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ISSN: (Print) (Online) Journal homepage: https://cogentoa.tandfonline.com/loi/iebt20

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To cite this article: Oscar G Segurado, Ruhong Jiang & Steven W Pipe (2022): Challenges and opportunities when transitioning from *in vivo* gene replacement to *in vivo* CRISPR/Cas9 therapies – a spotlight on hemophilia, Expert Opinion on Biological Therapy, DOI: 10.1080/14712598.2022.2090241

To link to this article: https://doi.org/10.1080/14712598.2022.2090241

Published online: 21 Jun 2022.

EXPERT OPINION ON BIOLOGICAL THERAPY
https://doi.org/10.1080/14712598.2022.2090241

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PERSPECTIVE

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Challenges and opportunities when transitioning from *in vivo* gene replacement to *in vivo* CRISPR/Cas9 therapies – a spotlight on hemophilia

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ABSTRACT

Introduction: Currently, a few *in vivo* gene replacement therapies are commercially available, with many in clinical development for the treatment of some inherited monogenic diseases. These disorders arise from mutations in genes encoding essential proteins with a well understood biological function. Wide adoption of gene replacement therapies requires solid safety and efficacy profiles with demonstrable long-term durability and cost-benefit advantages vs standard therapies.

Areas covered: This expert review outlines the challenges and opportunities in treating hemophilia, including the progression from *in vivo* gene therapies toward *in vivo* gene editing, focusing on preclinical and emerging clinical data for gene editing and addressing the need for sustained and durable gene expression during hepatocyte proliferation when the liver is unable to maintain steady gene expression and protein production.

Expert opinion: *In vivo* gene editing in liver tissues may be able to rescue patients younger than 18 years who are not eligible for gene replacement therapies, with hemophilia as a prime example.

ARTICLE HISTORY
Received 11 January 2022
Accepted 13 June 2022

KEYWORDS
Gene therapy; CRISPR/Cas9; gene editing; hemophilia

1. Introduction

Inherited monogenic diseases are the main targets for gene replacement therapies, as the mutations which cause the condition are located on a single gene [1]. A representative example is hemophilia, a rare inherited hemorrhagic disorder characterized by spontaneous bleeding caused by defects in vital coagulation factors. Both Hemophilia A (HemA) and Hemophilia B (HemB) are X-linked recessive disorders that primarily affect males and are caused by defects in clotting factor VIII (FVIII) and clotting factor IX (FIX), respectively. The severity of the bleeding phenotype is related to the residual factor activity levels and categorized as severe (<1% activity), moderate (1–5%) and mild (6–40%). Bleeding episodes characteristically occur in joints (hemarthroses) but may also be present in muscles, soft tissues, and intracranially. Hemophilia patients are also at a greater risk of severe bleeding following trauma or surgery. Repeated hemarthroses leads to progressive osteochondral degeneration and eventually contractures, fixed deformity of joints and reduced quality of life [2]. Accordingly, the standard of care is to reduce bleeding through prophylactic strategies.

According to the World Federation of Hemophilia (WFH), people with hemophilia have an array of needs that require a multidisciplinary team of health professionals with experience in treating hemophilia including optimizing prophylactic therapies [3–6]. Traditionally, prophylaxis has been provided through regular intravenous infusions of plasma derived or recombinant FVIII or FIX. However, due to their relatively short half-life, infusions are needed up to every other day with the aim to maintain residual factor levels in the non-severe range (>1%) [6]. Extended half-life (EHL) coagulation factors have been developed which can reduce the frequency of prophylactic infusions, extending the half-life of FVIII 1.3–1.5-fold and FIX 3-5-fold [7,8]. Prophylactic therapy is quite efficacious, eliminating nearly all episodes of spontaneous bleeding and reducing the risk of chronic joint disease. However, a substantial proportion of patients, particularly with HemA, develop inhibitory antibodies to the infused factor requiring alternative treatments. Recently, effective prophylaxis has been demonstrated with emicizumab, a human monoclonal bispecific antibody that recognizes both activated FIX and factor X, by substituting for the cofactor function of activated FVIII. Emicizumab has the advantage of subcutaneous administration, a long half-life with weekly, bi-weekly, and monthly dosing, and efficacy in the presence or absence of FVIII inhibitors [6,9]. Nevertheless, neither factor replacement therapy nor emicizumab are curative and patients will require adherence to regular IV infusions or subcutaneous dosing for their entire lives to prevent spontaneous bleeding and associated complications.

Hemophilia is an appealing target for liver-specific *in vivo* gene replacement therapies as endogenous expression of FVIII or FIX activity would resolve the symptoms of the disease [7,10]. The key premise for gene replacement therapies is to provide safe, durable, and stable transgene expression while avoiding the challenges of clotting factor replacement

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Article highlights

- Inherited monogenic diseases may be an ideal target for *in vivo* gene editing in pediatric and adult populations based on the progress achieved with *in vivo* gene replacement therapies in adults. However, additional clinical trials must be conducted to properly assess candidacy.
- In hemophilia, clinical trials using AAV vectors targeting liver cells can achieve levels of circulating clotting factors so that subjects no longer require replacement therapies.
- Gene replacement therapies in liver cells cannot maintain protein production once the cell divides limiting application to the pediatric population. Gene editing may be able to overcome this through integration into the nuclear genome.
- Improved methods for the design of guide RNAs and new Cas9 variants have dramatically reduced off-target and deleterious effects in pre-clinical models, which now are being translated into robust clinical development programs.
- The transition from gene replacement to gene editing therapies should focus on advancing methods to detect and prevent off-target activity which can result in unintended mutations.

therapies in patients with hemophilia [6,11]. Numerous clinical trials attempting to cure hemophilia are currently using an adeno-associated virus (AAV)-mediated cargo delivery system to deliver a FVIII or FIX transgene that remains primarily episomal with only a very small percentage of integration events. These trials have demonstrated clinically relevant expression of FVIII and FIX, within the normal or even supra-normal range, after a single treatment event with accompanying reductions in bleeding episodes, clotting factor utilization, and improved quality of life [12,13]. However, there has been high variability in the levels of achieved expression, challenges related to immune responses to the AAV capsid, and loss of expression over time, particularly with FVIII, raising concerns regarding long-term durability and whether this can be a truly curative approach. In addition, given the low level of integration, transgene expression would be lost with cell division which occurs in a growing liver, limiting this application to adult patients [6,16,14,15,17]. Following *in vivo* gene replacement therapy data, *in vivo* gene editing is an obvious next step for hemophilia, as we elaborate in the sections below.

1.1. Overview of CRISPR/Cas9 and other gene editing modalities

Most gene editing techniques focus on using a DNA nuclease to cleave a specific site in the genome. Previous techniques such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been effective in the past, but both come with caveats like challenging and time-consuming designs and large constructs with limited delivery potential, respectively. ZFNs and CRISPR/Cas9 have been combined with lentiviral and AAV vectors to improve efficacy and reduce off-target activity.

Delivery of the gene-editing machinery has been achieved using both viral and non-viral delivery systems. Viral delivery includes AAV vectors and lentiviral vectors, while non-viral delivery vehicles, like ribonucleoproteins (RNPs), often require electroporation to efficiently transfect the cells with a gene therapy [18–20]. Non-viral delivery is often complicated by electroporation techniques for *in vivo* delivery, as the high voltage required for electroporation results in cell toxicity [21]. An alternative vehicle is lipid nanoparticles (LNP), which can easily pass through cell membranes and, while targeting the liver without setting off the immune system, can allow for repeated administration. LNPs have been paired with close-ended DNA to dramatically increase the size of cargo that can be carried [20–22]. Additionally, magnetic nanoparticles have been shown to effectively deliver the gene-editing system with low off-target activity and no toxicity [23,24]. GeneRide is a nuclease-free therapeutic strategy that has been successfully used to cure HemB in mice following insertion into the albumin locus [25–27]. This nuclease-free system has also been paired with CRISPR/*Sa*Cas9 showing significant improvements in targeting effectiveness and what appears to be stable gene modification [24].

CRISPR/Cas9 has quickly become the go-to gene-editing technique around the world; this is due to the simplicity, low cost, and high efficacy of the system. Comprised of a guide RNA (gRNA) which is responsible for locating the loci of interest within the genome, and a Cas9 nuclease which causes a double-stranded break (DSB) in the DNA. The DSB causes cleavage of both strands of DNA which must be repaired, this is accomplished via non-homologous end joining (NHEJ) or homologous direct repair (HDR) depending on whether a knockout or a knock-in was desired [22]. The CRISPR/Cas9 system (Figure 1) has made genetic editing a possibility for nearly every researcher worldwide. Since the discovery of adaptive immunity in cells treated with CRISPR in 2007, there have been many improvements and modifications to the system [26].

Base editing is another approach to permanently correct base pair mismatches with fewer off-target effects; both cytosine and adenosine base editors have been used [28,29]. A newer method called Prime Editing combines prime editing gRNA (pegRNA) and Cas-nickase; this technique has an advantage over traditional base editing as it is capable of correcting any transition mutation, unlike base editing, which can only correct 4 of the 12 [30].

1.2. Recent progress on CRISPR/Cas9 gene editing

The sensitivity and specificity of CRISPR/Cas9 are dependent on the gRNA sequence specificity, as such much effort has been put into improving the gRNA currently being used in gene-editing therapies. The use of modified guide RNAs, like truncated gRNA, which has been shortened using a crRNA-derived sequence or gRNA that has been lengthened by two guanine (G) nucleotides, has seen increasing use in attempts to combat off-target effects [30–32]. The use of paired nickases, which combines a gRNA with paired Cas9 nickases, has been shown to greatly reduce off-target effects [17,23,31]. For example, Cas9 nickase, which is a mutated form of SpCas9, is responsible for creating a 'nick' in a single strand and thereby requiring two nickases to properly break both strands of DNA [23,32]. Several new Cas9 variants have been designed recently with hopes to improve on-target activity, reduce off-target effects, and create permanent gene modification.

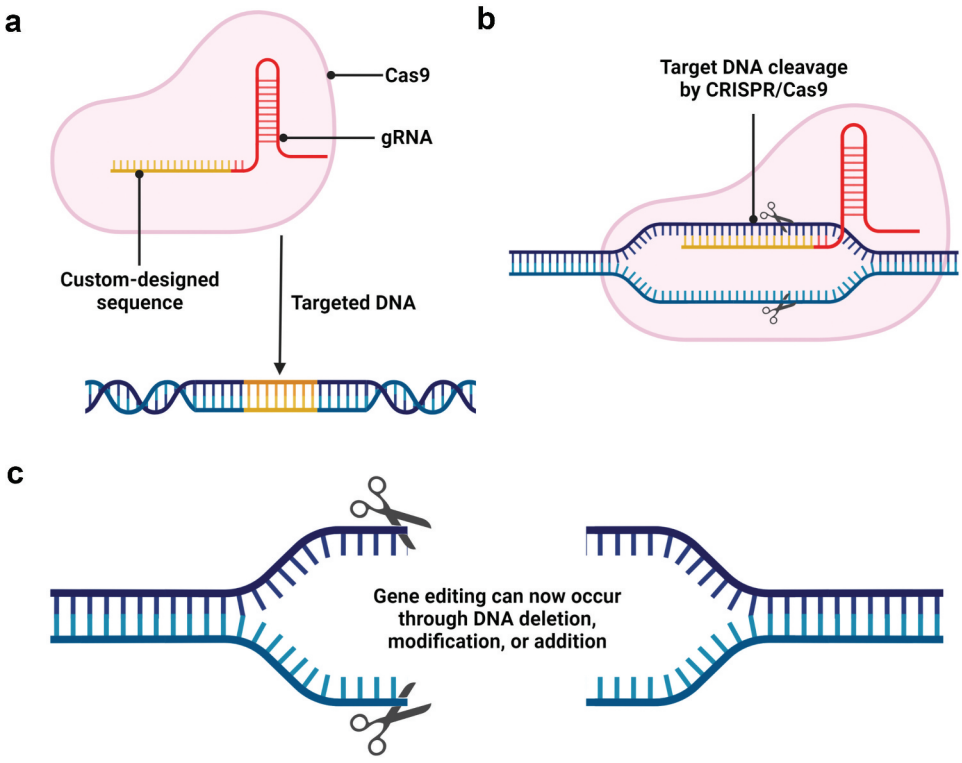

Figure 1. **Key elements of CRISPR/Cas9 gene editing**. The CRISPR/Cas9 system allows scientists to make targeted changes to an organism's DNA by following these major steps: **a)** Identification of target DNA by CRISPR/Cas9. **b)** CRISPR/Cas9 creates a double-stranded break. **c)** Following the double stranded break, gene editing takes place through DNA deletion, modification, or addition. Adapted from GAO-20-478SP and created with BioRender.com.

Engineered high-fidelity SpCas9 has been developed, followed by hyper-accurate Cas9 when it became clear that high-fidelity SpCas9 had poor activity at certain loci [33,34].

A Cas9 variant created using E. coli bacteria called Sniper-Cas9 combines RNP delivery with a truncated gRNA sequence. Sniper-Cas9 has shown greater efficacy and fewer off-target effects in human cells [35]. The SpCas9 variant packaged in RNP has been used to treat X-linked diseases, like Juvenile Retinoschisis in human cells [20]. Larger biochemical loads (i.e. SpCas9) will sometimes be split into two AAV vectors to get around the issue of limited carrying capacity [36–38].

1.3. Challenges of CRISPR/Cas9 gene editing

Cas9 evolved naturally in bacteria; therefore when placed into a human genome that is dramatically larger than that of bacteria, the chances of off-target effects increase significantly [39]. Most of the work done in improving the CRISPR/Cas9 system has been focused on reducing these off-target effects and on providing stable expression in the genome. Off-target effects are an inherent problem with gene editing, with detection in ZFNs, TALENs, and RNA interference, as well as CRISPR/Cas9 [40–42].

A crucial part of pre-clinical testing is the thorough analysis of potential off-target effects caused by any gene editing modality. To this end, many analysis software systems have been developed to aid in the detection of possible off-target effects. *In silico* predictive models allow for the detection of off-target effects prior to designing gRNA [43]. gRNA have shown greater efficacy when aligned with reference genome sequences based on homology through reduced off-target activity. *In silico* off-target prediction software like Flashfry, Bowtie, Cas-OFFinder, and CALITAS, among others, can be used when designing gRNA [44,45]. *In vivo* detection of off-target activity can be achieved using a variety of assays, chromatin immunoprecipitation and high throughput sequencing (Chip-seq), genome-wide, unbiased identification of DSBs enhanced by sequencing (GUIDE–seq), through capture of integrase defective lentiviral vectors (IDLVs), which are delivery vehicles for CRISPR/Cas9 that have weak integration abilities, or double-strand oligonucleotides (dsODN), to name a few. IDLV-capture was one of the first assays available to detect *in vivo* off-target effects following the use of ZFNs; GUIDE-seq is currently more widely used to identify off-target sites in cells treated with CRISPR/Cas9 [46–48]. Off-target detection tools are characterized as either biased or unbiased and are designed to predict either off-target or on-target activity. These off-target tools rely on *in silico* bioinformatics approaches to determine where off- or on-target activity will occur prior to setting up any experiments, allowing for reduced chances of deleterious off-target effects [23].

Another barrier to both genetic therapy and genome editing is the immune response, regardless of therapeutic route and biochemical modality. The immune response includes both innate and adaptive immune responses and can be directed against the viral vector capsid, the transgene, or the transgene product. In hemophilia gene therapy trials with AAV targeting the liver, this manifests clinically as a transaminitis and has been associated with loss of transgene expression. The primary strategy to attempt to circumvent this has been

to provide patients with a course of immunosuppression with corticosteroids, typically reactive upon demonstration of transaminase elevation or prophylactic administration prior to clinical manifestations of the immune response. This has contributed to significant side effects proportional to the required duration of treatment which can be weeks and even months [49].

While AAV vectors are technically capable of cargo delivery to mitotic and post-mitotic cells, in practice, the delivery is normally episomal, resulting in diminishing effects as cells continue to divide. This is particularly relevant in cases involving pediatric patients, where attempts to cure monogenic liver diseases have been met with concerns regarding diminished effectiveness due to hepatocyte proliferation [50–52]. Further complicating this matter, repeated injections of AAV vectors may be untenable due to the production of anti-AAV neutralizing antibodies following the first administration [53–56]. *In vivo* gene replacement therapies have limitations that can be overcome with *in vivo* gene editing, such as maintaining gene therapy expression in dividing cells. This review presents the key elements required for transitioning from gene therapy to gene editing, focusing on current approaches to overcome key challenges, such as off-target effects. However, for patients who have received AAV gene replacement therapy and developed anti-AAV antibodies, a gene editing therapy which utilizes AAV as a delivery system for CRISPR/Cas9 and donor genes will not be able to overcome the anti-AAV immune response.

1.4. CRISPR/Cas 9 in hemophilia pre-clinical studies

The FDA has shown particular interest in hemophilia-related gene therapy clinical trials, with one of the six draft guidance documents they released in 2019 being related to hemophilia. They highlighted the need for potential therapies to increase clotting factor levels to within normal levels [20]. A recent Expert Review focusing on HemA gene therapies listed biopharma companies in phase I (4 active), I/II (6 active), or III (6 active) clinical trials. In these studies, different AAV serotypes have shown clinical benefit in treating both HemA and HemB [57].

Pre-clinical studies in HemA have shown that injection of dual AAV vectors appears efficacious in treating HemA, as two AAV vectors containing *Staphylococcus pyogenes* Cas 9 (SpCas9) and guide RNA with human B-domain deleted FVIII were used to integrate the human FVIII into the albumin locus, resulting in production of FVIII by the liver in mice (Figure 2). The amelioration of the HemA phenotype persisted for at least 7 months with no obvious off-target effects or signs of liver toxicity, leading the authors to hypothesize that permanent FVIII replacement may be possible using this method [58]. This study provides the basis to initiate clinical development programs incorporating two vectors: The first vector incorporates the gRNA and CRISPR/Cas 9 machinery; the second vector incorporates the FVIII transgene precisely located in a so-called 'safe harbor' designed to leverage the promoter activity of the albumin locus [25]. For the treatment of genetic liver disorders, the albumin locus provides the best promoter for targeted integration of donor genes. A 'safe harbor' means that the location of genome integration will not pose a risk to the host and will perform predictably [59].

Critical advancements are being developed for HemA, like a second generation F8 transgene incorporating carefully selected porcine sequences that in pre-clinical studies have shown increased biosynthesis and secretion of FVIII in the blood [57,58]. Pre-clinical studies in HemB have shown mixed results with CRISPR/Cas9 applications in murine and canine models [11,15,16,60], as well as initial ZFN pre-clinical and

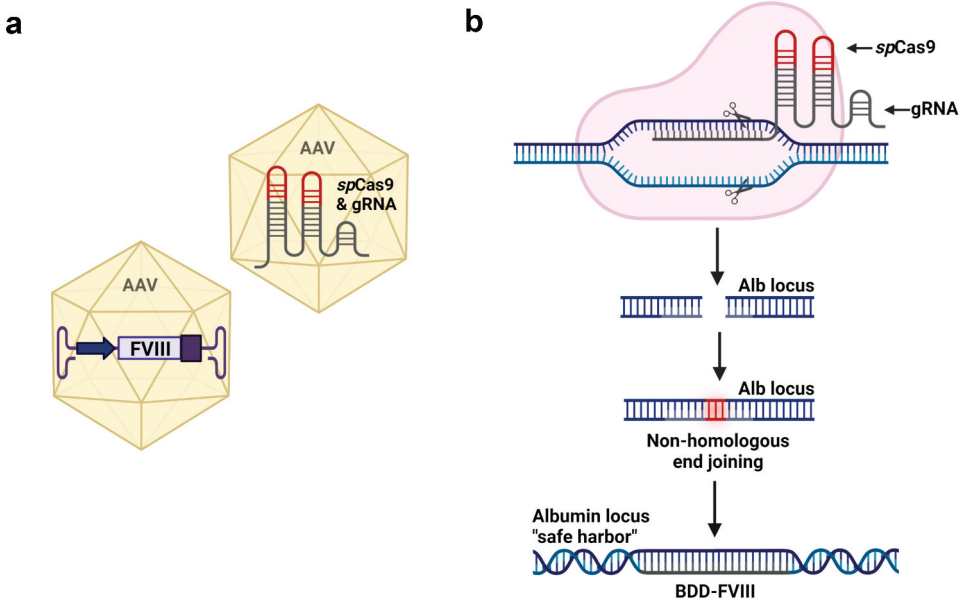

Figure 2. **Dual AAV vectors to integrate BDD-FVIII into the genome using CRISPR/Cas9.** a) Use of dual adeno-associated viral vectors to package *sp*Cas9, gRNA, and human B-domain deleted clotting factor FVIII. b) Graphic depictions of CRISPR use in Hem A/B; *sp*Cas9 and gRNA are used to insert BDD-FVIII into the genome, leveraging the promoter of a 'safe harbor,' such as the albumin locus for gene editing targeting the liver. Created with BioRender.com.

clinical studies by Sangamo Biosciences that were subsequently discontinued [61]. In other discontinued studies, GeneRide has been used to ameliorate bleeding in HemB mice by targeting the albumin locus [20]. Using HDR-mediated integration of a cDNA construct, researchers knocked in FVIII and FIX in the albumin locus [62].

Pre-clinical studies in HemB have reported the correction of the bleeding phenotype in newborn and adult factor IX (FIX) knockout mice through *in vivo* gene editing mediated by CRISPR/Cas9 gene editing technology, which appears the most versatile, with several recent reports claiming correction of HemB in murine and canine models [63]. The stable expression of FIX over an 8-month period in newborn and adult FIX-knockout mice following a single injection of dual AAV gene-targeting vectors has been reported [64]. This dual AAV8 vector system includes a donor vector that contains a promoterless, codon-optimized, partial human FIX complementary DNA (exons 2 to 8) carrying the hyperactive FIX Padua mutation and a single guide RNA vector to target exon 2 of murine FIX. Targeting this region of the FIX gene is expected to have the broadest applicability because 90% to 95% of HemB mutations are 3′ to exon 1.

2. Conclusion

Inherited monogenic rare diseases are an ideal target for gene editing based on the initial success of gene replacement clinical trials in the adult population. Pre-clinical studies have utilized ZFNs, TALENs, RNP, and CRISPR/Cas9 to deliver the defective or missing gene to animal models. Despite promising pre-clinical data, sustained, long-term expression of these gene editing therapies in human beings requires a lengthy road covering both sustained efficacy and a pristine off-target data assessment to provide a solid safety profile. Improved methods for the design of gRNAs and new Cas9 variants have dramatically reduced off-target and deleterious effects in pre-clinical models, which now should be translated into a robust clinical development program. AAV vectors are currently being utilized by numerous clinical trials to treat inherited diseases, such as hemophilia, with many adult patients in clinical trials maintaining normal levels of circulating clotting factors and no longer requiring replacement therapies. Gene editing will pave the way for achieving these results in the pediatric population.

3. Expert opinion

In vivo gene therapies targeting the liver to express targeted proteins can ameliorate the clinical manifestations of hemophilia and other diseases, but the current strategies have demonstrated a lack of predictability of response, concerns regarding long term durability of expression and significant adverse events from immune responses [65]. Among the most difficult challenges is the inability to achieve durable gene expression after gene replacement therapy if delivered when liver cells are replicating rapidly. This is especially significant in the pediatric population as hepatocyte proliferation would diminish the effects of the therapy over time. Yet, pediatric patients with hemophilia would have the most to gain from correction of their factor levels as this would likely abrogate their risk for bleeding and joint disease over a lifetime. Gene replacement strategies have not yet demonstrated that they can offer curative correction over the lifespan. Thus, efforts should be made to circumvent or resolve the potential need for repeated injections of gene therapies which are limited by the immune responses.

The translation of progress achieved with *in vivo* gene therapy in pre- and clinical development programs for hemophilia A and B in the academic and biopharmaceutical spaces are paving the way to substantial advances toward the application of *in vivo* gene editing modalities to treat these conditions. The goal of *in vivo* gene editing is to achieve a safe and sustainable efficacy profile that can surpass the initial data observed with *in vivo* gene therapy clinical programs, especially showing applicability to treat both the pediatric and adult populations.

Based on the techniques reviewed here, a targeted approach has been published using dual AAV delivery for *in vivo* gene editing of HemA and HemB. Prior to the selection of the clinical candidate gRNA, comprehensive off-target assessments using *in silico, in vitro,* and *in vivo* methodologies are required. For best results, use of the highly active albumin promoter should be considered for target integration. In addition to the fact that albumin is robustly expressed in the liver, integration of AAV vectors into the albumin locus can leverage its highly active promoter proving to be an effective treatment of hemophilia in animal models. The albumin locus also provides a 'safe harbor' that replaces the promoter indispensable with gene replacement therapies.

To achieve the full potential of *in vivo* gene editing, there are two main elements that need to be considered: first, to ensure that off-target effects are well understood and controlled; second, to ensure that neither the therapeutic carriers, usually AAV vectors, nor the clotting factor protein can generate intolerable immunogenicity for the patients. Regarding efficacy, the engineering of second generation high-activity transgenes for both HemA and HemB implies more potent levels of clotting factors, leading to an increase in efficacy, reduction of cost of goods through lowering of dosages and a decrease in the risk of toxicity. We expect that *in vivo* gene editing for the treatment of inherited monogenic diseases, led by promising work in the hemophilia field, should focus on improved methods to detect and prevent off-target activity which can result in unintended mutations, as this is one of the widest-ranging issues across the field of genetic editing.

In summary, *in vivo* gene editing has opened the door to seemingly endless possibilities, and while incredible progress has been made in the past 15 years, there are many areas that still need to be improved. Hemophilia A and B have received an enormous amount of attention with regards to gene editing therapies driven by the severity of disease and burden of treatment, but also because hemophilia is a monogenic disease with a well-defined biological function of defective clotting factors.

Abbreviations

AAV	Adeno-associated virus
BDD-FVIII	B-domain deleted clotting factor VIII
CALITAS	CRISPR-Cas-aware aligner and integrated off-target search algorithm
cDNA	Complementary DNA
Chip-seq	Chromatin immunoprecipitation and high throughput sequencing
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DNA	Deoxyribonucleic acid
DSB	Double-stranded break
dsODN	Double-strand oligonucleotides
EHL	Extended half-life
FIX	Clotting factor IX
FVIII	Clotting factor VIII
gRNA	Guide RNA
GUIDE-seq	Genome-wide, unbiased identification of DSBs enabled by sequencing
HDR	Homologous direct repair
HemA	Hemophilia A
HemB	Hemophilia B
IDLVs	Integrase defective lentiviral
IV	Intravenous
LNP	Lipid nanoparticles
NHEJ	Non-homologous end joining
pegRNA	Prime editing guide RNA
RNA	Ribonucleic acid
RNPs	Ribonucleoproteins
saCas9	Staphylococcus aureus Cas9
spCas9	Staphylococcus pyogenes Cas 9
TALENs	Transcription activator-like effector nucleases
WFH	World Federation of Hemophilia
ZFNs	Zinc-finger nucleases

Acknolwedgements

Mackenzie Hagan provided medical writing and figure generation support. Figures were created with BioRender.com.

Funding

This paper was funded by ASC Therapeutics Inc.

Declaration of interest

OG Segurado and R Jiang are employees of ASC Therapeutics. SW Pipe has received consultants fees from Apcintex, ASC Therapeutics, Bayer, BioMarin, Catalyst Biosciences, CSL Behring, GenVentiv, HEMA Biologics, Freeline, LFB, Novo Nordisk, Pfizer, Regeneron/Intellia, Roche/Genentech, Sangamo Therapeutics, Sanofi, Takeda, Spark Therapeutics and uniQure. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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