packaging capacity of around 4.7 kb, making it difficult to deliver large Cas9-like effector proteins such as the *Streptococcus pyogenes* Cas9 (SpCas9), with a size of around 4.2 kb, a single gRNA, and other components necessary for effective transcription.[57,58] In order to overcome this packaging issue, distinct solutions have been suggested. For instance, smaller Cas9 orthologs, such as *Straphylococcus aureus* Cas9 (SaCas9), with ~3.3 kb has been successfully packaged into a single AAV,[32] and another small ortholog, *Streptococcus thermophilus* (St1Cas9) with ~3.3 kb[59,60] could also potentially be packaged into a single AAV. However, a potential issue with smaller orthologs is it that they require more complex PAM sequences: SaCas9 recognizes the 5′-NNGRRT-3′ PAM and St1Cas9 recognizes the 5′-NNAGAAW-3′ PAM, which restricts the range of targetable sequences.[32] Moreover, these orthologs are not all equally robust. To circumvent this issue, the PAM sequence can be engineered utilizing structural information, such as been done in *Francisella novicida* FnCas9, in which its PAM 5′-NGG-3′ was altered to a more relaxed 5′-YG-3′ PAM.[61] In another study, SpCas9 was modified to recognize alternative PAM sequences via bacterial directed evolution, structural information, and combinatorial design.[62] Alternatively truncated SpCas9 proteins have been designed[63,64] utilizing the SpCas9 crystal structure,[65] however these do not retain the robustness of their wild-type counterpart. In an elegant study to circumvent this, a split-Cas9 system was recently designed which takes advantage of inteins from *Nostoc punctiforme*. This design utilizes a two-vector system where each half of Cas9 is fused to a corresponding split-intein moiety, and upon co-expression intein-mediated trans-splicing occurs and the full SpCas9 protein is reconstituted.[66] Additionally, some other studies have also utilized a dual-AAV system, where one AAV is utilized to deliver the sgRNA and the other to deliver the SpCas9.[38,67] The drawback to these dual-AAV systems, however, is that both AAVs must be delivered to the same cell to elicit an effect, thereby decreasing the efficiency or alternatively entailing the use of significantly higher viral titers. In summary, while AAVs are a preferred mode of delivery for Cas9-gRNA agents, further studies on serotype engineering and Cas9 orthologs must be done to

deliver all desired components and fusions thereof efficaciously.

Another versatile delivery system relevant to CRISPR-Cas are lentiviruses, which have also been widely utilized for gene therapy. Lentiviruses, originally adapted from HIV-1, are highly potent viral vectors due to their broad tropism, their large cargo capacity (9.7 kb), and the fact that they can infect post-mitotic cells.[68] Lentiviruses can thus be readily utilized to deliver Cas9 and other components necessary for genome editing via a single construct.[69] Additionally, the tropism of lentiviruses can be easily altered by the addition of envelope proteins targeting distinct cell surface receptors. This pseudotyping is ideal for transducing certain cell types or tissues that are otherwise difficult to transduce. Lentiviruses have also been widely utilized for *ex vivo* gene therapy, specifically in hematopoietic stem cells (HSCs) and T lymphocytes (T cells). In a recent review, some relevant genetic therapy clinical trials were highlighted in which lentiviruses were safely utilized to treat Wiskot-Aldrich syndrome, β-Thalassaemia, and various T-cell immunotherapy for cancer.[70] Additionally, some *in vivo* studies utilizing CRISPR-Cas systems have also demonstrated robust gene editing (Table 1). It should be noted nevertheless that while lentiviruses are preferred due to their high efficiency of delivery and long-term expression of payload, their genomic integration, however, is unfavorable due to its potential mutagenic effects, or silencing/activation of neighboring loci.[71] In this regard the newest generation of integrase-deficient lentivirus vectors (IDLV), which carry mutations in the integrase and viral LTRs greatly reduce the risk of insertional mutagenesis, however these do not completely eliminate this risk.[72–74] Additionally, immunogenicity is still a risk.[75] Furthermore, the consequences of Cas9 integration into the genome by lentiviruses are not yet fully understood, and although advances in lentiviruses have been made,[76] there may still be risks of having a nuclease integrate into the genome. Lentiviruses, however, could be utilized for the delivery of nuclease-deficient dCas9, or nonintegrating versions of lentiviruses may be utilized for hit-and-run nuclease applications.

Nonviral Delivery Systems

Complementary to the viral systems are a host of nonviral delivery systems that have been developed over the past few decades. These include methods utilizing nanoparticles such as cationic nanocarriers,[39] liposomes, and polymeric materials. An advantage to these systems is their overall safety, low immune response, large loading capacity, and general ease of production. On the other hand, nonviral methods utilized to deliver DNA have to surpass many physical barriers imposed by the cell to efficiently deliver genomes to the cell.[77] However, given the nearly complete control over their synthesis and constituents, one could potentially engineer nanoparticles or polymeric materials to target diseased cells specifically, for instance by coating them with ligands that are specific to receptors on target or diseased cells, as has been done for drug delivery and imaging.[78]

In this context, both Cas9 and gRNA are anionic, which allows their ready integration into cationic liposomes or polymers as a delivery system.[79] Correspondingly there have been several recent studies utilizing nanoparticles for CRISPR-Cas9 delivery. For instance, a recent study developed bioreducible lipid nanoparticles to deliver Cas9-sgRNA *in vitro* with genome efficiencies of 70%.[80] Additionally, a Cas9:gRNA targeting EGFP complex was delivered *in vivo* to Atoh1-GFP mice ear hair cells via cationic lipids, enabling up to 20% editing rates.[39] This study demonstrated that by modifying protein charges of Cas9-sgRNA complexes, one can successfully deliver these via cationic lipids. Additionally, due to the fact that the Cas9–sgRNA complex is delivered as a protein, with a short half-life, the risk of potential off-target effects can be significantly reduced.[81] These approaches thus have huge potential for utilization in gene therapy applications.

Another alternative approach commonly used for delivery is hydrodynamic injections – these are high volume injections (8–10% body weight of mice) delivered into the vasculature at high speeds (5–-7 seconds), to enable the delivery of naked DNA or siRNA. Hydrodynamic injections delivering CRISPR-Cas9 have since been utilized in several *in vivo* CRISPR-Cas9 studies targeting the liver.[23,24] Hydrodynamic injections, however, are not considered to be clinically feasible due to the potential damage these inflict on the liver and the heart.[82]

CHALLENGES AND FUTURE DIRECTIONS

While CRISPR-Cas systems have been widely utilized for genome engineering, several key challenges must to be addressed before these tools can be utilized for efficacious gene therapy. For instance, an important challenge for *in vivo* CRISPR-Cas therapy is the ability to achieve immune stealth in order to minimize dosage and enable re-administration of nucleic acid or protein therapeutics. Classic immunosuppression

for the duration of treatment is one option, but this will be less useful for long-term genome regulatory modifications. Therefore, the delivery vector of choice must be capable of high transduction efficiency while avoiding immune responses of both of the delivery system and the CRISPR payload. In this regard, Table 2 describes the distinct delivery systems, both viral and nonviral, and their known immunogenicity and levels of payload expression. For example, although adenoviruses (Ad) or retroviruses have the advantage of a high packaging capacity, their high immunogenicity may lead to unwanted side effects.[83,84] A study found that inflammatory cytokines and chemokines were activated by Kupffer cells in the liver and MARCO+ macrophages in the spleen in as early as ten minutes post intravenal injection of adenoviruses in mice.[85] On the other hand, AAVs have lower immunogenicity in comparison, but have a small packaging limit. Although viruses are a preferred delivery system due to their efficacy in infecting cells and tissues, the low immunogenicity of nonviral vectors, such as nanoparticles could be a preferred method in the future. Nonviral vectors, however, require further engineering to improve levels of transduction and transient expression *in vivo*.[86,87] It is important to note that not only is the immune response an important consideration for delivery systems, but for the CRISPR-Cas9 payload as well. For instance, Cas9 is a foreign prokaryotic protein, and could potentially elicit a strong immune response. A recent study demonstrated that Cas9 indeed evokes cellular and humoral immune responses, with Cas9-specific antibodies elicited post exposure.[88] Therefore, if a patient were to necessitate repetitive treatments, CRISPR-Cas efficacy might be reduced by the body's immune response.

Yet another important challenge to overcome is specificity, or reducing off-target genomic effects. Specifically, in order to conduct safe clinical treatments, off-target mutations must be reduced in order to avoid perturbation of areas of the genome with unknown effects. Various groups have correspondingly characterized SpCas9 specificity[89–91] and several strategies to improve Cas9 specificity have been reported, including minimizing the amount of Cas9 in the cell,[89,92] utilizing a nickase Cas9,[43,93] and utilizing dCas9 fused to a FokI nuclease domain.[94,95] An important factor that can contribute to reducing off-target effects is also the design of gRNAs. Studies on the specificity of SpCas9 have demonstrated that mismatches between gRNA and target DNA can be tolerated.[43,57,89,95] Moreover, the farther mismatches are from the PAM sequence, the more tolerated that they are.[57,91] Additionally, several *in silico* methods have been developed to design gRNAs[96–99] including easily accessible online sources, for example, Benchling (http://benchling.com), which detail the number of mismatches each gRNA will have, in order to aid the design of the optimal gRNAs for genome engineering. Importantly, one also needs to balance gRNA activity with specificity. For example research has indicated that guanine rich gRNA sequences, specifically close to the PAM sequence, are favorable, while cytosines are unfavorable.[99] Furthermore, truncated gRNAs of ~17 nt have been shown to have lower off-target effects than a ~20 nt gRNA.[100] Another strategy that has been utilized to increase specificity is using a nickase version of Cas9. Briefly, SpCas9 creates double stranded breaks through two catalytic domains: RuvC and HNH domains. By editing one of these two domains, one can create a nickase, which only creates a single-strand break. By designing two gRNAs, one in the sense and one in the antisense direction, one can then create targeted double-stranded breaks. Since two gRNAs are involved, the probability of off-target effects is greatly reduced, as off-target single nicks are typically repaired scarelessly by the cell.[43,101] Another alternative is to utilize an engineered Cas9, such as the high-fidelity Cas9 (SpCas9-HF1)[102] or 'enhanced specificity' SpCas9 (eSpCas9).[103] The SpCas9-HF1 was engineered based on the hypothesis that there is excess energy between the SpCas9–gRNA complex and the DNA target, and that disrupting the Cas9–target DNA interactions might minimize off-target effects. To this effect, an SpCas9 with the four amino acid mutations, N497A, R661A, Q695A, and Q926A, deemed SpCas9-HF1, reduced off-target effects, while maintaining on-target activity.[102] In another study, the eSpCas9 was rationally designed based on the crystal structure of SpCas9, with the hypothesis that disrupting positive charges between the domains that are involved in stabilizing the nontarget DNA strand, will weaken nontarget binding. Correspondingly they disrupted interactions between SpCas9 and the nontarget DNA strand by neutralizing the positive charges between these domains. Their results demonstrated reduced off-target activity while maintaining on-target activity.[103] In addition to Cas9 engineering, one can also deliver Cas9 as a protein instead of as a vector, since reducing the temporal pulse of Cas9 expression can greatly reduce off-target effects.[81] Finally, one can also utilize genetic circuits to enable temporal control of Cas9, such as small-molecule regulated approaches.[33,104–108] In addition to temporal control, tissue-specific expression, or spatial control, is another important challenge. Here tissue-specific promoters can be utilized.[109,110]

Additionally, one can engineer targeted integrations at a specific locus, for example, the albumin locus, which leads not only to high transgene expression, but also is tissue specific to the liver.[111] Moreover, by engineering viral vectors to target specific tissues, the input titers utilized could be minimized, which in turn could also potentially minimize the immunogenic response. One can therefore decrease the chance of off-target effects (genomic and tissue specific) by effective design of gRNA, and spatiotemporal control of Cas9 or gRNA expression.

Finally, basic science insights into the mechanisms of DNA repair will also be critical: specifically biasing HR versus NHEJ outcomes is a major challenge that needs to be addressed. This is critical since for safe gene repair one needs to enable primarily HR events with essentially no concurrent NHEJ events (which could instead mutagenize the target region of interest). This aspect is further complicated by the fact that the majority of the human body is post-mitotic and correspondingly HR machinery and hence activity is significantly diminished. Inspiration for new technologies to address this may come from nature, for instance in *E. coli* the lambda red system can be harnessed to enable DSB independent recombineering,[112] or by coopting transposase or recombinase mediated approaches. On the other hand, growing mechanistic insights into underlying pathways will also be critical. Indeed in a pioneering study, investigation of the mechanism for suppression of HR in G1 phase deduced that the mechanisms which inhibit HR in G1 include suppression of DNA-end resection coupled with a block in recruitment of BRCA2 to DNA sites with damage, and leveraging this knowledge the authors were able to create conditions to stimulate HR in G1.[113] In another strategy, Cas9 was controlled in a cell-cycle temporal manner by fusing its N-terminal to human Geminin, converting it into a substrate for APC/Cdh1, the E3 ubiquitin ligase complex, thereby regulating Cas9 expression to S/G2/M phases, by degrading Cas9 in the G1 phase.[114] Another interesting recent approach here was to inhibit NHEJ by targeting of the DNA ligase IV enzyme with the inhibitor Scr7.[115] Alternatively, as indicated earlier one can also forego HR/NHEJ pathways altogether. A recent paper developed 'base editing,' which enables point mutations without relying on innate HR/NHEJ pathways. This method utilizes dCas9 fused to a cytidine deaminase enzyme, that mediates direct conversion of cytidine to uridine.[9] In another study, the interaction of Cas9–sgRNA complex with the target DNA was harnessed in a clever approach. The authors while measuring the Cas9 dissociation rates found that both Cas9 and dCas9 dissociate very slowly from their target. Upon investigating the release of DNA target post-cleavage closely, they observed that Cas9 held tightly to one of the two strands of the duplex, leaving the other free to anneal to complementary ssDNA. They then tested ssDNA donors and confirmed that single stranded DNA that was complementary to the released strand supported higher frequencies of gene editing, and that one could thereby systematically increase HR frequencies by tailoring the orientation, polarity, and length of the donor ssDNA to match the properties of the Cas9–DNA complex.[116]

CONCLUSION

Taken together, we believe that with the rapid progress in genome engineering toolsets based on CRISPR-Cas systems, coupled with the development of new generation of viral and nonviral delivery approaches will spur many new gene therapeutic applications. As outlined earlier, several important challenges need to be surmounted towards enabling safe and efficient gene therapy. For instance, studies need to be performed to better understand Cas9 immunogenicity. Additionally, finding the ideal delivery system for CRISPR-Cas systems with the optimal packaging limit, and efficiency also need to be taken into consideration. Finally, finding ways of eliminating NHEJ and boosting HR efficiency will further increase our abilities to target human disease, including in adults. In addition, discovery of new Cas9-like proteins from diverse organisms might further expand our toolset towards addressing some of the current challenges. We foresee that further development of CRISPR-Cas systems will eventually pave the way for gene therapeutic applications.

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