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Genome editing, a superior therapy for inherited retinal diseases

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Abstract

Gene augmentation and genome editing are promising strategies for the treatment of monogenic inherited retinal diseases. Although gene augmentation treatments are commercially available for inherited retinal diseases, there are many shortcomings that need to be addressed, like progressive retinal degeneration and diminishing efficacy over time. Innovative CRISPR-Cas9-based genome editing technologies have broadened the proportion of treatable genetic disorders and can greatly improve or complement treatment outcomes from gene augmentation. Progress in this relatively new field involves the development of therapeutics including gene disruption, ablate-and-replace strategies, and precision gene-correction techniques, such as base editing and prime editing. By making direct edits to endogenous DNA, genome editing theoretically guarantees permanent gene-correction and long-lasting treatment effects. Improvements to delivery modalities aimed at limiting persistent gene-editor activity have displayed an improved safety profile and minimal off-target editing. Continued progress to advance precise gene correction and associated delivery strategies will establish genome editing as the preferred treatment for genetic retinal disorders. This commentary describes the applications, strengths, and drawbacks of conventional gene augmentation approaches, recent advances in precise genome editing in the retina, and promising preclinical strategies to facilitate the use of robust genome editing therapies in human patients.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

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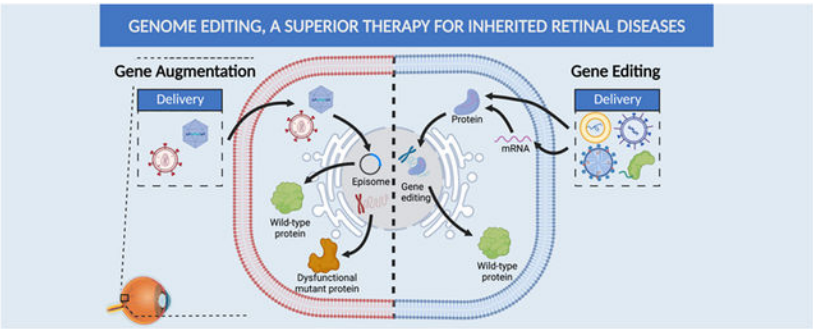

Keywords

retinal degeneration; genome editing; vision; retinopathies; Leber congenital amaurosis; retina physiology

1. INTRODUCTION

Vision is the dominant modality by which we perceive the world. Throughout our daily lives, we are culturally dependent on vision to read, write, and navigate, among many other functions. Thus, people with visual impairment are often severely disadvantaged. Inherited retinal diseases (IRDs) are a class of diseases that damage the retina, often leading to severe visual deterioration or blindness. IRDs affect approximately 200 thousand people in the United States and about 4.5 million people around the world. The genetic diversity of IRDs is broad, as they are caused by mutations in over 280 different genes, with multiple mutations in each gene capable of causing severe disease (Fig. 1A) (Hohman, 2017). Mutations associated with key enzymes of the retinoid cycle are especially devastating, as they lead to impaired synthesis of the visual chromophore or accumulation of cytotoxic retinoid byproducts, which cause retinal dystrophy (Palczewski & Kiser, 2020, Travis, et al., 2007). Hundreds of these different mutations are implicated in several distinct diseases, such as Leber congenital amaurosis (LCA), retinitis pigmentosa (RP), Stargardt disease, choroideremia, and age-related macular degeneration (AMD) (Cremers, et al., 2018). Although all characterized as IRDs, these diseases manifest with various modes of inheritance and times of onset and affect different areas of the retina, which makes them especially challenging to treat (Sahel, et al., 2015, Suh, et al., 2022).

Thus far, treatments for the vast majority of IRDs are only supportive, which require expensive and persistent care (Benati, et al., 2020). Recently, much progress involving gene augmentation therapy and genome editing has been made towards treating IRDs. The retina is a particularly attractive tissue for developing gene augmentation therapy and genome editing treatments, as it is relatively accessible by surgery and can be imaged noninvasively to assess treatment safety and efficacy (Suh et al., 2022). Most genetic therapies for IRDs currently in development consist of gene augmentation strategies, which involve delivering a wild-type cDNA to retinal cells (Georgiou, et al., 2021). Yet, due to rapid advances in genome editing strategies, several potential gene disruption and gene correction treatments have been taken to clinical trials. Currently, clinical trials using CRISPR-Cas technology

to treat various cancers, sickle cell disease, misfolded protein diseases, and cardiovascular disease have already begun (Frangoul, et al., 2021, Gillmore, et al., 2021, Ou, et al., 2021). With the advent of even newer genome editing technologies such as base editors and prime editors, which can install permanent precise gene corrections, CRISPR-Cas technologies have become more attractive as approaches to treat IRDs (Anzalone, et al., 2020). Herein, we discuss the main challenges of gene augmentation therapy and the promise of genome editing as a more effective and robust therapy for IRDs.

2. Challenges of Gene Augmentation Therapy for the Treatment of IRDs

In 2017, the U.S. Food and Drug Administration (FDA) approved voretigene neparvovec (brand name Luxturna) as a gene augmentation therapy for patients with biallelic missense or nonsense mutations in the RPE65 protein (retinal pigment epithelium-specific 65 kDa protein), a key protein in the pathway for regenerating visual pigment (Russell, et al., 2017). As the first FDA approved gene therapy, Luxturna engendered a new wave of optimism for gene augmentation strategies to treat monogenic IRDs. Currently, most clinical trials for IRDs involve gene augmentation, spanning a broad range of disorders including LCA, achromatopsia, choroideremia, Stargardt disease, and RP (Nuzbrokh, et al., 2021). Despite the enthusiasm for gene augmentation therapy, there are several serious challenges, including: 1) delivery effectiveness, 2) variable expression of the transgene, and 3) waning expression of the transgene over time (Greig, et al., 2022). These shortcomings have raised concerns regarding the potential of gene augmentation therapy as a truly curative treatment.

The efficacy of gene therapy depends on the effectiveness of the delivery vector to reliably transduce target cells. The most promising vehicle for transgene delivery is the adeno-associated virus (AAV) due to its favorable safety profile, low immunogenicity, and broad tropism (Surace & Auricchio, 2008). Luxturna is administered *via* a subretinal injection of recombinant AAV serotype 2 (rAAV2) containing a wild-type copy of the *RPE65* gene; the loaded rAAV2 then transduces the *RPE65* transgene into retinal pigment epithelium (RPE) cells (Fig. 1B). Two clinical trials for X-linked retinoschisis (NCT02317887 and NCT02416622) are exploring intravitreal delivery of AAV, which would theoretically allow for a broader region of therapeutic effect and supplant the need for the invasive surgery involved in subretinal injections. However, early clinical data suggest that intravitreal injection of rAAV2 has limited therapeutic benefit, and intravitreal injection of rAAV8 produced ocular inflammation (ClinicalTrials.gov, 2015, Cukras, et al., 2018). Moreover, Xiong *et al.* recently found that certain AAV promoters are correlated with RPE toxicity; in particular, the cytomegalovirus immediate-early promoter (CMV) and chicken beta actin promoter (CAG), which are the promoters used by Luxturna to drive *RPE65* expression (Xiong, et al., 2019).

Due to the ocular inflammation that can result from AAV administration, Luxturna is limited to a small dose that minimizes toxicity and potential efficacy (Bainbridge, et al., 2015). Moreover, typically only half of the delivered volume is available to transduce the retina, because the subretinal injection forms a bleb which depresses the retina into the vitreous (Ladha, et al., 2022). As a result, Luxturna administration results in delivery to only ~10% of retinal cells (Xiong et al., 2019). Although *RPE65* is highly expressed

in RPE cells and the rAAV2 targets RPE cells, Luxturna also delivers the transgene to some photoreceptors (Gao, et al., 2020). As *RPE65* was previously shown to be naturally expressed in mammalian cones, but not in rods, there is concern that unnatural expression of *RPE65* in rods may have adverse effects (Znoiko, et al., 2002).

Moreover, another shortcoming of AAVs is their limited packaging capacity (5 kb). Consequently, gene augmentation therapies seeking to deliver larger cDNA sequences, such as the *ABCA4* gene (6.8 kb) implicated in some forms of Stargardt disease must utilize other delivery vectors or strategies. In the past, a phase I/II clinical trial (NCT01367444) using a lentiviral vector to deliver the *ABCA4* transgene was terminated due to loss of sponsorship, but a long-term safety study is presently ongoing (Parker, et al., 2022). However, delivery by lentiviral vectors is not ideal for gene therapy, as it risks insertional mutagenesis of the transgene (Arsenijevic, et al., 2022).

Despite evident short-term improvement in visual acuity for Luxturna-treated patients, clinical follow-up studies of these patients for 3 to 6 years after treatment reported diminution of visual sensitivity and unabated advancement of photoreceptor deterioration (Bainbridge et al., 2015, Cideciyan, et al., 2013, Jacobson, et al., 2015). Given the short history of Luxturna use, the reason for continuous retinal degeneration is yet unknown. In 2013, Cideciyan *et al.* published a 3-year follow-up study of patients who received *RPE65* gene augmentation therapy, which showed sustained improvements in visual function, but also progressive retinal atrophy and photoreceptor death (Cideciyan et al., 2013). The researchers postulated that biochemical changes in photoreceptors as a result of *RPE65* deficiency place cells into a spectrum of functionally silent pre-apoptotic states, which suggests that advancing retinal generation results from photoreceptors that are already on the verge of cell death (Cideciyan et al., 2013). In another 3-year study of *RPE65* gene augmentation patients, Bainbridge *et al.*, reported a decline in retinal sensitivity 12 months after treatment along with uninterrupted retinal degeneration (Bainbridge et al., 2015). They also found that maximal retinal sensitivity was only achieved after extended periods of dark adaption, hypothesizing that *RPE65* expression is insufficient to meet the threshold of 11-*cis*-retinal supply necessary for normal visual function (Bainbridge et al., 2015). Similar visual decline in *RPE65* gene augmentation patients was reported in a 6 year follow up study by *Jacobson et al,* which found that after reaching a peak visual sensitivity 1-3 years after treatment, there was a progressive diminution in retinal sensitivity in parallel with retinal thinning (Jacobson et al., 2015). While it is not known why there is an eventual contraction in visual function, Jacobson and coworkers speculate that many factors contribute: 1) many photoreceptors were already in a pre-apoptotic state at the time of treatment and degenerated shortly after; 2) waning transgene expression due to methylation of the exogenous promoter failed to meet the physiological requirement; and 3) insufficient transgene expression in RPE cells at the edges of the subretinal bleb, which causes them to degenerate (Jacobson et al., 2015). More recent clinical trials concluded that improved visual acuity is sustained for at least 4 to 7.5 years, but the evidence for eventual relapse provided by the previous studies is especially concerning (Chung, et al., 2019, Maguire, et al., 2021). Nevertheless, the variable durability of transgene expression and episomal persistence that accompany existing gene therapy approaches make gene augmentation a less favorable treatment strategy for inherited diseases.

3. Classical Genome Editing with CRISPR-Cas9 Nucleases

Considering the many drawbacks of gene augmentation therapy, genome editing therapies have emerged as the most promising treatments for IRDs. Although genome editing with zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have existed for some time, they are limited by many disadvantages, such as time-consuming and expensive construct design as well as large assemblies that form barriers to certain delivery modalities (Segurado, et al., 2022). However, the discovery of RNA-guided CRISPR (clustered-regularly interspaced short palindromic repeats) nucleases in 2012 has made ZFNs and TALENs largely irrelevant for further *in vivo* genome editing applications. The most commonly used CRISPR platform for genome editing therapeutics is the CRISPR associated protein 9, Cas9, which when complexed with an engineered single guide RNA (sgRNA), can recognize specific DNA sequences and generate a double-strand break (DSB) at that sequence (Doudna & Charpentier, 2014). Although ZFNs and TALENs also generate DSBs, the advantage of CRISPR-Cas9 is that only the sgRNA sequence needs to be changed to recognize a different genetic locus, rather than completely reengineering the protein domains (Hsu, et al., 2014, Sander & Joung, 2014).

After Cas9 introduces a double stranded break, there are two main methods of repair – non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Fig. 2A) (Hustedt & Durocher, 2017). End-joining pathways directly re-ligate the double stranded break and often result in misaligned repair which includes a random mixture of insertions and deletions (indels), which often lead to frameshift mutations and gene knockout (Hsu et al., 2014). On the other hand, the HDR repair pathway inserts a donor DNA molecule containing the correct gene sequence directly into the genome as the cell repairs the DSB. In theory, HDR can precisely correct mono-allelic mutations involved in IRDs, and researchers have demonstrated that targeted *in vivo* gene-integration can rescue mutations in the *PDE6B, NR2E3, RPGR, USH1C, RHO,* and *RPE65* genes (Bassuk, et al., 2016, Bohrer, et al., 2019, Cai, et al., 2019, Greenwald, et al., 2010, Jo, et al., 2019, Overlack, et al., 2012, Vagni, et al., 2019, Yanik, et al., 2017). However, HDR has several drawbacks that make it less desirable for introducing precise gene corrections in the retina. Cas9-induced DSBs often result in substantial indels that nullify the potential therapeutic benefits of HDR (Sander & Joung, 2014). In addition, the HDR pathway is predominantly active in dividing cells, so the efficiency of homologous recombination in post-mitotic cells, such as photoreceptors and RPE cells, is drastically reduced (Cox, et al., 2015). Thus, HDR is largely unsuitable for precise genome editing in the eye.

On the other hand, end-joining methods have considerable therapeutic potential for treating inherited retinal diseases. NHEJ is active throughout the cell cycle and across several different cells, which is especially useful for applications of gene disruption, where indels are desired (Cox et al., 2015). Using NHEJ repair, Suzuki *et al.* developed a strategy for homology-independent targeted integration (HITI), which results in targeted transgene integration (Fig. 2A) (Suzuki, et al., 2016). The DNA template used in HITI is flanked by Cas9 cleavage sites rather than homology domains as in HDR. Using this approach, researchers improved rod-cone response by restoring a 1.9 kb deletion from the *Mertk* gene in a mouse model of autosomal dominant retinitis pigmentosa (adRP) (Suzuki et al., 2016).

Because NHEJ is active in post-mitotic cells and HDR is not, HITI could greatly expand the scope of nuclease-mediated genome editing in the retina.

An alternative DSB repair pathway, microhomology-mediated end-joining (MMEJ), functions by annealing microhomologies (5-25 complimentary base pairs) upstream and downstream of the DSB (McVey & Lee, 2008). The MMEJ pathway most often results in short deletions of the microhomologous sequence, which makes it effective for gene knockout. Recently, Sakuma *et al.* developed a system for MMEJ-assisted gene knock-in by delivering Cas9 nuclease in conjunction with a donor DNA vector including two different microhomology arms (Sakuma, et al., 2016). Cleaving between the microhomologies allows targeted gene integration by MMEJ. Although MMEJ has not yet been applied in the eye, it serves as a promising alternative pathway to HDR and NHEJ for gene knock-in treatments.

Researchers have also developed an approach to preferentially ablate the mutant *Rho* allele carrying either a P23H or S334ter mutation in mouse models of adRP, thereby preserving the wild-type allele and ameliorating disease phenotypes (Bakondi, et al., 2016, Li, et al., 2018). In some cases, this gene disruption approach induces haploinsufficiency, which then requires cells to be supplemented with an exogenous cDNA copy of the wild-type gene. The main drawback of this approach is the genetic heterogeneity in *Rho* that causes adRP, which poses an economic challenge when designing sgRNAs to target each mutation, especially in conjunction with the delivery of exogenous cDNA. To alleviate these costs, researchers have used Cas9 to eliminate both copies of the endogenous *Rho* gene before supplementation with an exogenous wild-type copy of *Rho* (Tsai, et al., 2018). This ablate-and-replace strategy rescued photoreceptor structure and function in adRP mice (Tsai et al., 2018). Although this approach eliminates potential cellular stress from products of the mutant allele, there are still concerns of waning transgene expression over time, the same as those in gene augmentation therapy, as well as genotoxicity from DSB formation.

Multiplex nuclease-mediated editing is enabled by delivery of several sgRNAs targeting different sites. This approach was used by Maeder *et al.* to correct a deep-intronic recessive mutation (IVS26) in the *CEP290* gene, which results in erroneous protein splicing and is implicated in LCA type 10 (LCA10) (Maeder, et al., 2015). Employing *Staphylococcus aureus* Cas9 (SaCas9), the group was able to restore normal splicing of *CEP290* transcription products by excising or inverting the mutation (Maeder et al., 2015). In experiments involving human retinal explants, editing efficiency reached the desired level for therapeutic effect and there was no detected off-target editing (Maeder, et al., 2019). In addition, this study demonstrated that human G protein-coupled receptor kinase 1(*GRK1)*-promoter-driven expression of SaCa9 is specific to photoreceptors, and there appears to be limited immunogenicity towards SaCas9. Based on the favorable nature of these results, Editas Medicine initiated a phase 1/2 trial of EDIT-101 to correct a *CEP290* mutation in patients with LCA10. EDIT-101 uses a single AAV to deliver *GRK1*-driven SaCas9 along with two sgRNAs to target the IVS26 point mutation in intron 26 of the *CEP290* gene (NCT03872479). By introducing DSBs at both ends of the mutation, the mutated sequence is excised, and normal protein splicing is restored. While post-treatment assessment is still ongoing, EDIT-101 repairs the mutation and solves many of the problems of gene augmentation, as the effects of editing are permanent and there is little risk with

cellular toxicity of mutant gene products. Overall, nuclease-mediated editing has pushed gene therapy away from gene augmentation and towards genome editing approaches.

4. Extending Cas9 Functionality in the Eye with Base Editors and Prime Editors

Since the first demonstration of targeted genome editing of Cas9 nuclease, explosive growth has occurred in the development of CRISPR-Cas9-derived precision genome engineering technologies. Base editors install targeted base changes without generating DSBs, and with significantly reduced indel frequency compared to Cas9 nuclease (Gaudelli, et al., 2017, Komor, et al., 2016). Base editors are constructed by fusing a catalytically inactive Cas9 or Cas9 nickase, which only nicks one DNA strand, to either a cytidine deaminase (cytosine base editor) or a laboratory-evolved deoxyadenosine deaminase (adenine base editor). Cytosine base editors (CBEs) catalyze the conversion of C•G base pairs to U•G base pairs and adenine base editors (ABEs) catalyze the conversion of A•T base pairs to I•T base pairs by deamination of the cytosine or adenine residues, respectively (Fig. 2B) (Gaudelli et al., 2017, Komor et al., 2016). Using a Cas9 nickase to nick the non-deaminated strand encourages DNA repair, which codifies the U•G and I•T base pairs into permanent T•A and G•C transition mutations, respectively (Gaudelli et al., 2017, Komor et al., 2016). CBEs and ABEs can theoretically correct all transition mutations within the human genome, which account for approximately 30% of all known human pathogenic variants (Anzalone et al., 2020). Recent developments of C•G to G•C base editors (CGBEs) have further expanded the scope of mutations that base editors can target (Fig. 2B) (Kurt, et al., 2021, Zhao, et al., 2021). As a result, base editors are an extremely attractive technology for the treatment of IRDs, especially when gene augmentation therapies fall short. Base editors are particularly suitable for correcting mutations in large genes that exceed the AAV cargo limit for exogenous cDNA delivery, like *ABCA4* (6.8 kb) and *USH2A* (15.5 kb); these are the two most implicated genes across all IRD cases in the U.S. (Stone, et al., 2017).

Base editing has significant advantages over conventional gene augmentation in the treatment of IRDs. Because base editors directly correct endogenous DNA, there is no expression of the mutant protein, which greatly reduces the potential toxicity of the dysfunctional protein. This is especially important in the context of IRDs with dominant modes of inheritance, as gene augmentation may not be sufficient to negate the effect of the mutant allele. In addition, correcting the endogenous locus allows expression of the corrected gene to be driven by the endogenous promoter, which enables cell-specific, physiological regulation of expression. Gene augmentation strategies often rely on exogenous promoters to drive gene expression, which faces the risk of DNA methylation and waning transgene expression over time (Jacobson et al., 2015, Xiong et al., 2019). Such risks are alleviated in base editing treatments, as base editors install permanent corrections in the genome which are regulated by endogenous transcription factors (Gaudelli et al., 2017).

In 2021, Suh *et al.* demonstrated the first *in vivo* application of base editing to treat an IRD. Through subretinal delivery of a lentiviral vector expressing an ABE and sgRNA, the research group corrected the nonsense mutation in the *Rpe65* gene of the

autosomal recessive *rd12* mouse model for LCA type 2 (LCA2) (Suh, et al., 2021). With the appropriate sgRNA, the target mutation was precisely corrected with up to 29% efficiency, with less than 0.5% indel formation and no off-target editing at the top ten potentially mutable genomic sites (Suh et al., 2021). Base editing rescued *Rpe65* expression and restored visual function, as the treated mice exhibited nearly 50% recovery of retinal function in scotopic electroretinography (ERG) recordings and demonstrated strong responses to visual changes in orientation, spatial and temporal frequency, size, and contrast (Suh et al., 2021). A later study found that using base editing to restore *Rpe65* expression in *rd12* mice resolved many of the problems associated with *RPE65* gene augmentation therapy. Choi *et al.* showed that base editing could induce long lasting improvements in cone function while preventing photoreceptor degeneration (Choi, et al., 2022). As opposed to near complete degeneration of the retina in untreated mice, mice treated with the base editor displayed improved cone function and survival 6 months following the treatment. Further, the researchers found that restoring *Rpe65* gene expression through base editing downregulated the expression of genes that potentially confer cell death (Choi et al., 2022). Thus, base editing has demonstrated its potential to overcome the shortcomings of today's gene augmentation therapies and provide a method for permanent gene correction, which raises the prospect of permanent improvement in visual acuity (Bainbridge et al., 2015, Cideciyan et al., 2013, Jacobson et al., 2015). In a separate study of genome editing in the same *rd12* mouse model of LCA, Jang *et al.* demonstrated non-viral delivery of base editors *via* subretinal injection. By injecting lipofectamine in conjunction with ABE/sgRNA ribonucleoproteins (RNPs), they observed delivery of the ABE-sgRNA complexes and up to 5.7% correction efficiency, which resulted in rescued *Rpe65* mRNA and protein expression without risk of lentiviral integration into the genome. (Jang, et al., 2021). These studies raise the prospect of permanent rescue of photoreceptor degeneration *via* genome editing, which was demonstrated in the mouse model of LCA2. While these results are not directly translatable to human LCA2 patients, genome editing seems to provide a clear path to restoration of photoreceptor health that is unaddressed by Luxturna, though further investigation is needed to assess long-term outcomes of base editing. However, restoration of physiological protein expression may be sufficient to overcome these challenges.

Prime editors (PEs) are the most recent advances among the CRISPR-Cas9-derived genome editing technologies. Consisting of a reverse transcriptase fused to a Cas9 nickase, PEs can theoretically correct all transition and transversion mutations as well as generate small indels (Fig. 2C). PEs use prime editing guide RNAs (pegRNAs), which serve both as the sequence that guides Cas9 to the target genomic locus as well as the RNA template for reverse transcription (Anzalone et al., 2020). This innovation allows PEs to precisely write mutations directly into genomic DNA, without the risk of bystander editing.

The first *in vivo* application of prime editing in the eye was demonstrated shortly after these initial base editor studies. In this case the *rd12* mouse model of LCA2 was again used, although it was already shown to be precisely corrected by ABE. However, PEs hold a significant advantage over BEs, as BEs can potentially deaminate multiple bases within its catalytic window while PEs exhibit very low bystander editing. Jang *et al.* subretinally administered dual-AAVs of PE and pegRNA, which resulted in about 6.4% correction efficiency without any detectable indels, unintended substitutions, bystander effects, or

off-target effects (Jang, et al., 2022). They also observed improved dark-adapted ERG responses, up to 67% of the wild-type amplitude. As PEs also install permanent corrections directly in the genome, the rescued visual function is expected to be long-lasting. In addition to their ability to generate all point mutations as well as short indels, successful delivery by AAV, a proven clinical approach, makes prime editing an extremely promising new strategy for the treatment of IRDs.

5. Moving Genome Editing to the Clinic

Newly innovated CRISPR-Cas9 derived precision genome editing agents have proven to be highly viable potential therapies for monogenic diseases. Compared to AAV-mediated gene augmentation therapy, Cas9 systems have the potential to treat a broader range of diseases with longer lasting effects. However, genome engineering *in vivo* carries several concerns regarding safety and efficacy. Thus, the risks and benefits of genome editing therapies need to be evaluated to ensure their therapeutic viability in the clinic.

5.1. Immunogenicity

Minimizing the immune response to Cas9-derived therapeutics in the eye is essential for future clinical applications. Intraocular inflammation is a considerable risk-factor for vision-threatening complications (E.Cunningham & Zierhut, 2021). Because CRISPR-Cas9 machineries originate from common bacteria, there is the possibility of immune responses to intraocular injection of these proteins, which could result in inflammation and less efficient editing. Probing human donor serum randomly selected from healthy participants at the Stanford Blood Center revealed detectable anti-Cas9 antibodies against SaCas9 and *Streptococcus pyogenes* Cas9 (SpCas9) in 78% and 56% of samples, respectively (Charlesworth, et al., 2019). A study of anti-Cas9 antibodies in the eye found that while there was a high prevalence of preexisting anti-Cas9 antibodies in serum, there was no expression in the eye (Toral, et al., 2022). However, they detected anti-SpCas9 antibodies in the vitreous after mice were treated with an intraocular injection of SpCas9 (Toral et al., 2022).

In a genome editing study using an AAV5 vector encoding a SaCas9 construct targeting *GUYC2D* in non-human primates (NHPs), researchers observed that animals with the highest preexisting T-cell response to Cas9 showed the greatest SaCas9 editing efficiency (Tran, et al., 2019). Further, they observed that there was no need for systemic immunosuppression in NHPs injected with AAV5-SaCas9, and no significant intraocular inflammation (Tran et al., 2019). These results indicate that the appearance of anti-Cas9 antibodies in the serum may not limit the capabilities of intraocular injections of Cas9-derived therapeutics, but the evidence of intraocular expression of anti-Cas9 antibodies after intraocular injection is concerning.

In addition, there may be potential immune responses to the delivery vehicle used in Cas9-derived therapies. As is inherent with gene augmentation methods, gene delivery and expression *via* AAVs can potentially induce ocular inflammation (Cukras et al., 2018, Xiong et al., 2019). AAV vectors have been shown to elicit immune responses in the eye in NHPs and greater dosing of AAVs was found to cause gene therapy-associated uveitis (Reichel,

et al., 2017, Timmers, et al., 2019). Further research is needed to determine the effects of the intraocular immune response to Cas9 delivery, and if there is an inflammatory effect or diminished efficacy.

5.2. Controlling Cas9 Activity While Improving Safety and Efficiency

Although major advancements have been made in the development of precision genome editing tools, there are risks of deleterious off-target effects. Many preclinical and all clinical studies have used viral vectors to deliver cDNAs encoding genome editing proteins and guide RNA. Lentiviral and AAV-mediated delivery creates risks of insertional mutagenesis of the cDNA cargo with potential oncogenic effects (Chandler, et al., 2017, Wu & Dunbar, 2011). Another complication with AAV-delivered gene-editors is that constitutive expression of the gene-editors could result in off-target editing (Wang, et al., 2020). Moreover, CBEs and ABEs have been shown to target both DNA and RNA, introducing transcriptome-wide off-target RNA editing (Grünewald, et al., 2019). These safety concerns of *in vivo* genome editing have motivated researchers to develop different delivery cargos and vehicles, as well as modes of drug delivery, to control persistent gene-editor expression and increase editing efficiency.

To overcome the risks of transgene integration and prolonged expression, researchers have explored different delivery cargos. Genome editing agents are delivered as DNA, mRNA, or RNP, each with their advantages and disadvantages (Fig. 3A). In addition to the concerns with DNA delivery, the long coding sequence of PE and BE machinery makes delivery difficult, as viral vectors have a limited packaging size. The dual split-AAV approach has been used to deliver BEs and PEs to target cells, but this results in lower editing efficiency (Levy, et al., 2020, Liu, et al., 2021). Researchers have recently developed compact ABEs that are compatible with single-AAV delivery (Davis, et al., 2022). However, delivery of the PE by single-AAV remains a challenge.

At present, several chemical methods for non-viral delivery strategies are being developed for both mRNA and RNP delivery to shorten the lifetime of gene-editors in the cell. Lipid nanoparticles (LNPs) are relatively non-immunogenic, highly scalable, and have large delivery capacities capable of encapsulating gene-editor cargos. LNP systems have successfully delivered and expressed Cas9 mRNAs/sgRNA to rodent skeletal muscle and liver tissue (Fig. 3B) (Finn, et al., 2018, Han, et al., Kenjo, et al., 2021, Qiu, et al., 2021). In addition, mRNAs encoding ABEs and sgRNA targeting the *PCSK9* gene were delivered *via* LNP to the livers of NHPs in two separate studies (Musunuru, et al., 2021, Rothgangl, et al., 2021). Using two different LNP formulations, *PCSK9* expression was significantly knocked down, and researchers were able to achieve on average 66% and 28% A-to-G editing with 0.2% and 0.3% indel frequency, respectively (Musunuru et al., 2021, Rothgangl et al., 2021). In one study, the induced phenotypic and genomic changes remained stable up to 8 months after treatment (Musunuru et al., 2021). mRNA delivery overcomes the risk of transgene integration present in DNA delivery and often has higher efficiency, as it avoids the necessity of nuclear entry. Also, mRNA-driven gene-editor expression is faster relative to DNA, although the expression is still prolonged. Further, lipofectamine has been shown to deliver Cas9 RNPs and, as previously discussed, ABE RNPs *in vivo* with clinically

significant efficiency (Jang et al., 2021, Zuris, et al., 2015). RNPs are an ideal cargo for genome editing applications, as there is no risk of gene insertion and RNPs typically degrade after 3 days in the RPE (Kim, et al., 2017). Despite the many advantages of RNP delivery, the large size and negative charge of the RNP-sgRNA makes intracellular delivery cumbersome. Cell-penetrating peptides (CPPs) can also transiently deliver protein or nucleic acid cargos to the retina with even lower immunogenicity (de Cogan, et al., 2017, Johnson, et al., 2008, Pescina, et al., 2018). CPPs and gene-editors can potentially be conjugated or expressed as fusion proteins, but CPP-mediated delivery is often achieved through endocytosis, which results in entrapment of the protein cargo in endosomes and subsequent lysosomal degradation (LeCher, et al., 2017). A new strategy to avoid endosomal entrapment was recently developed, which involves the coupling of CPPs to endosomal leakage domains that destabilize endosomal membranes, allowing direct translocation of the gene-editor to the cytosol (Del'Guidice, et al., 2018). Further development of CPP-mediated delivery is exciting due to its favorable safety profile, rapid delivery, and convenient packaging, which circumvents the need to encapsulate the gene-editor cargo in a viral capsid or liposome.

A recently developed strategy combines the advantages of viral and non-viral delivery vehicles to deliver mRNA and RNPs to the retina. Thus, virus-like particles (VLPs) are derived from viral molecules that have the ability to self-assemble around either mRNA or RNPs and mimic the form of a virus particle. VLPs lack viral genetic material, so they are unable to infect cells. In 2015, Prel *et al.* demonstrated efficient *in vivo* delivery of mRNAs by VLPs to mouse liver and muscle tissue (Prel, et al., 2015). They showed that effective delivery could be achieved with RNA molecules approaching 10 kb and that RNA cargo could be feasibly swapped given the presence of a 19 nucleotide stem loop, thus paving the way for VLP-mediated delivery of genome editing mRNAs (Prel et al., 2015). More recently, researchers generated VLPs from a mammalian retrotransposon-derived protein (Segel, et al., 2021). The protein PEG10, homologous to core retroviral structural proteins, was previously shown to bind RNA and form capsids (Abed, et al., 2019). By flanking target genes with *PEG10*-untranslated regions, researchers showed that human and mouse orthologs of PEG10 protein were able to deliver functional SpCas9 mRNA *in vitro,* achieving 30% and 40% indels respectively (Segel et al., 2021). Because PEG10 is an endogenously expressed protein, PEG10 VLPs may have much lower immunogenicity compared to other CRISPR delivery vehicles. Similarly, VLPs can be used to deliver Cas9 proteins. Banskota *et al.* demonstrated *in vivo* delivery of base editor RNPs using engineered virus-like particles (eVLPs) (Fig. 3B) (Banskota, et al., 2022b). Subretinal injection of eVLPs containing ABE/sgRNA RNPs significantly improved visual function in *rd12* model mice, with similar editing efficiency and reduced off-target effects relative to viral vector delivery (Banskota et al., 2022b). eVLPs do not exhibit the same risks as other delivery strategies, such as insertional mutagenesis and overexpression of the gene-editor. Importantly, when comparing genome- and transcriptome-wide off-target effects of eVLPs and LVs, only ABEs delivered by LVs exhibited off-target effects, highlighting how novel delivery modalities can improve on the safety of genome editors by minimizing off-target effects (Banskota, et al., 2022a). In addition, modifications to the eVLP glycoproteins can alter tropisms for different cell types (Banskota et al., 2022b). These results highlight the

remarkable potential of eVLPs as a novel approach to the *in vivo* delivery of therapeutic genome editing proteins.

The genes implicated in IRDs span across all retinal cells, including ganglion cells, bipolar cells, photoreceptors, and RPE cells (Fig. 1A). However, the vast majority of IRD cases involve mutations in either the photoreceptors or the RPE (Perea-Romero, et al., 2021). The standard mode of drug delivery to these cells is subretinal injection, but these injections are invasive surgeries that can lead to complications such as retinal detachment and collateral damage to the retina (Fig. 3C) (Peng, et al., 2017). As previously discussed, subretinal injection is also inefficient, as only about half of the injected volume is able to transduce cells and only the cells covered by the subretinal bleb are transduced (Ladha et al., 2022). Thus, there is demand for less invasive modes of delivery that can efficiently target photoreceptors and the RPE. Different AAV serotypes have shown preferential transduction of photoreceptors and RPE cells, whereas non-viral vehicles have a weaker tropism for cells (Gonzalez-Cordero, et al., 2018). Continued directed evolution has enabled researchers to generate numerous novel AAV capsids with specific characteristics, including AAV serotypes capable of transducing photoreceptors and RPE cells *via* the less invasive intravitreal injection (Byrne, et al., 2020, Dalkara, et al., 2013, Giannelli, et al., 2018, Keeler & Flotte, 2019). Intravitreal injection can be done in the medical office as opposed to an operating room and often results in less complications compared to subretinal injection, so it is a desirable mode of delivery for genome editing therapeutics (Suh et al., 2022) (Fig. 3C). An emerging retinal drug administration route uses transscleral microneedles to inject therapeutic agents into the subretinal or suprachoroidal space, between the choroid and sclera of the eye (Fig. 3C). Yiu *et al.* demonstrated transscleral microneedle injections of AAV8 encoding eGFP to these two spaces. In the case of suprachoroidal injection, they observed that only RPE cells were transduced, whereas robust gene delivery into the RPE and photoreceptors was observed with transscleral subretinal delivery (Yiu, et al., 2020). Transscleral microneedle injections are also an office-based procedure, so these methods of gene-editor delivery show great potential for the administration of genome editing therapeutics for IRDs.

5.3. Future Clinical Developments for Retinal Genome Editing

Genome editing is a promising strategy for treating IRDs, especially as it overcomes the shortcomings of gene augmentation therapy. However, most preclinical studies of *in vivo* genome editing have been conducted in rodent models of IRDs. Translating these treatments from rodents to humans remains a challenge, as the development of primate-specific sgRNAs will require even more preclinical testing. Thus, induced pluripotent stem cell (iPSC)-derived organoids will be extremely useful for limiting the costs of these preclinical studies and accelerating the development of genome editing therapies. iPSC-derived organoids capture the original structure and function of their counterpart organs, which allows researchers to observe treatment effects on intercellular interactions (Kim, et al., 2020).

Despite advancements in retinal organoids, it is not economically feasible to optimize sgRNA sequences *in vitro*, as a completely unique sgRNA must be developed for

each individual mutation. This drawback invites the development of *in silico* sgRNA design strategies, such as machine learning models, to design a gene-editor plus sgRNA combination that maximizes on-target editing and minimizes off-target effects (Suh et al., 2022). The highly personalized nature of genome editing, despite its advantageous therapeutic effects relative to gene augmentation, raises a fiscal barrier that must be solved for genome editing to become clinically relevant.

6. CONCLUSION

Genome editing for the treatment of IRDs is a highly feasible alternative to gene augmentation therapy. While gene augmentation strategies have demonstrated efficient gene delivery to the retina, precise Cas9 genome editing systems can treat a broader range of diseases and circumvent many of the drawbacks of gene augmentation therapy, such as waning transgene expression and inability to slow retinal degeneration. Importantly, precision genome editing can be applied to IRDs with dominant inheritance, as they can silence the mutant allele. It is unclear how generalizable gene augmentation could be for application to dominant negative diseases, even if the ratios of wildtype to mutant alleles are increased with augmentation; therefore, gene editing would be the preferred strategy for this type of mutation. Currently, rapid parallel development of several genome editing strategies is occurring, including ablate-and-replace, targeted transgene integration, base editing, and prime editing. Each strategy offers different trade-offs of efficiency, safety, and adaptability. Translation of these preclinical approaches to the human eye will be challenging, but *in silico* sgRNA design could accelerate the development of patient-specific treatments.

The first genome editing clinical trial, EDIT-101, dosed its first patients in 2020, and the results of this phase I/II trial will be essential to guide the development of safer high-efficiency modes of delivery to minimize the temporal activity window of gene-editors and the need for invasive surgery. Optimizing therapies for different routes of administration will also ensure sufficient drug delivery to enough target cells. By adopting these strategies, it is possible to broaden the therapeutic range of genome editing and unleash their true potential. The development of genome editing therapies will pave the way for achieving truly innovative, permanent cures for IRD patients that gene augmentation alone cannot provide.

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HIGHLIGHTS

- Limitations of current gene augmentation therapies need to be addressed.
- Advances in CRISPR-Cas9 systems have potential to treat inherited retinal diseases.
- Base editing and prime editing enable precise mutation corrections in the eye.
- Advances in delivery methods have improved safe, effective precision gene editing.
- Delivery of ribonucleoproteins or mRNA is ideal for therapeutic CRISPR treatments.

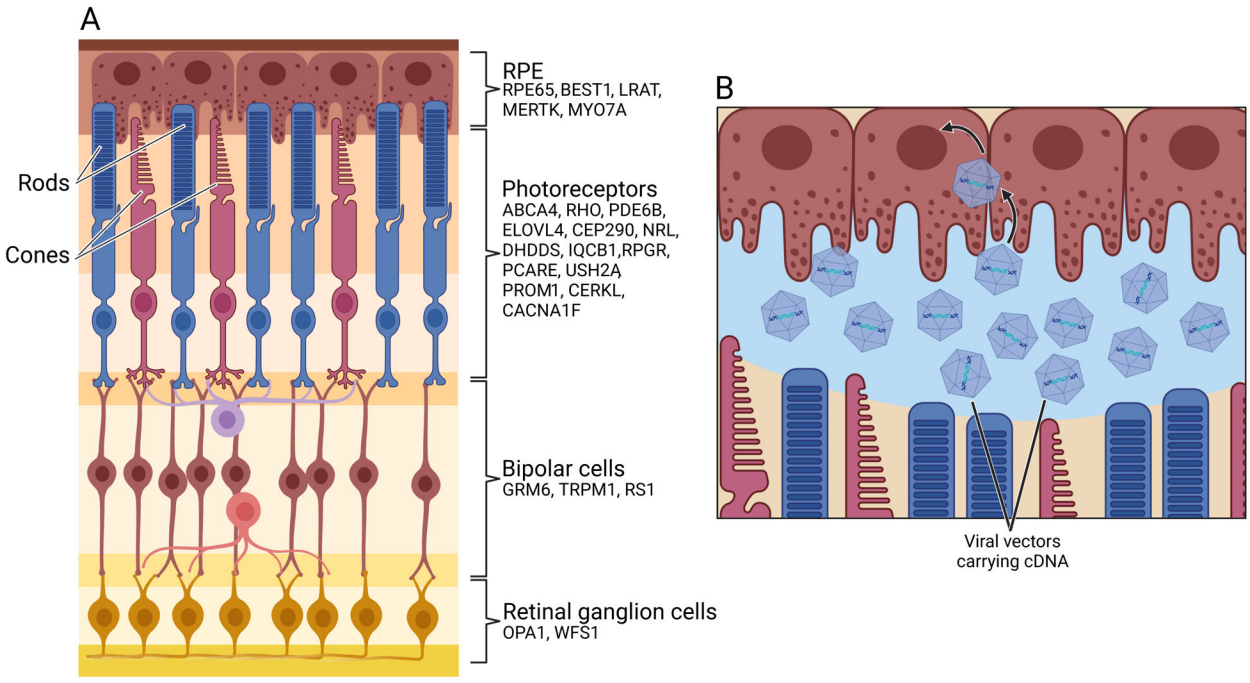

Figure 1. Selected mutations in the eye that lead to inherited retinal diseases targetable by gene augmentation.

The retinal pigment epithelium (RPE) and neural retina compose numerous cell types that support image-forming vision. (A) Selected cell types and inherited mutations in cell-specific genes that lead to inherited retinal diseases (IRDs) are highlighted here. Numerous mutations have been identified in the RPE, rod and cone photoreceptors, bipolar cells, and retinal ganglion cells. Furthermore, different mutations in the same gene can lead to different IRDs (*e.g.*, *RPE65* mutations lead to either Leber congenital amaurosis or retinitis pigmentosa). (B) Gene augmentation for inherited retinal diseases is performed by the delivery of wildtype gene cDNA delivered to the target cell *via* administration of a viral vector.

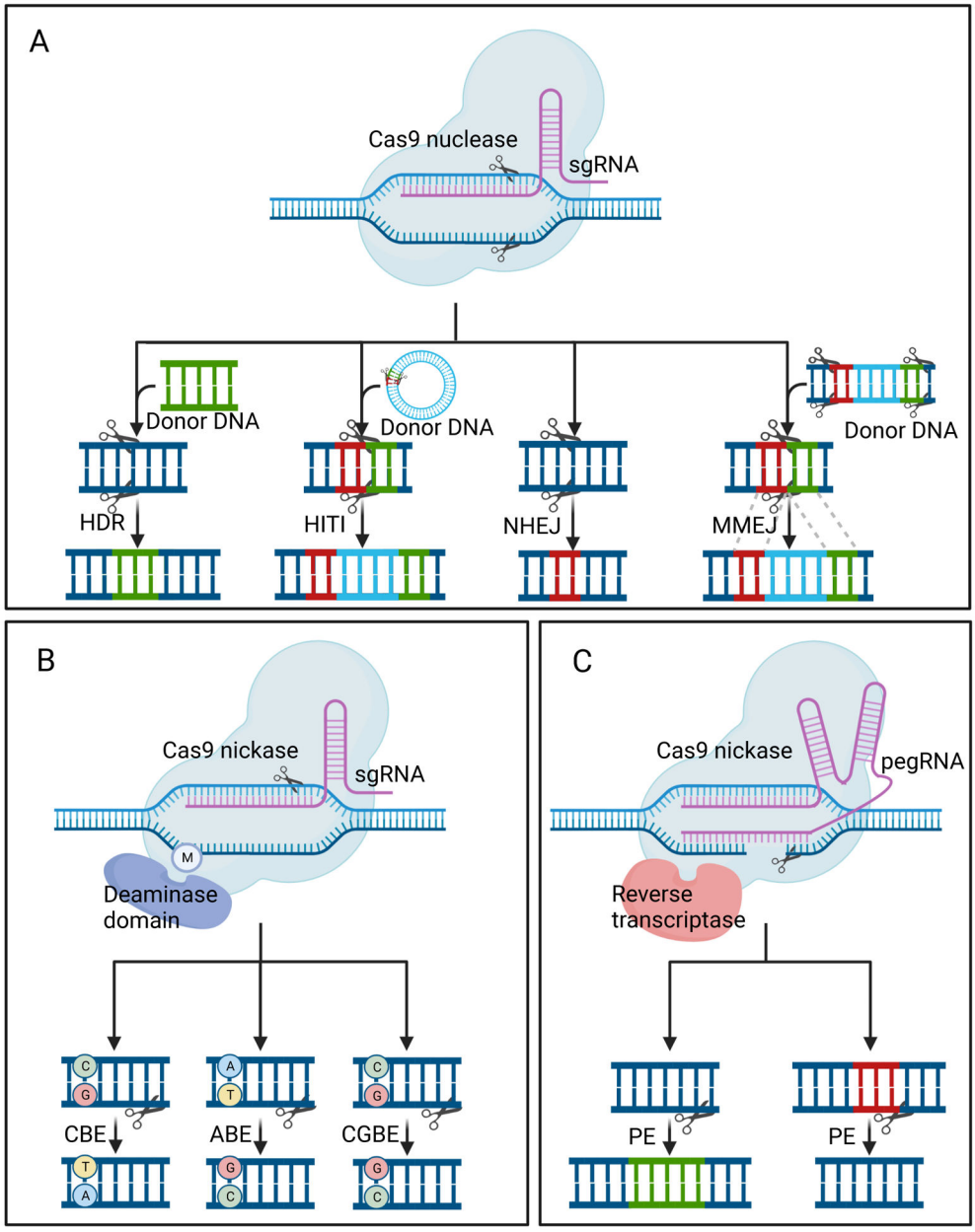

Figure 2. Summary of common genome editing strategies.

(A) CRISPR/Cas9 nuclease treatment uses a single guide RNA (sgRNA) to direct a Cas9 nuclease to a selected site in the genome. Cas9 then induces a double-stranded DNA break, which the cell repairs *via* two dominant mechanisms, homology-directed repair (HDR) and non-homologous end joining (NHEJ). HDR is commonly employed to edit DNA through donor DNA incorporation, and NHEJ is often used to knockout gene expression. Another reported strategy to incorporate a transgene using Cas9 nuclease is homology-independent targeted integration (HITI). (B) Base editing utilizes a sgRNA and Cas9 to target specific sites in the genome, but the Cas9 is mutated to cleave only the targeted strand (Cas9 nickase). The fused deaminase domain deaminates target bases on the non-target strand, and cellular repair machinery finishes the conversion. Cytosine base editors (CBEs, left) convert

cytosines to thymines, adenine base editors (ABEs, center) convert adenines to guanines, and cytosine to guanine base editors (CGBEs, right) convert cytosines to guanines. (C) Prime editing also utilizes a Cas9 nickase, but the sgRNA is further modified into a prime editing guide RNA (pegRNA) that binds to the cleaved strand and provides a template encoding the desired edit. Instead of a fused deaminase domain, a reverse transcriptase is fused to Cas9 nickase, and reverse transcribes the pegRNA; the created DNA flap is either ligated into the genome for successful editing or excised when editing is unsuccessful. Prime editing can notably be used to correct all point mutations, insert DNA (left, green), or remove DNA (right, red).

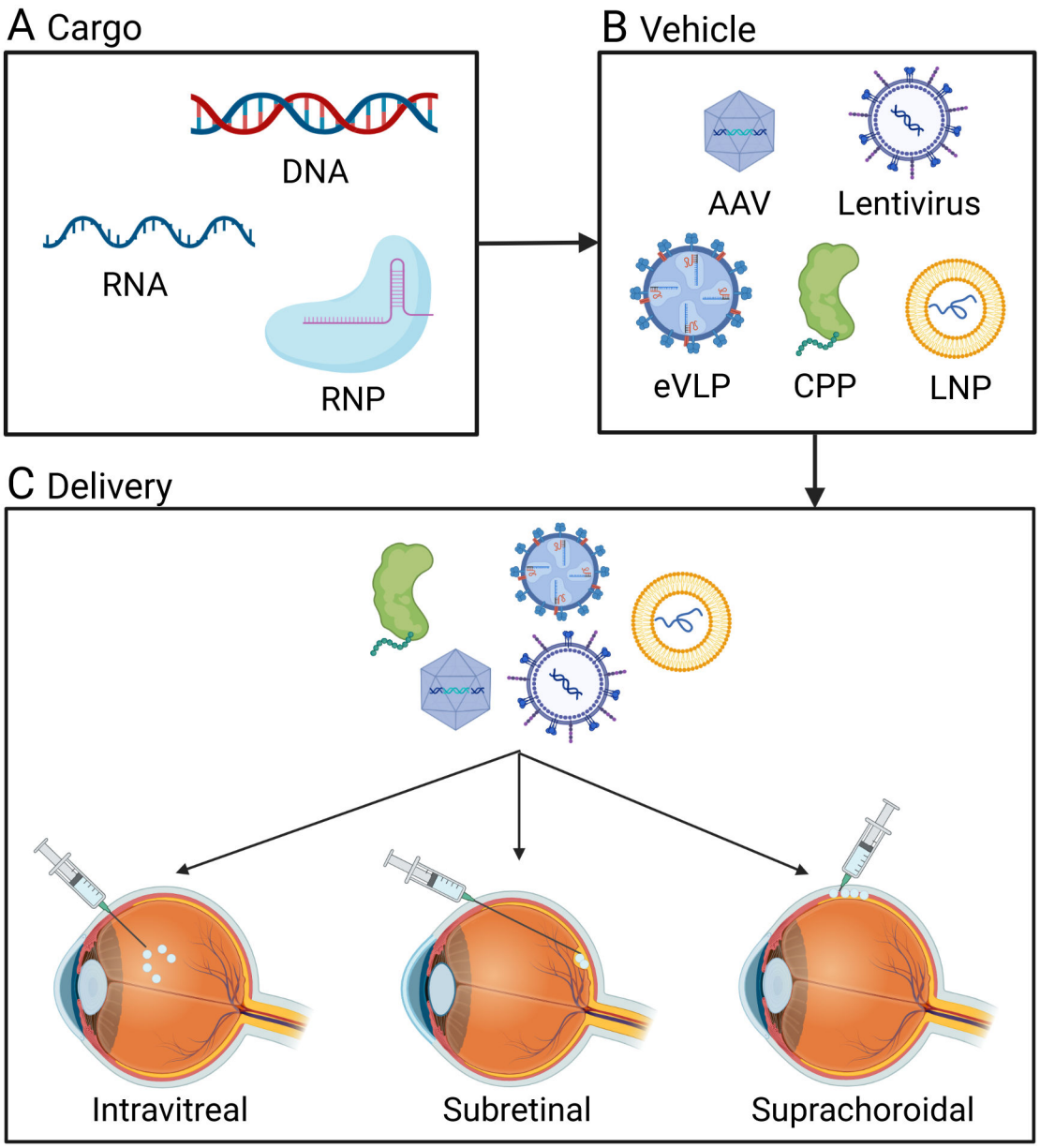

Figure 3. Precision genome editing formulation and delivery.

(A) Cas9 nucleases, base editors, and prime editors can be delivered as DNA in the form of plasmids or viral genomes, synthetic mRNA and sgRNA, or ribonucleoproteins (RNPs), composed of Cas9 protein precomplexed with an sgRNA. (B) Depending on the formulation, different delivery vehicles for genome editing cargos can be considered. These include adeno-associated viruses (AAVs) and lentiviruses (LVs) for DNA delivery, engineered virus-like particles (eVLP) and cell-penetrating peptides (CPPs) for RNP delivery, and lipid nanoparticles (LNPs) for mRNA delivery, among others. (C) Depending on the formulation and vehicle, different routes of administration can be considered. For instance, AAV capsids which have been reported to transduce the outer retina from an intravitreal injection can be injected intravitreally, while other delivery modalities would

likely be injected subretinally. Alternatively, treatments which target the RPE or the choroid could be injected suprachoroidally.