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Viral Vector Technologies and Strategies: Improving on Nature

Roxanne H. Croze, PhD
Melissa Kotterman, PhD
Christian H. Burns, MS
Chris E. Schmitt, PhD
Melissa Quezada, BS
David Schaffer, PhD
David Kirn, MD
Peter Francis, MD, PhD

Introduction

Gene therapy has been known for decades but only recently has its true potential started to be realized as a growing number of therapies are reaching the clinic.[1] This is in part due to the increased understanding of viral biology and advances in genetic engineering.[2] In particular, the use of adeno-associated virus (AAV) is an emerging gene therapy field that holds great potential to treat a wide range of diseases because of its proven safety profile.[3–5] Glybera (alipogene tiparvovec) for the treatment of lipoprotein lipase deficiency, was the first AAV gene therapy approved in the world and showed no safety concerns.[6] Two AAV gene therapies, Luxturna (voretigene neparvovec-rzyl) and Zolgensma (onasemnogene abeparvovec-xioi) have been approved in the United States, for the treatment of RPE65 biallelic mutations causing retinal dystrophy and spinal muscular atrophy, respectively. These treatments utilize naturally occurring AAV serotypes and aim to express a gene that fits within the size constraints of an AAV packaging capacity.[7–9] Luxturna and Zolgensma

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both have been successful in part due to certain low-risk aspects of treatment. For Luxturna, the subretinal route of administration is considered safe and decreases the barriers the AAV face from infecting the target cell.[10] Zolgensma requires a low dose because of the target patient population and therefore the manufacturing burden, normally large for a systemic therapeutic, was decreased.[11]

Ophthalmic diseases are an excellent therapeutic area to employ gene therapy due to the small organ size (low manufacturing burden), accessible route of administration, clear clinical endpoints, a relatively immune-privileged compartment, and a multiplicity of ocular genetic and complex diseases with high unmet medical need.[12–14] Spurred by evidence of an excellent safety profile and durable treatment effect, there are many ongoing clinical trials using viral vectors to treat various retinal diseases both monogenic conditions (choroideremia, retinitis pigmentosa, achromatopsia) and large-market complex diseases (age-related macular degeneration, Stargardt disease, and Usher syndrome).[14] This, together with ever-blossoming preclinical literature supports clinical translation. The scope of this review focuses on viral gene therapy, specifically AAV gene therapy which has proven to be the main choice of delivery vehicle for several reasons including, its inability to replicate without a helper virus or integrate, the adaptability of the viral genome, and that AAV are not known to cause human disease.[3,4] Other avenues of gene therapy including nonviral approaches for delivery are not covered in this review.

There is, however, a clear need for improvement on currently available gene therapy technology, both in terms of capsid engineering and payload design. The main issues the viral gene therapy field is looking to address are efficiency and specific delivery and expression, and payload size limitations. In earlier times, concern had been expressed about the length of time gene therapy for ocular conditions could last. To date, reassuringly, gene therapy appears to be highly durable and long-lasting as demonstrated in both preclinical studies and clinical trials. Novel technologies and strategies are being developed to address the remaining concerns based on the continual advancement in our knowledge of viral vehicles and payload cassettes. Through ingenuity and sophisticated engineering techniques, novel approaches to improve capsid specificity to diseased cell types and to modify promoters and transgenes, are under development in many groups.[8] Addressing these issues will lead to our ability to target an even broader range of diseases with viral gene therapy.

This review will cover 2 main strategies to improve viral gene therapy: viral capsid engineering and payload design. Approaches to viral capsid engineering, including rational design and directed evolution are aimed at overcoming natural barriers to delivery, improved and more selective tropism, and evading preexisting immunity. Payload design will cover strategies of dual vector approaches, mini-promoter

design, tissue/cell-specific promoters, minigene construction, and emerging technologies to correct underlying pathogenic mechanisms such as RNA interference (RNAi) and gene editing. This review will synthesize the progress of researchers in the academic, pharmaceutical, and biotechnology spheres and describes the many novel engineering techniques being utilized to date.

■ Engineering the Next Generation of AAV Capsids

Early AAV-based gene therapy products utilized naturally occurring [wild-type (WT)] AAV capsids, which have been extensively studied and showed efficacy in mouse models. The different protein capsids of naturally occurring AAV are typically referred to as serotypes, based on the presence of distinct antigens and a lack of antibody cross-reactivity. These serotypes have differences in their capsid protein sequences, which manifest as structural differences in key surface loop regions. Recently, the US Food and Drug Administration (FDA) approved Luxturna (voretigene neparvovec-rzyl), an AAV2-based product carrying the RPE65 transgene for the treatment of inherited blinding diseases caused by mutations in the *RPE65* gene.[10,15–18] In addition, promising results in clinical trials have been reported for an AAV2-based product carrying the Rep1 transgene for the treatment of choroideremia,[19] and AAV8-based products carrying the RPGRorf15 transgene and the CNGA3 transgene for the treatment of X-linked retinitis pigmentosa (XLRP) and Achromatopsia, respectively.[20,21] Table 1 outlines all the ocular gene therapy clinical trials using AAV capsids. While these early programs have shown proof-of-concept for AAV-based retinal gene therapy, they also highlight the need for better delivery vehicles, which can achieve broad, efficient transduction that will result in safe, effective gene therapy products for both monogenic and complex diseases of the retina. These better delivery vehicles would allow for the development of gene therapy products for a larger number of disease targets, as well as increase the addressable patient population to treat patients at any stage of disease progression.

Key areas for gene delivery improvement within the retina are ease of route of administration and obtaining broad delivery to either multiple cell types across the entire tissue or specific cell types in the outer retina. Current clinical trials have most often resorted to the use of subretinal injection.[10,15–17,22–25] Although subretinal delivery results in efficient transduction of cells within and closely surrounding the bleb region, this region is only a small portion of the entire retinal area.[18,19] Furthermore, the subretinal injection can cause retinal tears or detachments and other safety risks (including cataracts and outer nuclear layer thinning).[10,17,26]

Another potential route of administration for retinal gene therapy is suprachoroidal, an injection between the sclera and the choroid. Following

Table 1. *AAV Capsids Used in Clinical Ocular Gene Therapy*

Disease Target	Capsid	Product Name	Route of Administration	Clinical Phase	Trial Identifier
RPE65 mutations (LCA2)	AAV2	Luxturna (voretigene neparvovec-rzyl)	Subretinal	FDA approved	
Achromatopsia	AAV2tYF	AGTC-402	Subretinal	Phase I/II	NCT02935517
Achromatopsia	AAV8	AAV-CNGBA	Subretinal	Phase I/II	NCT03758404
Achromatopsia	AAV2tYF	AGTC-401	Subretinal	Phase I/II	NCT02599922
Achromatopsia	AAV8	AAV-CNGB3	Subretinal	Phase I/II	NCT03001310
Choroideremia	AAV2	AAV2-REP1	Subretinal	Phase III	NCT03496012
				Phase I/II	NCT01461213
Choroideremia	AAV2	SPK-7001	Subretinal	Phase I/II	NCT02341807
Choroideremia	R100	4D-110	Intravitreal	Phase I	NCT04483440
Diabetic macular edema	AAV7m8	ADVM-022	Intravitreal	Phase II	NCT04418427
Diabetic retinopathy	AAV8	RGX-314	Suprachoroidal	Phase II	NCT04567550
RPE65 mutations	AAV5	AAV-RPE65	Subretinal	Phase I/II	NCT02781480
Retinitis pigmentosa	AAV5	HORA-PDE6B	Subretinal	Phase I/II	NCT03328130
Retinitis pigmentosa	AAV7m8	GS030-DP	Intravitreal	Phase I/II	NCT03326336
Retinitis pigmentosa	AAV2	RST-001	Intravitreal	Phase I/II	NCT02556736
Retinitis pigmentosa	AAV2	rAAV2-VMD2- hMERTK	Subretinal	Phase I	NCT01482195
XLRP	AAV8	AAV8-RPGR	Subretinal	Phase II/III	NCT03116113
XLRP	AAV2tYF	AGTC-501	Subretinal	Phase I/II	NCT03316560
XLRP	AAV5	AAV-RPGR	Subretinal	Phase I/II	NCT03252847
XLRP	R100	4D-125	Intravitreal	Phase I	NCT04517149
XLRS	AAV8	AAV8-scRS/IRBPhRS	Intravitreal	Phase I/II	NCT02317887
XLRS	AAV2tYF	rAAV2tYF-CB-hRS1	Intravitreal	Phase I/II	NCT02416622
Wet AMD	AAV8	RGX-314	Subretinal	Phase I/II	NCT03066258
			Suprachoroidal	Phase II	NCT04514653
Wet AMD	AAV7m8	ADVM-022	Intravitreal	Phase I	NCT03748784

AMD indicates age-related macular degeneration; AAV, adeno-associated virus; CNGB, cyclic nucleotide–gated channel beta subunit; DP, drug product; FDA, United States Food and Drug Administration; hMERTK, human tyrosine protein kinase Mer; LCA, Leber congenital amaurosis; PDE, phosphodiesterase; RPE65, retinal pigment epithelium-specific 65 kDa protein; REP1, rab-escort protein 1; RPGR, X-linked retinitis pigmentosa GTPase regulator; RS, retinoschisis; VMD, vitelliform macular dystrophy; XLRP, X-linked retinitis pigmentosa; XLRS, X-linked retinoschisis.

injection into this region, the fluid flows from the administration site around the orb of the eye and can infect a broader region of the retina than with a subretinal injection, and appears to be less likely to lead to retinal detachment compared with subretinal delivery.[27] Clinical trials using this technique to treat retinal vein occlusion[28] (NCT02303184) and macular edema[29,30] (NCT02255032, NCT02595398) have shown promising results. Early preclinical work has shown this to be an effective method of delivering an AAV8-based gene therapy in rats.[31] Further investigation into this method for retinal gene therapy is needed to fully understand its potential.

Over the past 2 decades, the intravitreal injection has proven to be a safe, routine route of administration used for the delivery of protein therapeutics to the retina that can be delivered in the doctor's office. In addition, gene therapeutics can be readily formulated and suitably concentrated for intravitreal injection. Wild-type AAV capsids are not capable of the efficient trafficking through the inner limiting membrane (ILM) and ganglion cell layer necessary to achieve broad, efficient delivery to photoreceptors and retinal pigmented epithelia (RPE) to the posterior of the retina via an intravitreal injection. An engineered AAV capsid with the ability to traverse the ILM following an intravitreal injection is an ideal delivery modality for broad retinal distribution, and this work is ongoing (Kotterman M, Beliakoff G, Croze R, Vazin T, Schmitt C, Szymanski P, Leong M, Quezada M, Holt J, Barglow K, Hassanipour M, Schaffer D, Francis P, Kirn D, unpublished data, 2021).

The concept behind AAV capsid engineering is based on the observation that the differences in protein sequence and structure between the WT capsid lead to differences in the cell surface receptors utilized for entry, the transduction efficiency for various cell types, the relative biodistribution within an organism, and the affinity for antibodies. It is thus hypothesized that additional capsid protein changes can lead to improvements to overcome 1 or more main challenges to gene delivery. The approaches used for AAV capsid engineering fall into 3 categories: isolation of naturally occurring capsids, rational design, and directed evolution. Isolation of naturally occurring capsids involves the use of polymerase chain reaction or other amplification methods to extract latent or persistent AAV genomes from tissues of organisms that have been naturally exposed to AAV. The rational design combines knowledge of AAV biological properties, known functions for specific residues or regions, and structural analyses to inform capsid alterations. Directed evolution involves iterative high-throughput screening of libraries generated through genetic diversification and subjected to selective pressure to identify capsid variants that have accumulated modifications that improve function. A key feature of directed evolution is that it can successfully identify enhanced variants in the absence of complete knowledge of underlying structure-function relationships in the capsid proteins and the target cells.

Isolation of Naturally Occurring AAV Capsids for Retinal Gene Therapy

AAV2 and other AAV serotypes were originally isolated as contaminants within a laboratory adenovirus stock.[32] The successful cloning, sequencing, and use of the AAV2 capsid as a recombinant gene delivery vehicle subsequently led researchers to the identification of other serotypes of AAV, such as AAV5, AAV8, and AAV9 from tissue samples from humans and non-human primates (NHPs).[33–35] As the clinical use of non-AAV2 serotypes such as AAV1, AAV5, AAV8, and AAV9 has grown, the use of these capsids has spurred attempts to isolate and characterize the gene delivery properties of additional naturally occurring AAV capsids present in a human, NHP, and even nonprimate species.[36] To date, these capsids have been evaluated primarily for systemic applications, but there are a few examples of initial evaluation within the retina.

Smith et al[36] isolated a panel of naturally occurring variants most closely related to AAV9 from human $CD34^+$ hematopoietic stem cells. During a study to evaluate transduction efficiency in the central nervous system following intravenous administration, Ellsworth et al[37] determined that AAVHSC15 and AAVHSC7 capsids were also capable of transducing cells within the retinal ganglion cell layer and inner nuclear layer. However, genome biodistribution to the eye was orders of magnitude lower compared with peripheral organs.[37]

AAV44.9 was originally identified as a contaminant in a laboratory stock of adenovirus, and it is most closely related to AAVrh8.[34] Boye et al[38] evaluated the retinal transduction of AAV44.9 and a variant AAV44.9(E531D) following subretinal administration in both mice and NHP. In mice, both AAV44.9 and AAV44.9(E531D)-mediated transduction at efficiencies higher than WT AAV comparators. In NHP, both AAV44.9 and AAV44.9(E531D) transduced cells inside and outside of the subretinal bleb region.

Rational Design of AAV Capsids for Retinal Gene Therapy

The rational design utilizes knowledge of elucidated crystal structures,[39–46] cell surface receptors,[43,47–54] infectious pathways,[55] antibody binding epitopes,[56–59] and immune system activation,[60] as well as the known relationships between the primary protein sequence, higher order protein structure, and protein function to generate alterations to the capsid to improve performance.

Research into the biology of AAV transduction identified that the presence of inhibitors of tyrosine kinases increased AAV transduction efficiency.[61,62] Further experiments determined that AAV capsids were phosphorylated at tyrosine residues, which provided a ubiquitination signal and targeted the capsids for degradation.[63] On the basis of these results,

Zhong et al[64] generated versions of the AAV2 capsid in which a surface-exposed tyrosine residue was mutated to phenylalanine (referred to as the "tyrosine mutant" capsids). In particular, Y444F and Y730F mutations resulted in significantly improved transduction in vitro and in vivo, by decreasing phosphorylation and subsequent ubiquitination of the capsids.[64] Markusic et al[65] showed that combinations of tyrosine mutations could further improve transduction. In particular, mutations to 3 surface tyrosine residues (Y444F, Y500F, Y730F; subsequently referred to as AAV2tYF) resulted in the highest transduction of murine hepatocytes in vivo.[65] These capsids also showed enhanced transduction efficiency in mouse retinas following subretinal and intravitreal administration, with the AAV2tYF capsid being capable of mediating strong and consistent transgene expression.[66] Additional modifications have also been evaluated in the retina, including mutations of surface threonine residues to valine and changes to serotypes other than AAV2.[67–69] As an example, an engineered AAV2 capsid containing mutations to 4 surface tyrosine residues and 1 surface threonine residue (Y272F, Y444F, Y500F, Y730F, T491V) efficiently transduced mouse photoreceptors following intravitreal administration.[68]

The AAV2tYF capsid is currently being utilized in preclinical and clinical gene therapy programs. Safety and efficacy studies in murine and canine models of XLRP demonstrated that the AAV2tYF capsid carrying a codon-optimized RPGR transgene driven by a GRK promoter resulted in dose-dependent RPGR protein expression and rescue of photoreceptor function.[70,71] These results supported the initiation of a phase I/II clinical trial for XLRP patients (NCT03316560). Preclinical evaluation of the AAV2tYF capsid carrying a codon-optimized RS1 transgene driven by a CMV enhancer/chicken beta-actin (CBA) promoter resulted in high levels of vector genomes in the injected eye and RS1 expression in the fovea of NHP,[72] which supported evaluation in a phase I/II clinical trial for X-linked retinoschisis patients (NCT02416622). Safety and efficacy studies evaluating products based on the AAV2tYF capsid carrying transgenes driven by a PR1.7 (photoreceptor-specific) promoter in the CNGA3 achromatopsia sheep model[73] and the CNGB3 achromatopsia mouse model[74] also lead to the initiation of phase I/II clinical trials for achromatopsia (NCT02935517 and NCT02599922).

The cell surface glycans used for cellular entry and the amino acid residues on the capsid which facilitate binding have been identified for most of the natural serotypes of AAV.[43,47–54] Boye et al[75] generated AAV capsid variants from different serotypes which modulated affinity for heparan sulfate and subsequently determined that heparan sulfate binding is necessary but not sufficient for retinal transduction following intravitreal administration and is not required for transduction of photoreceptors or RPE following subretinal administration in mice. These results provide an interesting initial insight into the mechanisms by which AAV capsids mediate transduction into the retina.

Phylogenetic modeling and ancestral sequence reconstruction have been used to generate AAV capsid sequences that were likely evolutionary predecessors of current naturally occurring AAV serotypes. One such effort, undertaken by Zinn et al,[76] focused on a node of the AAV phylogeny (Anc80) to study structure-function relationships between different residues and regions of the AAV capsid. Sequences generated from this *in silico* prediction were evaluated individually for packaging and transduction efficiency, and Anc80L65 emerged as the best variant.[76] Anc80L65 was capable of efficient transduction of mouse RPE and photoreceptor cells following subretinal administration.[76,77] Anc80L65 also demonstrated a capacity to transduce cells within the inner nuclear layer, including Müller cells.[76,77] Anc80L65 also had a faster onset of expression in the mouse retina following subretinal administration, with initial expression seen as early as 1 day postadministration and near-maximal expression 3 days postadministration.[77] The rapid onset of gene expression and transduction of RPE and photoreceptors following subretinal administration was also demonstrated in NHP.[77]

Directed Evolution of AAV Capsids for Retinal Gene Therapy

Despite the growing knowledge base of information regarding the structure and function of the AAV capsid, researchers are still left with gaps in knowledge and an incomplete understanding of the relationship between the protein sequence, structure, and function, as well as interactions with the target cell type and surrounding cells and matrix. In situations such as this, directed evolution can be used as a powerful, high-throughput approach to identify new capsids. Directed evolution is a strategy to harness genetic diversification and selection processes to enable the creation and discovery of novel synthetic biologics with desired characteristics.[78] The 2 key components to a successful directed evolution screen are the use of high quality, high diversity libraries, and the choice of an appropriate model system. Researchers in the AAV capsid engineering field have generated a multitude of libraries using in vitro and in vivo techniques for viral DNA mutagenesis, including random mutagenesis of the AAV *cap* gene, insertion of random peptide sequences, randomized hypervariable loop regions, shuffled DNA from 2 or more WT serotypes, and ancestral reconstructions.[79–84] These libraries can be used individually, but they are more commonly used in combination in an effort to more fully interrogate the diversity landscape across the entire AAV capsid. Furthermore, mutagenesis techniques can be combined during initial library creation or during the selection process to allow for additional genetic diversification and a true evolutionary process. Model systems used during capsid development for applications in the retina have ranged from primary human cells in vitro to rodents, to NHPs.

Several capsid variants were originally identified by Koerber et al[81] through selection on human astrocytes in vitro and rat astrocytes in vivo. Following the discovery of these variants, Klimczak et al[85] hypothesized that these variants may also be capable of enhanced transduction of Müller cells, due to shared properties between astrocytes and Müller cells within the retina. Additional characterization was performed in the retina using intravitreal injection to explore the potential transduction of Müller cells. Of the variants evaluated, ShH10 (a shuffled variant based on AAV6) demonstrated enhanced specificity and efficiency for rat Müller cells in vivo, with ~94% of cells transduced corresponding to Müller cells.[85] The enhanced transduction was determined to result from a combination of mutations that improved affinity for heparan sulfate proteoglycans and an N451D substitution which conferred Müller cell tropism.[85] A subsequent study showed the ShH10 capsid containing an additional tyrosine to phenylalanine substitution (ShH10Y) coupled with a 2.8 kbp fragment of the human RLBP1 promoter mediated high levels of GFP expression within murine Müller cells in vivo and human Müller cells in vitro. The ShH10 capsid has been used preclinically to show photoreceptor protection by expressing secreted neurotrophic factors in rodent models of retinal degeneration and optic nerve injury.[86–88]

Dalkara et al[89] used an in vivo selection process in which AAV capsid libraries were administered to transgenic mice expressing a rhodopsin-GFP fusion protein by intravitreal administration. Fluorescence-activated cell sorting was used to isolate rod photoreceptors, and capsid variants were amplified from these cells.[89] The dominant capsid variant which emerged from the selection, AAV7m8, was capable of highly efficient transduction of all layers of the mouse retina following intravitreal administration.[89] The AAV7m8 capsid further showed higher transduction efficiency in vitro compared with WT AAV capsids in human induced pluripotent stem cell–derived RPE and 3-dimensional cone-enriched retinal organoid cultures.[90] Comparisons have been performed between the AAV7m8 capsid and the rationally designed AAV2 tyrosine mutant capsids in mice, NHP, and ex vivo retinal explants, and the AAV7m8 capsid is capable of higher transduction efficiency in all cases.[86,89,91]

Gene therapy products using the AAV7m8 capsid have demonstrated preclinical proof-of-concept for treatment in several mouse models of inherited retinal diseases, including retinoschisis,[89,92] Leber congenital amarousis,[89] and retinitis pigmentosa.[93] Preclinical evaluation of AAV7m8 also demonstrated efficacy and long-term durability of expression of a product carrying the aflibercept transgene in an NHP model of laser-induced choroidal neovascularization.[94] These results supported evaluation in a phase I/II clinical trial for wet age-related macular degeneration, which has currently demonstrated potential efficacy in patients (NCT03748784). Additional evaluation in a phase II clinical trial for diabetic macular edema is also ongoing (NCT04418427).

While AAV7m8-based products have resulted in functional correction in a number of mouse models of inherited retinal disease, AAV7m8 required a high dose of 5×10^{12} vg/eye to achieve transduction in NHP, was not capable of transducing RPE and did not robustly transduce outside of the fovea.[89] Additional studies by Ramachandran et al[95] reported that AAV7m8 was capable of efficient transduction of multiple cell types by subretinal injection in NHPs, but transduction was mostly restricted to the inner retina following intravitreal administration. This decrease in transduction in the NHP compared with the mouse is further evidenced by the use of a relatively high 2×10^{12} vg/eye dose of a secreted transgene to show efficacy for an anti-VEGF product.[94] These results highlight that although capsids engineered using lower-order species can be useful, in particular for applications where a secreted protein can be expressed, differences in efficiency observed between mice and NHP justify the need for additional directed evolution in large animal models.

Byrne et al[86] applied directed evolution to identify variants capable of enhanced transduction of the NHP retina following intravitreal administration. The 2 most promising variants, following the initial selection and subsequent evaluation of a subset of capsid variants and controls using a barcoded reporter transgene, were evaluated for transduction efficiency in NHPs. The top-ranked variant, named NHP#26, could only be evaluated at a low dose due to manufacturing issues. However, low dose administration of NHP#26 still resulted in strong transduction of cells within the fovea, including Müller cells, cells of the inner nuclear layer, and some foveal cones.[86] The second-ranked variant, named NHP#9, transduced foveal cone photoreceptors more efficiently compared with AAV7m8 when paired with photoreceptor-specific transgene expression driven by the PR1.7 promoter.[86] As another example of the use of directed evolution in NHPs, researchers developed an AAV capsid variant, R100, capable of superior transduction of human retinal cells compared with WT AAV and highly efficient pan-retinal expression of intracellular or secreted transgenes throughout the NHP retina following intravitreal administration (Kotterman M, Beliakoff G, Croze R, Vazin T, Schmitt C, Szymanski P, Leong M, Quezada M, Holt J, Barglow K, Hassanipour M, Schaffer D, Francis P, Kirn D, unpublished data, 2021). Preclinical evaluation supported the recent translation of products including the R100 capsid into clinical trials for choroideremia (NCT04483440) and X-linked retinitis pigmentosa (NCT04517149).

Clinical advancement of products using engineered capsids demonstrates both the utility of capsid engineering for the development of gene therapy products and the potential for further clinical benefit with the next wave of advancement. As such, additional capsid engineering efforts have been undertaken to build upon these initial advances and utilize emerging tools and technologies. The incorporation of computational design strategies can be leveraged to enable higher-throughput,

systematic optimization of the AAV capsid through rational engineering.[96] Likewise, pairing directed evolution principles with the continued incorporation and expansion of the use of bioinformatics and machine learning, through bioinformatically designed libraries[82–84,97] and the use of next-generation sequencing (NGS) for identification of top capsid variants will lead to new capsids poised for therapeutic benefit.[86] In all cases, the use of large animal models and human cell models early and often within the discovery and development pathway will provide confidence in the translation to human therapeutics.

Managing Immune and Inflammatory Responses to Ocular Gene Therapy

The therapeutic efficacy of viral gene therapy can be dampened following an immune-response targeted against either the AAV capsid or cargo.[98,99] Neutralizing antibodies (NAbs) against natural AAV serotypes are acquired following exposure within the first years of life. These preexisting NAbs recognize the viral capsid, block infection, and initiate clearance of the virion.[100] AAV seropositive individuals are typically excluded from clinical trials of AAV-based gene therapy because even relatively low titers of NAbs can abolish transduction, especially in systemic delivery.[99] However, patients that are seronegative can still mount an immune response to the virus following infection. Once the capsid is internalized, the cell begins to degrade the protein coat and peptides of the capsid can be presented as antigens to cytotoxic T-lymphocytes.[101] This triggers the immune system to eliminate the transduced cells, therefore dampening the therapeutic effect.[102] Early clinical studies involving iterative improvements to immunomodulatory regimens have resulted in the implementation of steroidal immunosuppression protocols that can help alleviate this response, allowing the capsid proteins to be fully degraded without triggering cytotoxic T-lymphocytes, maintaining the efficacy of the therapy.

The innate response can also be triggered against the viral genome following capsid degradation. This response is mediated by pathogen recognition receptors (PRRs) which recognize foreign material including viral DNA. Upon PRR stimulation, toll-like receptor (TLR) 9 and TLR2-MyD88 are activated and trigger an interferon response.[103] Although the PRR of the AAV genome are minimal, as AAV have continuously been shown to have a minimal impact on human health, this innate response is still known to exacerbate the adaptive immune response outlined above. Similarly to the humoral immune response, immunosuppression has been shown to mitigate the PRR response to viral cargo.[104,105] In gene therapy, the viral genome is replaced with a mainly eukaryotic payload which explains why most work has been surrounding techniques to mitigate the immune response to the capsid.[106] However, this is an evolving field and

there are a number of mammalian immune-response mechanisms that are not well understood including the numerous receptors responsible for DNA sensing.[107] For example, work suggests the presence of CpG motifs in the payload can trigger an immune response.[108,109] Despite these mechanisms, the eye exists in a relatively immune-privileged state, as few immune cells are found in the healthy retina and allografts usually survive well.[110]

The route of administration also plays a role in the strength of an immune response.[111,112] In the retina, 3 routes are under investigation, subretinal, suprachoroidal and intravitreal, each with their own challenges and advantages. Subretinal administration, being used in the FDA-approved Luxturna therapy,[10] yields the lowest immune response; however, treatment is limited to a small area within the retina.[17] In addition, in a clinical trial supporting the Luxturna product, readministration was successful in the contralateral eye with minimal immune response, supporting the notion of immune privilege in the eye using this route.[17] Work to understand the immune implications of a suprachoroidal injection is ongoing, however, the viral particles need to traverse the choroid to infect the retina, which some preliminary studies show elicits a stronger immune response than subretinal administration.[113] In a recent study, Chung et al[114] discovered that injection of AAV8-GFP via the suprachoroidal route did result in an immune response against the transgene but showed less humoral immune response to the capsid compared with an intravitreal route. The intravitreal injection has the potential for a larger immune response, however, can treat a broader region on the eye. Interestingly, recent work from AGTC has shown that NAbs do not appear to affect the efficacy of an intravitreally administered AAV gene therapy.[115] Overall, the eye appears to maintain its immune-privileged state following administration of AAV gene therapy products and remains a reason why ocular disease has been a successful target for novel gene therapies.[110]

Nevertheless, there is still a well-characterized retinal inflammatory response that can be triggered, that contributes to several ocular diseases.[116] In addition, following the treatment of a seronegative patient, seroconversion can occur with the potential for reduced efficacy if redosing is contemplated, especially within the same eye.[117] Therefore, a need for immune-evading retinal viral variants remains.

Aside from immunosuppression during administration, several techniques are being utilized to engineer capsids and cargos that can evade NAbs and limit immune response to ensure a strong therapeutic effect following treatment.[98,118]

Directed evolution can be used to select for a novel, immune-evading capsid. Library creation is a critical step in directed evolution to ensure numerous variants with unique potential. Chimeric variants identified from directed evolution screens have shown strong immune-evading potential against NAbs in a CNS selection.[84] Therefore, including

libraries designed with chimeric AAV variants could translate to selection for retinal capsids that evade the immune response.[119] In addition, preincubating libraries before administration in selection rounds with increasing concentrations of human intravenous immunoglobulin can select for NAb evading variants.[78]

Researchers are also beginning to map the antibody epitopes on AAV capsids. Increasing our understanding of how NAbs bind and clear AAV capsids will allow the rational design of these regions to minimize the immune response.[58] Work by Tse et al[120] has already discovered that NAbs target conserved residues along the 3-fold axis of symmetry. Site mutagenesis of these epitopes can modify these regions to yield an immune evading variant. However, caution must be taken to ensure mutations do not disrupt proper protein folding. As we understand more about how the immune system responds to both the capsids and cargo, we can continue to employ computer modeling and algorithms to generate immune-evading variants.[96,97]

In conclusion, understanding the interaction between gene therapies and the immune system is a substantial hurdle, even in the immune-privileged ocular environment. Strategies exist for the evasion of both innate and adaptive immune responses, including both directed evolution and rational design combined with immunosuppression to ensure the largest therapeutic effect. The coming years will surely unveil exciting, targeted, immune-evading gene therapies for ocular disease.

■ Payload Design

The standard AAV cargo capacity is 4.7 kbp with the viral genome consisting of a single-stranded DNA genome. This coding region of the genome is flanked by inverted terminal repeats at the ends that mainly serve as origins of replications.[21] For utilization in gene therapy, the viral coding region is excised, and a promoter, human gene coding sequence, polyadenylation signal, and regulatory elements are cloned in its place (Fig. 1).[21] This section will describe various engineering techniques to modify the promoter region for size, specificity, and strength, and the coding sequence to yield gene replacement, knockdown, gene editing, or protein production for secretion.

A Dual Vector Approach

With the AAV cargo capacity being 4.7 kbp, it means that $>20\%$ of human genes are too large to fit in a single AAV.[121] From a retinal perspective, diseases with genes that are too large to fit in AAV include Stargardt disease ATP-binding cassette transporter protein (*ABCA4*), Usher syndrome, type 1B (*MYO7A*), and Usher syndrome, type 2A

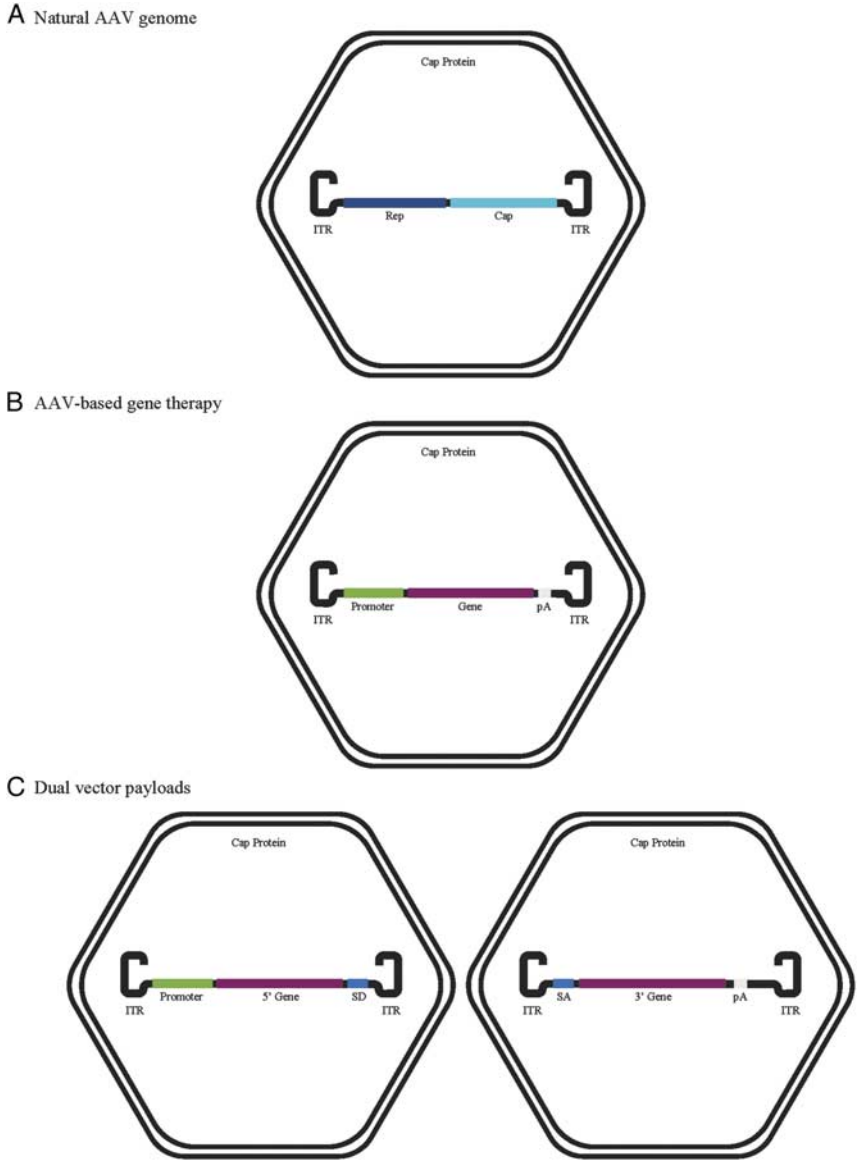

Figure 1. *Adeno-associated virus (AAV) payload schematic. The natural AAV genome is diagramed in the first image (A). The second image (B) represents a typical gene therapy payload with the rep and cap genes excised and a promoter, gene of interest and poly A (pA) tail cloned in between the inverted terminal repeats (ITRs). The third image (C) represents a dual vector gene therapy approach, where 2 capsids encoded the gene of interest in 2 parts with splice donor (SD) and splice acceptor (SA) regions to enable homologous recombination after infection of a cell and deencapsidation.*

(*USH2A*). In addition, not only the human coding sequence needs to fit within the 4.7 kbp limit, but the promoter driving the expression as well. Many gene therapy products utilize large ubiquitous promoters such as CBA and a cytomegalovirus enhancer fused to CBA (CAG), which further limits the available space for the actual gene of interest.[1]

Even with this limitation, AAV viral gene delivery is still a highly desirable and favorable delivery vesicle in comparison to lentivirus and other retrovirus whose cargo capacity is significantly larger than that of AAV. The disadvantages of utilizing lentivirus and retrovirus in the retina are they have less ability to diffuse through the ILM and the multilayers of the retina and have increased potential to cause an unwanted immune response.[122] Therefore, being able to overcome the limitation of the small cargo space of AAV is a highly attractive concept. A dual vector, as well as triple vector strategies, hold high promise in addressing this obstacle.

The general principle of the dual vector system is co-transduction of a cell with 2 distinct AAV vectors each carrying a piece of a divided transgene which expands the AAV cargo capacity to 9 kbp. Following virus uptake and unsheathing, the 2 pieces of the transgene are combined. This approach has been previously reported to be efficient[123] to expand the AAV cargo capacity. This method takes advantage of the concatemerization of AAV genomes.[124] Different strategies have been explored to achieve this, including overlapping, trans-splicing, hybrid, and fragmentation of each transgene. In the overlapping strategy, both vectors display a region of sequence homology to promote intermolecular homologous recombination generating the larger transgene, but this is highly dependent on recombination efficiency of the overlapping sequence. In the trans-splicing strategy, the transgene is split where one vector, typically described as the upstream portion, contains the promoter, a 5′ portion of the gene, followed by a splice donor sequence. The downstream vector contains a splice acceptor sequence, the remaining 3′ gene and a poly A tail (Fig. 1). The hybrid strategy is a combination of the overlapping and trans-splicing strategies. The technique primarily uses trans-splicing vectors with the inclusion of an overlapping sequence within the intron of both the 5′ and 3′ vectors. The fragmented technique forces the larger transgene in a single AAV vector and is often used as a comparative baseline to the alternative dual vector approach.[125]

The development of an AAV gene therapy for Usher syndrome, type 1B was investigated by Lopes et al.[126] To deliver the Usher 1B gene, myosin VIIA (MY07A, 8.7 kbp) was packaged in a dual vector system of 2 overlapping halves in AAV2 vectors, as well as single transgene in AAV2 and AAV5 vectors, as oversized constructs. Although the packaging size exceeded the single AAV2 and AAV5 vectors, successful transduction of photoreceptor and RPE cells in vitro and in vivo were observed. The overlapping dual vector construct showed detection of the full-length MYO7A protein, but the level of protein expression was variable and

phenotype correction was minor.[126] Alternatively, Trapani et al[127] performed extensive studies to also address Usher 1B syndrome and Stargardt disease applying multiple dual vector techniques of AAV2/AAV8 overlapping, trans-splicing, hybrid and fragmented to deliver the Usher 1B gene, *MY07A* and the Stargardt gene, *ABCA4* in vitro and in vivo. The dual vector overlapping construct gave better expression levels than the fragmented and hybrid approach in vitro. Although this did not directly translate in vivo, trans-splicing and the hybrid approach were observed to transduce mouse and pig photoreceptors efficiently and significantly improved the retinal phenotype of mouse Stargardt and Usher syndrome, type 1B models.[127]

A triple vector approach has also been investigated to address additional retinal degeneration diseases with larger transgenes and can extend the AAV cargo capacity up to 14 kbp. Maddalena and colleagues studied the triple vector approach for Usher syndrome, type 1D, and Alström syndrome type 1. The triple vector system composed of either 5′ coding region of the gene (CDS), body CDS, or 3′ CDS of either Usher syndrome, type 1D gene, *CDH23* (10.1 kbp) or Alström syndrome type 1 gene, *ALMS1* (12.5 kbp) was then delivered to mouse and pig retina. Expected full-length products along with several unwanted transcripts were detected but demonstrated only the full-length transcripts translated in vivo. A triple vector approach for *ALMS1* delivery yielded transduction of 4% of photoreceptors and demonstrated correct localization in photoreceptors of the mouse retina. Interestingly, in the pig retina, 40% transduction was observed in comparison to a single vector approach.[128]

Overall, these studies suggest dual vector and triple vector systems can be promising in AAV gene therapy as the successful expression has been observed with the appropriate construct designs and general cautions to carefully assess preclinical safety outcomes to avoid additional unwanted products.

Minigenes

Minigenes are truncated versions of large genes that maintain function. Minigenes have been utilized heavily in several indications within the gene therapy space. As an alternative to dual vector approaches, such as trans-splicing, or high capacity AAV vectors, which are still under development,[129] minigenes have allowed not only an increased understanding of the protein structure-function relationship but also the functional requirements for effective gene therapy.

The bulk of the work surrounding minigenes in the context of gene therapy has been done with the engineering of the dystrophin protein in Duchenne muscular dystrophy (DMD). Current gene therapy approaches for DMD all utilize different variations of microdystrophin, a highly truncated dystrophin gene that can fit within the limits of AAV genome capacity. Preclinically, these microdystrophins have shown strong efficacy in the MDX mouse model of DMD,[130] and several variations of

microdystrophin have been implemented into current and ongoing clinical trials.[131,132] In addition to DMD, work has been done to identify minigene constructs for the treatment of cystic fibrosis (CF). Several papers identified a disposable element of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, to decrease the gene size to fit within the AAV packaging limits.[133–135] The *CFTR* minigene is currently under investigation for a potential CF AAV gene therapy.[136]

Within the retina, *CEP290* minigenes have been explored preclinically for Leber congenital amaurosis (LCA) 10. Using the ability of fibroblast to form cilia, researchers screened multiple truncations of *CEP290* expressed from an AAV vector, from which they identified a substantial truncation of *CEP290* that resulted in the rescue of cilia formation in vitro. In the LCA10 mouse model, intravitreal administration of AAV encoding truncated *CEP290* was able to significantly delay photoreceptor degeneration and maintain function compared with control mice.[137] These developments, among others, have dramatically increased the potential of AAV-mediated gene therapy to go after large gene diseases such as DMD, CF, and LCA10.

Promoters

Among the top considerations for developing an effective and specific gene therapy, is the regulation of the transgene through the promoter. Standard practices in the gene therapy field for regulating transgene expression include cell-type specific, minimal, and strong ubiquitous promoters. However techniques such as directed evolution and identification of transcriptional regulatory elements by NGS are being researched as the next generation of promoters to increase specificity and strength of transgene expression.

The rational design of novel promoters from core enhancer and regulatory elements defined by NGS is a newer field that has the potential to dramatically increase the regulation of transgenes to near endogenous levels. This again has been a huge area of focus in the CF field. Zhang et al[138] identified 2 predominant airway-specific DNase hypersensitive sites in the *CFTR* locus at the −35 and −40 kbp sites (relative to the promoter). Cobbling together these peaks from DNase hypersensitivity analysis and chromatin immunoprecipitation sequencing based on marks of active transcription, in front of a minimal *CFTR* core promoter increased activity almost 30-fold. Individual regulatory elements had smaller, yet still quite significant effects, although they function better in concert. Applying these techniques and pairing identified regulatory elements in the context of retinal gene therapy is still in its early phases, however, this approach is highly applicable, and in fact favorable, in indications where the regulation of the levels of the therapeutic transgene are essential like rhodopsin-mediated autosomal

dominant retinitis pigmentosa (adRP), where a 20% overexpression of the rhodopsin gene (*RHO*) has been shown to be cytotoxic to photoreceptors, necessitating the tight regulation of transgene expression.[139]

The directed evolution of promoters is a relatively new area with great potential and much left to be explored. Early work focused on large libraries of tandem transcription factor binding sequences in tandem with a CMV ubiquitous promoter.[140] This library was put through screening in a variety of cell lines to identify strong ubiquitous promoters and identified promoters with repeats that enhanced promoter activity > 2-fold. In a follow-up, Yan and colleagues utilized this approach to identify small lung-specific promoters for CF gene therapy, by running these libraries through several lung lines, including primary airway cells isolated from the ferret. From these selections, they identified tandem enhancer elements that increased the activity of a small core promoter by > 50-fold in primary lung cultures.[141] Applying this directed evolution strategy to retinal promoters could feasibly lead to the identification of novel retinal specific transcription factor binding motifs and small enhancer elements that could greatly enhance the activity and the specificity of minimal retinal promoters or to generic core promoters.

RNAi

Since the discovery of RNAi technology over 20 years ago, many attempts at harnessing this technology have been made, but only recently have serious breakthroughs in using the technology in vivo been made. RNAi, in the context of gene therapy, consists of the expression of an exogenous RNA molecule, usually as a short-hairpin or micro-RNA (shRNA or miRNA), which targets a gene's mRNA transcript to suppress translation of the protein. RNAi can be conceptualized with 2 differing approaches: broad suppression of the target gene (irrelevant of any potential toxic disease-causing mutations) or highly specific, allele-targeted suppression (targeting the disease-causing mRNA only). Within the clinical space, significant effort has been dedicated to targeting diseased gene transcripts with RNAi, including the current ongoing trials of ProQR injectable RNAi for the P23H rhodopsin-mediated adRP (QR-1123), and the AAV gene therapy utilizing miRNA targeting the mutant huntingtin transcript by UniQure (AMT-130) recently dosing Ph1/2 trials.

The allele-specific RNAi approach is one that has been explored widely, although it has proven technically difficult. The rationale with allele-specific–mediated RNAi, is that suppression of the disease allele is maximized, while the functional WT transcript from the other allele is untouched; allowing it to perform its normal function and translate into protein. This is especially relevant in the context of dominant diseases, like adRP, where the mutant protein has a negative impact on the function of the WT protein. The ProQR therapy (QR-1123) trial, although not gene therapy, has shown strong

preclinical efficacy in humanized adRP mouse models and was a proof-of-concept for RNAi-based technologies in the retina.[142] The authors also show that repeated injections of QR-1123 reduced rhodopsin mRNA transcript by up to 40% in mice and increased outer nuclear layer thickness compared with the sham injections. In addition, they showed only a slight reduction in WT rhodopsin transcript levels in WT mice at the highest dose, emphasizing the specificity of their P23H targeted RNAi. Although it has been observed that overexpression of the RNAi can ultimately lead to off-target effects towards the WT mRNA due in part due to mass action effects, this can potentially be overcome by modifications to the target sequence (increasing mutations and mutant location within target sequence).

Alternatively, broad suppression of both alleles, with or without the secondary replacement of the WT gene, is another exciting but equally challenging approach in RNAi mediated gene therapy. This approach utilizes the knockdown of both alleles and can include the simultaneous replacement of the target gene driven by an additional promoter, termed "suppress and replace." Preclinical work and an ongoing Ph1/2 clinical trial on Huntington disease utilizes a miRNA targeting the disease-associated huntingtin (*HTT*) transcript broadly, which has been shown to be nondeleterious in adult neurons when ablated completely. In their preclinical work in the mini-pig Huntington model,[143] the *HTT* targeted miRNA reduced mutant HTT protein and mRNA expression dramatically at the highest dose level. Clinical dosing of the Ph1/2 trial is currently in progress (NCT04120493). Although not clinically implemented, suppress and replace RNAi technology has had success in the preclinical space. Cideciyan et al[144] implemented the suppress and replace strategy in the dog model of rhodopsin-mediated RP. Utilizing an AAV delivered shRNA targeting rhodopsin and a minimal rhodopsin promoter driving an shRNA-resistant rhodopsin cDNA, researchers demonstrated long-term efficacy in the reduction of rhodopsin, and replacement of endogenous rhodopsin up to 30% of WT levels. Importantly, they observed profound retention of photoreceptor integrity, suggesting this is a promising gene therapy strategy for the broad treatment of rhodopsin-mediated RP.

Within the ophthalmic space, significant strides have been made to implement RNAi, as an injection (ProQR) in adRP (rhodopsin suppression), antisense oligonucleotide mediated exon skipping in LCA10 (ProQR), and as gene therapy for treatment for adRP[144] and wet age-related macular degeneration.[145] Outside the retina, viral delivery of RNAi using AAV8 showed promising results in NHP preclinical studies and proceeded into clinical trials for Hepatitis C virus treatment without safety concerns (NCT01899092).[146,147] Several nonviral modes of delivery have also been examined including lipoprotein conjugation, liposomes, and various nanoparticles with varying degrees of success.[148]

Although the clinical approval of RNAi-mediated treatments as a biologic or gene therapy have been limited since its discovery over 20 years

ago, recent progress in limiting the toxicity of RNAi, and optimal delivery and specificity have ignited newfound hope for the utility of the technology.

CRISPR-Cas9

The discovery of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins has dramatically expanded the reach of gene therapy, no longer limiting us to the expression of a gene packaged in an AAV, or to knockdown of toxic disease-associated transcripts using RNAi. Using CRISPR-Cas9 it is possible to change the genetic code to reverse disease by altering the mutations that cause disease (gene editing) or simply preventing the production of toxic proteins in disease (knockout). However, CRISPR-Cas9 comes with its own unique set of challenges, including the size of Cas9 in the context of an AAV gene therapy and the immunogenicity of bacterial proteins being expressed in vivo. Despite these challenges, CRISPR-Cas9 has moved into the clinic paving the way for the future of genetic disease treatment.

Within the eye, significant efforts have been made to optimize CRISPR-Cas9 for gene therapy, including the recent dosing of the first CRISPR therapy for LCA from Allergan (NCT03872479). Allergan and Editas, whose EDIT-101 gene therapy has initiated first-in-human trials, has several AAV gene therapies utilizing the CRISPR-Cas9 system to correct diseased genes, such as *CEP290* in LCA, *USH2A* in Usher Syndrome, and *RHO* in retinitis pigmentosa type 4.

Although significant progress has been made, there exist several obstacles to normalizing CRISPR-based gene therapy. For example, there is still ongoing work to determine the immunogenic effects of CRISPR-Cas9, a bacterial protein complex, when expressed in a human. A recent study from Charlesworth et al[149] demonstrated that preexisting antibodies to Cas9 in adult humans is upwards of 70%. In addition, Cas9 therapies are currently limited in part by the size of the Cas9 protein. Studies have been undertaken to identify smaller, active Cas proteins for gene editing that maintain high activity. Recent work from Liu et al[150] identified another genome editor, CasX (986bp), that is smaller than Cas9 and Cas12 and could be more amenable to different types of gene therapy approaches. Combining the power of CRISPR/Cas gene editing with gene therapy is a natural progression for these 2 fields and holds great promise for many patients suffering from disease.

ZFNs and Meganucleases

In addition to CRISPR-Cas9 gene editors, additional technologies have emerged to bring gene editing into the gene therapy space, including zinc-finger nucleases (ZFNs) and meganucleases. Each with advantages and distinct progression through preclinical and clinical-stage programs.

ZFNs are the first gene-editing technology to make an appearance in the clinic, with Sangamo Therapeutics SB-913 for Hunter syndrome (*MPSII*), a neurological disorder, dosing its first patient in early 2018 (NCT03041324). Initial, in vivo work from Sangamo,[151] showed promising results using their 3-vector system to introduce 2 ZFNs, targeting the albumin locus, and a homology template to place the iduronate-2-sulfatase (*IDS*) gene coding sequence in frame with the albumin intron 1. In *MPSII* mutant mice, they show that all dose ranges had integration of the *IDS* CDS in the liver, and the mid-dose and high-dose animals achieved super-physiological *IDS* expression in the plasma of treated mice. However, the recent release of interim clinical trial data for the Ph1/2 CHAMPIONS trial showed no measurable increase in the low-dose and high-dose cohorts and only transient increases in IDS protein plasma levels in the high-dose cohort.[152] However, second-generation ZFN products have shown promise in the preclinical studies run by Sangamo[153] where they developed new architectural linkers for addressing the need to achieve both highly dense targeting of a chosen genomic locus, as well has highly active and specific cleavage. Additional considerations surrounding ZFNs that we can take away from Sangamo's extensive portfolio of ZFN work include the expression of ZFNs off a single vector is limited to applications of gene suppression and the expression of multiple ZFNs off a single vector can prove problematic.[154] In the eye, ZFN technology could be used to repair many of the point mutations that plague retinal disease. Nonviral work on Usher syndrome in simple in vitro models, show the ability of ZFNs to repair mutations in the context of retinal disease.[155] Although the road to effective gene therapy using ZFNs still presents several obstacles, initial data from preclinical and clinical studies shows promise ahead.

Meganucleases, also known as "homing-nucleases," are another class of DNA endonucleases that have a large recognition site, which occurs rarely, even in entire genomes, which allows them to be used as highly specific tools in gene editing. Work by Precision Biosciences has developed the use of the ARCUS meganuclease technology in AAV gene therapy. ARCUS implements safety among all else, relying on self-inactivation of the nuclease over time such that persistent expression of the nuclease and the potential off-target effects and toxicity are eliminated.[156] Precision Biosciences has utilized their ARCUS technology in the reduction of *PCSK9* in hypercholesterolemia. In a preclinical study in NHPs,[157] meganuclease targeting *PCSK9* were administered using AAV targeting the liver. The authors observed potent decreases in *PCSK9*, with only transient expression, which was dramatically reduced shortly thereafter. This left the animals with a stable population of hepatocytes which had dramatically decreased *PCSK9* levels, with undetectable levels of meganuclease present (having been self-reduced by several orders of magnitude). Although this technology has not been clinically investigated, it is progressing rapidly across multiple

indications. This technology provides a potent gene-editing technology that shows great promise preclinically and could be applied to several indications in the eye, including adRP (targeting toxic rhodopsin expression) or even to repair mutations in diseases such as Usher syndrome.

Gene editing with CRISPR, ZFNs, or meganucleases has been limited, but relatively successful preclinically.[151,157–159] This technology can ideally be applied in the ophthalmic space, especially as an immune-privileged organ that could likely avoid the immune-response problems discussed.

RNA Editing in Retina Disease

As an alternative to CRISPR-Cas9, which alters DNA permanently and has unpredictable off-target effects, genome editing on the intermediate species, RNA, holds promise for better regulation and fewer off-target effects. RNA editing approaches are currently focused on 2 different approaches, including adenosine deaminase acting on RNA (ADAR) and RNA targeting Cas protein variants (Cas13). Although RNA editing approaches in gene therapy are relatively new, significant advances in the field have led to several strong preclinical candidates.[160–162]

ADAR technology has expanded significantly in recent years, leading to the implementation of this technology preclinically in AAV gene therapy (ShapeTx and KorroBio) and small-molecule delivery (ProQR). Although this technology has not been used extensively in the ophthalmic space, the preclinical work of others has laid the groundwork for the transition of this technology into the eye. Sinnamon et al,[163] previously demonstrated the efficacy of AAV-mediated delivery of ADAR2 into Rett syndrome (RTT) derived mouse neurons, to edit and restore the function of the MeCP2 protein in vitro. In this model of the disease, AAV1/2 harboring ADAR targeted to the MeCP2 RNA mutants underpinning RTT was used to infect neurons from the diseased mouse model where they showed roughly 72% of MeCP2 mRNA was repaired and protein levels of MeCP2 were significantly increased. Promising uses like this have encouraged the application of AAV-mediated ADAR delivery for RNA editing in diseases across multiple therapeutic areas, including some early preclinical work by KorroBio in the eye.[161]

Cas13, the RNA-specific nuclease relative of Cas9, was recently identified as a more programmable alternative to ADAR alone in RNA editing. Similar to Cas9, Cas13 requires a guide RNA to properly identify its cleavage target, which it does with a high degree of scrutiny. Pairing this with ADAR resulted in what Cox et al[164] deemed REPAIR [RNA editing for programmable A to I (G) replacement], which they show to have >900-fold increased specificity for the target RNA. Using dead-Cas13b-ADAR fusions packaged into AAV, they show that simple disease correction can achieve upwards of 28% correction of the diseased allele RNA in vitro with minimal off-target effects.

Although it comes as a new player to the field, RNA editing shows great promise for the future of gene therapy. ADARs and Cas13-ADAR fusions are already making their way through preclinical studies (ShapeTx and KorroBio). Additional work, including the directed evolution of ADARs with other specificities[165] and hijacking of the native ADAR system in vivo[166] are also new players to the RNA editing pipeline that are sure to impact the field. RNA editing in retinal disease is an approach that can reach a wide range of patient populations in diseases such as adRP P23H rhodopsin mutations where the mutant allele dramatically reduces normal gene function, Usher syndrome, type 2A, or Stargardt disease where the gene is too large for AAV genome limits and a wide range of other ocular diseases.

■ Conclusions

The FDA has approved 2 viral gene therapies for patient treatment, one of which treats a rare retinal disease by subretinal injection and many more are in clinical trials.[1] This review has outlined several creative and sophisticated techniques which are being studied and used to develop second-generation viral gene therapy products for clinical trials. These strategies engineer capsids and payloads to modify nature's blueprint for targeted delivery, immune evasion, and selective expression to modify gene and protein expression. Our continual increase in viral vector knowledge will allow further development of novel technologies to provide safer and more specific products to a wider range of patients.

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