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Retina-directed gene therapy: Achievements and remaining challenges

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ABSTRACT

Gene therapy is an innovative medical approach that offers new treatment options for congenital and acquired diseases by transferring, correcting, inactivating or regulating genes to supplement, replace or modify a gene function. The approval of voretigene neparvovec (Luxturna), a gene therapy for *RPE65*-associated retinopathy, has marked a milestone for the field of retinal gene therapy, but has also helped to accelerate the development of gene therapies for genetic diseases affecting other organs. Voretigene neparvovec is a vector based on adeno-associated virus (AAV) that delivers a functional copy of *RPE65* to supplement the missing function of this gene. The AAV-based gene delivery has proven to be versatile and safe for the transfer of genetic material to retinal cells. However, challenges remain in treating additional inherited as well as acquired retinopathies with this technology. Despite the high level of activity in this field, no other AAV gene therapy for retinal diseases has been approved since voretigene neparvovec. Ongoing research focuses on overcoming the current restraints through innovative strategies like AAV capsid engineering, dual-AAV vector systems, or CRISPR/Cas-mediated genome editing. Additionally, AAV gene therapy is being explored for the treatment of complex acquired diseases like age-related macular degeneration (AMD) and diabetic retinopathy (DR) by targeting molecules involved in the pathobiology of the degenerative processes. This review outlines the current state of retinal gene therapy, highlighting ongoing challenges and future directions.

Contents

Abbreviations: AAV, Adeno-Associated Virus; AAVR, Adeno-Associated Virus Receptor; AMD, Age-Related Macular Degeneration; ARR3, Arrestin 3; BD, Binding Domain; CMV, Cytomegalovirus; CEP290, Centrosomal Protein 290; CpG, Cytosine-phosphate-Guanine; DR, Diabetic Retinopathy; ERG, Early Growth Response; epegRNA, Extended prime editing guide RNA; GOI, Gene of Interest; HDR, Homology-Directed Repair; HITI, Homology-Independent Targeted Integration; hGRK1, Human G-protein-coupled Receptor Kinase 1; hRHO, Human Rhodopsin; IRDs, Inherited Retinal Diseases; ITR, Inverted Terminal Repeat; KRAB, Krüppel-Associated Box; LHON, Leber's Hereditary Optic Neuropathy; LNP, Lipid Nanoparticle; LV, Lentivirus; miRNA, MicroRNA; ML, Myeloid Leukemia; Mfrp, Membrane Frizzled-Related Protein; NA65p, Nucleic Acid 65 Protein; NHEJ, Non-Homologous End Joining; Nrl, Neural Retina Leucine Zipper; PE, Prime Editors; PR1.7, Photoreceptor 1.7; pA, Polyadenine (PolyA tail); RNAi, RNA Interference; RPE, Retinal Pigment Epithelium; RT, Reverse Transcriptase; SAS, Splice Acceptor Site; SCD, Sickle Cell Disease; sgRNA, Single-guide RNA; siRNA, Small Interfering RNA; STGD, Stargardt Disease; TLR9, Toll-like Receptor 9; USH1F, Usher Syndrome Type 1F; USH2A, Usher Syndrome Type 2A; VMD2, Vitelliform Macular Dystrophy 2; VEGF, Vascular Endothelial Growth Factor; VP1/2/3, Viral Protein 1/2/3.

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1. Introduction

Vision impairment has severe mental and socio-economic consequences. According to the World Health Organization 2.2 billion people globally suffer from visual disability (Burton et al., 2021). In industrialized countries acquired retinal diseases such as age-related macular degeneration (AMD), diabetic retinopathy (DR) as well as inherited retinal disorders (IRDs) are among the most common causes of irreversible vision impairment and blindness (Hartong, Berson, & Dryja, 2006; Wong et al., 2014).

Unlike acquired retinopathies, IRDs often impact a patient's life from early childhood and can lead to severe visual impairment or even complete blindness. In fact, IRDs are the main cause of vision loss in people between the ages of 15 and 45 and they affect approx. 5.5 million people worldwide (Chen et al., 2021; Cremers, Boon, Bujakowska, & Zeitz, 2018). More than 300 genes have been associated with various forms of IRDs so far (Liu et al., 2024). This heterogeneous group of diseases is caused by genetic defects that lead to structural and/or functional abnormalities in retinal cells and in most cases to a progressive loss of photoreceptors. The proteins encoded by the affected genes are involved in a wide variety of cellular biochemical mechanisms in retinal or retinal pigment epithelium cells. This includes, for example, signal transduction proteins, transcription factors, structural and cytoskeletal components, reactants of oxidative phosphorylation and energy balance, ion channels, as well as transport and phagocytosis proteins. The majority of pathogenic genetic defects affect the RPE and/or the photoreceptors (Verbakel et al., 2018).

Despite the large number of IRD-associated genes, there is currently only an approved therapy for the RPE65 gene, which is associated with Leber congenital amaurosis (LCA) IRD-linked genes.

Other than acquired retinopathies, IRDs cannot be adequately treated with conventional therapeutics like small molecules or antibodies. Gene therapy has the potential to provide a causal therapeutic option for IRD patients, possibly as a one-time treatment. In the past, various gene therapy approaches have been developed for the treatment of IRDs. A basic prerequisite for the use of gene supplementation therapy is the presence of a sufficient number of morphologically preserved target cells (e.g., RPE, rod or cone photoreceptors) for the expression of the transgene. The most commonly used treatment concept is gene supplementation (also known as gene augmentation or gene replacement), which is typically applied for monogenetic, autosomal recessive forms of IRD. Since the administration of 'naked' nucleic acids is not efficient in retinal cells, various viral and non-viral vector systems have been explored for the effective and durable expression of therapeutic genes. Ideal vectors should penetrate sufficiently into the retinal tissue and allow efficient transduction of specific target cells without causing adverse immune reactions and toxicity.

The strategy of gene supplementation is often no longer applicable in late-stage IRD patients because the degeneration of the retina has already progressed too far. Other treatment concepts, such as optogenetic therapy or cell therapy, are being explored to treat late-stage IRDs (Khaboushan et al., 2024; Yan et al., 2023). Moreover, gene supplementation therapy is less suitable for addressing autosomal dominant IRDs, particularly those caused by dominant-negative or gain-of-function mutations (Da Meng, Ragi, & Tsang, 2020). In such cases, the diseased allele or the corresponding protein must be removed and the intact version of the affected gene supplemented at the same time. Alternatively, the endogenous pathogenic gene mutation could be corrected using genome editing technologies (Li et al., 2020). Another restraint of gene supplementation is the limited loading capacity of the most commonly used gene therapy vectors (Bulcha, Wang, Ma, Tai, & Gao, 2021). Alternative approaches and technologies are therefore needed to treat as many forms of IRD as possible (Fig. 1).

AMD affects around 200 million individuals worldwide (Vyawahare & Shinde, 2022; Wong et al., 2014). Although the disease progresses slowly and rarely leads to complete blindness, patients have increasing

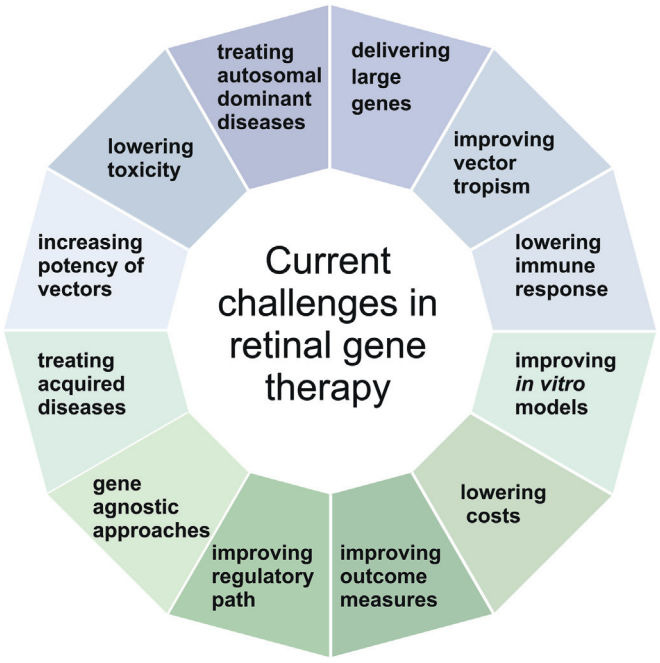

Fig. 1. Current challenges in retinal gene therapy. These include technical hurdles such as delivering large genes, improving vector tropism, and lowering immune response, as well as broader issues like regulatory pathways, costs, and outcome measures. Addressing these challenges is crucial for advancing gene therapy for retinal diseases.1.2 Gene delivery technologies for retinal gene therapy.

difficulty with everyday activities such as reading and recognizing faces. Despite coordinated efforts, the pathophysiology of AMD is not yet fully understood. An early characteristic of AMD is the presence of extracellular drusen deposits between the RPE and Bruch's membrane, a thin layer separating the neural retina from the underlying choroidal capillaries. Chronic inflammation and oxidative stress in this area appear to be the cause of primary RPE dysfunction and degeneration followed by progressive photoreceptor loss. Besides this nonexudative ("dry") form, AMD can also develop into a neovascular ("wet") form that is characterized by the ingrowth of leaky choroidal and/or retinal blood vessels into the neuronal retina (Anderson et al., 2010). While these can cause retinal edema and rapid vision loss, they only occur in approx. 10 % of AMD patients (Ambati & Fowler, 2012). In wet AMD, hypoxia-inducible factors, along with inflammation resulting from cellular damage, trigger immune responses that promote the production of angiogenic factors, most notably vascular endothelial growth factor (VEGF) (Mammadzada, Corredoira, & André, 2019).

DR develops as a vision impairing complication of diabetes mellitus that is currently affecting more than 120 million people worldwide (Teo et al., 2021; Zheng, He, & Congdon, 2012). Retinal damage results from chronic hyperglycemia and hypertension causing blood-retinal barrier breakdown, retinal ischemia, and pathological retinal neovascularization. The disease progresses through non-proliferative and proliferative stages, marked by increasing vascular abnormalities and the eventual formation of fragile, leaky blood vessels (Yadav et al., 2023). Pathophysiological mechanisms that trigger these changes encompass genetic and epigenetic factors, elevated free radical production, inflammatory agents, and VEGF expression (Brownlee, 2001; Kowluru, Kowluru, Mishra, & Kumar, 2015; Perrone, Matrone, & Singh, 2014).

In recent decades, significant progress in key antibody and protein engineering technologies has enabled the development of advanced biologics for the treatment of symptoms of acquired retinal diseases like AMD and DR. One such advancement is the prevention of neovascularization by VEGF inhibitors like antibodies (Bevacizumab), antibody

fragments (Ranibizumab, Brolucizumab) or fusion proteins (Aflibercept) (Pożarowska & Pożarowski, 2016; Śpiewak, Drzyzga, Dorecka, & Wyględowska-Promieńska, 2024). However, none of these treatments is curative and able to reverse or even halt the progression of the disease. Moreover, the available treatments need to be repeatedly (e.g. monthly or bimonthly) administered via intraocular injections (Moshfeghi, Pitcher, Lucas, Boucher, & Saroj, 2021). Accordingly, there is an unmet need for longer-acting therapies for AMD and DR (Fig. 1). Application of gene therapy concepts could overcome the limitations of available symptomatic AMD and DR treatments and lead to the development of curative treatments for these civilization disease (Cabral de Guimaraes, Daich Varela, Georgiou, & Michaelides, 2022; Pechan, Wadsworth, & Scaria, 2014).

In addition, gene therapy holds great promise for the treatment of acquired retinal diseases such as AMD and DR, as it offers the opportunity to tackle the underlying genetic, inflammatory, and degenerative processes with a one-off treatment. Instead of disease-specific gene supplementation, gene therapy approaches for these common diseases deliver molecules that interfere with pathophysiological signaling to provide clinical benefit to patients. One commonly explored gene therapy strategy is to convert target cells in the retina into so-called 'cell factories' that produce and secrete anti-angiogenic and/or anti-inflammatory molecules to halt or reverse disease progression. The aim is to achieve a long-term benefit with a one-off treatment and to lengthen the treatment interval, or even eliminate the need for conventional AMD/DR therapy. While challenges remain, ongoing research and clinical trials suggest that gene therapy could become a transformative approach in managing these complex retinal diseases (Fig. 1).

In recent decades, various viral and non-viral vector technologies have been explored to deliver therapeutic cargo to the target cells as efficiently and specifically as possible. A major challenge of gene therapy is the precise targeting and efficient transduction of specific cells while avoiding immune system detection and response to foreign material. Viral vectors have distinct modular features that make them well-suited for versatile genetic manipulation, high-throughput analytical techniques, large-scale production, and customizable cell or tissue-specific targeting. Non-viral vectors and vectors based on adenoviruses or lentiviruses were extensively studied in the early years of gene therapy. Most of these vector technologies are highly efficient in vitro, but in vivo they either exhibit toxicity or lack the efficiency and/or durability for gene transfer into retinal cells, especially photoreceptors. In contrast, vectors based on adeno-associated virus (AAV) have emerged in recent decades as a highly-efficient, versatile and relatively low-toxic vector technology for retinal gene delivery. Recombinant AAVs are currently the predominant vehicle for retinal gene therapy with only 3 of the more than 130 recent clinical trials on retinal gene therapy registered at clinicaltrials.gov relying on another viral vector (Table 1, https://clinicaltrials.gov/, 08/2024). For this reason, the focus of this review is on the properties and capabilities of AAV vectors.

2. The AAV vector technology

AAVs belong to the family of human parvovirus that require the presence of a helper virus for productive replication. Structurally, 60 copies of the three AAV capsid-forming viral proteins (VP1, VP2, and VP3) assemble in a symmetrically arranged icosahedral structure of approximately 20–25 nm in diameter (Xie et al., 2002). The capsid coats an ~4.7 kb single stranded DNA (ssDNA) genome that can be either plus or minus strand. Upon infection, the AAV vector enters the nucleus where the capsid uncoats to release the ssDNA genome (Fig. 2). The host cell's replication machinery converts the ssDNA into double-stranded DNA (dsDNA), which is then transcribed into mRNA. The mRNA is subsequently translated into the therapeutically active protein. For AAV-mediated gene therapy, the viral genome is replaced by a therapeutic gene expression cassette containing i) a promoter that regulates expression strength and tissue specificity, ii) the therapeutic gene of interest, and iii) other regulatory elements, such as a polyadenylation signal.

Various AAV variants have been isolated from different species, which differ in the primary amino acid sequence of the viral proteins of their capsid and the sensitivity to neutralizing sera and are therefore referred to as 'serotypes'. The 13 most commonly used serotypes (AAV1–13) exhibit distinct interaction profiles with carbohydrates and receptors on the cell surface, which define their specific tropism (Issa, Shaimardanova, Solovyeva, & Rizvanov, 2023). Serotype-specific differences in cellular transduction levels and varying immunogenicity have been described (Asokan, Schaffer, & Samulski, 2012). Alongside the naturally occurring AAV serotypes, novel chimeric or synthetic AAV vectors have been created through various bioengineering techniques to modify vector properties for therapeutic needs (Bryant et al., 2021; Büning & Srivastava, 2019; Grimm et al., 2008; Hauck, Chen, & Xiao, 2003). Recent fundamental research on the mechanisms of AAV transduction enabled scientists to adjust the vector systems for the use in specific gene therapy (Pavlou et al., 2021; Wang, Gessler, Zhan, Gallagher, & Gao, 2024).

2.1. AAV transduction modes of target cells

AAVs have been shown to interact with specific carbohydrates like galactose, heparan sulfate and sialic acid for cell attachment in a serotype-specific manner (Dhungel, Bailey, & Rasko, 2021). Cell entry of AAVs involves interaction with various transmembrane receptors and subsequent endocytosis (Bartlett, Wilcher, & Samulski, 2000). The broadly expressed transmembrane protein KIAA0319L has been identified as a critical factor for the rapid endocytosis of AAV1, AAV2, AAV3B, AAV5, AAV6, AAV8, and AAV9 through a clathrin-dependent mechanism (Pillay et al., 2016; Uhrig et al., 2012) and has been named AAV receptor (AAVR). AAVR is located on the cell surface as well as in the perinuclear trans Golgi, but its physiological function is not known. In addition, several AAVR independent pathways for cell entry of the AAVs have been described (Dudek et al., 2018). In total, more than 40 genes have been identified so far that play a role in cell entry, endocytosis, escape from the endosome, transport to the perinuclear region and uncoating of AAV (Meyer & Chapman, 2021).

Upon successful binding and endocytosis, AAV particles escape from endosomes and are transported through the nuclear pore complex into the nucleus where the capsid uncoats to release the genomes (Nicolson & Samulski, 2014) (Fig. 2). The AAV genome contains two 145 bp inverted terminal repeats which are important for replication and packaging of the AAV genome and can regulate transcription (Fisher et al., 1996). The ITRs also facilitate the formation of stable circular AAV genomes, so-called episomes, which do not integrate into the host cell genome, but associate with the chromatin in nucleosome structures (Chan et al., 2017; Penaud-Budloo et al., 2008) (Fig. 2). In post-mitotic cells, this episomal state ensures long-term expression of the therapeutic gene without the risk of insertional mutagenesis or transmission through the germline. While AAVs can infect both dividing and post-mitotic cells, gene expression in dividing cells becomes weaker over time due to the dilution of episomes by cell division. Due to the small AAV capsid diameter of 20–25 nm the packaging capacity of a single-stranded DNA genome is limited to about 5 kb (Dong, Nakai, & Xiao, 2010; Lai, Yue, & Duan, 2010). Indeed, wild-type AAV genomes are about 4.6–4.7 kb in size, and the risk of packaging truncated genomes increases when this size limit of the vector genome is exceeded. This limited packaging capacity poses a challenge for delivering larger genes. Strategies to overcome this limitation include approaches that allow for reconstitution of full-length genome, transcript or protein from dual AAV vectors delivered together into the target cell.

The broad application of AAV vectors in clinical trials, particularly for retinal diseases, underscores their potential in gene therapy. Ongoing

Table 1
Current interventional clinical trials for retinal gene therapy (https://clinicaltrials.gov/, search terms: *Retina, Gene Therapy*; time frame: 08/2021-08/2024).

Conditions	Target gene	Drug name	Vector type	Injection method	Sponsor	Phase	Study status	NCT number
Achromatopsia	CNGA3	AAV - CNGB3	AAV	intravitreal	MeiraGTx UK II Ltd	1/2	completed	03001310
Achromatopsia	CNGA3	AAV - CNGA3	AAV	subretinal	MeiraGTx UK II Ltd	1/2	completed	03758404
Achromatopsia	CNGA3/CNGB3	AAV - CNGB3 | AAV - CNGA3	AAV	subretinal	MeiraGTx UK II Ltd	1/2	completed	03278873
Achromatopsia	CNGA3	rAAV.hCNGA3	AAV8	subretinal	STZ eyetrial	1/2	active - not recruiting	02610582
Biallelic RPE65 Mutation-associated Retinal Dystrophy	RPE65	FT-001	AAV	subretinal	Frontera Therapeutics	1/2	recruiting	05858983
Biallelic RPE65 Mutation-associated Retinal Dystrophy	RPE65	voretigene neparvovec-rzyl	AAV2	subretinal	Novartis Pharmaceuticals	3	active - not recruiting	04516369
Biallelic RPE65 Mutation-associated Retinal Dystrophy	RPE65	LX101	AAV2	subretinal	Innostellar Biotherapeutics Co., Ltd	1/2	active - not recruiting	06196827
Bietti Crystalline Dystrophy	CYP4V2	VGR-R01	AAV8	subretinal	Beijing Tongren Hospital	1	unknown	05399069
Bietti Crystalline Dystrophy	CYP4V2	VGR-R01	AAV8	subretinal	Shanghai Vitalgen BioPharma Co., Ltd.	1	not yet recruitung	05694598
Bietti Crystalline Dystrophy	CYP4V2	rAAV2/8-hCYP4V2	rAAV2/8	subretinal	Beijing Tongren Hospital	1	unknown	04722107
Bietti Crystalline Dystrophy	CYP4V2	ZVS101e	AAV	subretinal	Chigenovo Co., Ltd	1/2	active - not recruiting	05832684
Bietti Crystalline Dystrophy	CYP4V2	ZVS101e	AAV	subretinal	Peking University Third Hospital	1	recruiting	05714904
Choroideremia	CHM	4D-110	AAV R100	intravitreal	4D Molecular Therapeutics	1	active - not recruiting	04483440
Choroideremia	CHM	AAV2-hCHM	AAV2	subretinal	Spark Therapeutics, Inc.	1/2	completed	02341807
Choroideremia	CHM	AAV-mediated REP1 gene replacement	AAV	subretinal	University of Oxford	2	completed	02407678
Choroideremia	CHM	BIIB111	AAV2	subretinal	Biogen	2	completed	03507686
Choroideremia	CHM	BIIB111	AAV2	subretinal	Biogen	3	completed	03496012
Choroideremia	CHM	rAAV2.REP1	AAV2	subretinal	University of Miami	2	completed	02553135
Choroideremia	CHM	rAAV2.REP1	AAV2	subretinal	University of Oxford	1/2	completed	01461213
Choroideremia	CHM	rAAV2.REP1	AAV3	subretinal	STZ eyetrial	2	completed	02671539
Choroideremia	CHM	rAAV2.REP1	AAV4	subretinal	University of Alberta	1/2	completed	02077361
Choroideremia|X-Linked Retinitis Pigmentosa	CHM	BIIB111| BIIB112	AAV2/8	subretinal	NightstaRx Ltd., a Biogen Company	3	enrolling by invitation	03584165
Diabetic Macular Edema	–	FT-003	AAV	intravitreal	Frontera Therapeutics	1/2	recruiting	06492876
Diabetic Macular Edema	–	FT-003	AAV	intravitreal	Frontera Therapeutics	1	recruiting	05916391
Diabetic Macular Edema	–	SKG0106	AAV	intravitreal	ENT Hospital of Fudan University	1	recruiting	06237777
Diabetic Macular Edema| Diabetic Retinopathy	–	4D-150	AAV R100	intravitreal	4D Molecular Therapeutics	2	recruiting	05930561
Diabetic Macular Edema| Diabetic Retinopathy	–	ADVM-022	AAV.7 m8	intravitreal	Adverum Biotechnologies, Inc.	2	completed	04418427
Diabetic Retinopathy	–	RGX-314	AAV8	subretinal	AbbVie	2	recruiting	04567550
Diabetic Retinopathy	–	RRG001	AAV	subretinal	Li Xiaorong	1	recruiting	06412224
Dry AMD	–	AAVCAGsCD59	AAV	intravitreal	Janssen Research & Development, LLC	1	completed	03144999
Dry AMD		AAV5-hRORA	AAV5	subretinal	Ocugen	1/2	recruiting	6,018,558
Dry AMD	–	GT005	AAV2	intravitreal	Gyroscope Therapeutics Limited	2	enrolling by invitation	05481827
Dry AMD	–	AAVCAGsCD59	AAV	intravitreal	Hemera Biosciences	2	withdrawn	04358471
LCA	RPE65	AAV RPE65	AAV	subretinal	MeiraGTx UK II Ltd	1/2	completed	02781480
LCA	RPE65	AAV2-hRPE65v2,voretigene neparvovec-rzyl	AAV2	subretinal	Spark Therapeutics, Inc.	3	active - not recruiting	00999609
LCA	RPE65	HG004	AAV2	subretinal	Xinhua Hospital, Shanghai Jiao Tong University School of Medicine	1	recruiting	06088992
LCA	RPE65	rAAV2/4.hRPE65	rAAV2/4	subretinal	Nantes University Hospital	1/2	completed	01496040
LCA	RPE65	rAAV2-CB-hRPE65	AAV2	subretinal	Applied Technologies Corp	1/2	completed	00749957
LCA	RPE65	rAAV2-hRPE65	AAV2	subretinal	Hadassah Medical Organization	1	completed	00821340
LCA	RPE65	voretigene neparvovec-rzyl	AAV2	subretinal	Spark Therapeutics, Inc.	1	completed	00516477
LCA	RPE65	voretigene neparvovec-rzyl	AAV2	subretinal	Spark Therapeutics, Inc.	1/2	active - not recruiting	01208389
LCA	RPE65	HG004	AAV2	subretinal	HuidaGene Therapeutics Co., Ltd.	1/2	recruiting	05906953
LCA	RPE65	rAAV2-CBSB-hRPE65	AAV2	subretinal	University of Pennsylvania	1	active - not recruiting	00481546
LCA 10	CEP290	EDIT-101	AAV	subretinal	Editas Medicine, Inc.	1/2	active - not recruiting	03872479
LCA 10	CEP290	QR-110	ASO	intravitreal	ProQR Therapeutics	1/2	terminated	03913130
LCA 10	CEP290	sepofarsen	ASO	intravitreal	ProQR Therapeutics	2/3	unknown	04855045
LCA 10	CEP290	sepofarsen	ASO	intravitreal	ProQR Therapeutics	2/3	active - not recruiting	03913143
LCA 10	CEP290	QR-110	ASO	intravitreal	ProQR Therapeutics	1/2	completed	03140969
LCA1	GUCY2D	ATSN-101	AAV5	subretinal	Atsena Therapeutics Inc.	1/2	active - not recruiting	03920007

Table 1 (*continued*)

Conditions	Target gene	Drug name	Vector type	Injection method	Sponsor	Phase	Study status	NCT number
LCA5	LCA5	AAV8.hLCA5	AAV8	subretinal	Opus Genetics, Inc	1/2	recruiting	05616793
LHON	MT-ND4	GS010	AAV	intravitreal	GenSight Biologics	3	unknown	03293524
LHON	MT-ND4	NFS-02	AAV2	intravitreal	Neurophth Therapeutics Inc	1/2	recruiting	05820152
LHON	MT-ND4	NR082	AAV2	intravitreal	Neurophth Therapeutics Inc	1/2	active - not recruiting	05293626
LHON	MT-ND4	rAAV2-ND4	AAV2	intravitreal	Huazhong University of Science and Technology		unknown	03428178
LHON	MT-ND4	rAAV2-ND4	AAV2	intravitreal	Huazhong University of Science and Technology	2/3	active - not recruiting	03153293
LHON	MT-ND4	GS010	AAV	intravitreal	GenSight Biologics	3	completed	02652780
LHON	MT-ND4	GS010	AAV	intravitreal	GenSight Biologics	3	completed	02652767
nAMD	–	4D-150	AAV (R100)	intravitreal	4D Molecular Therapeutics	1/2	recruiting	05197270
nAMD	–	AAV2-sFLT01	AAV2	intravitreal	Genzyme, a Sanofi Company	1	completed	01024998
nAMD	–	ADVM-022	AAV	intravitreal	Adverum Biotechnologies, Inc.	1	completed	03748784
nAMD	–	ADVM-022	AAV	intravitreal	Adverum Biotechnologies, Inc.	2	active - not recruiting	05536973
nAMD	–	EXG102–031	AAV	subretinal	Exegenesis Bio	1	recruiting	05903794
nAMD	–	FT-003	AAV	intravitreal	Frontera Therapeutics	1	not yet recruitung	05611424
nAMD	–	FT-003	AAV	intravitreal	Frontera Therapeutics	1/2	recruiting	06492863
nAMD	–	HG202	AAV	subretinal	HuidaGene Therapeutics Co., Ltd.	1	recruiting	06031727
nAMD	–	KH631	AAV8	subretinal	Chengdu Origen Biotechnology Co., Ltd.	1/2	recruiting	05672121
nAMD	–	KH631	AAV8	subretinal	Chengdu Origen Biotechnology Co., Ltd.	1	recruiting	05657301
nAMD	–	KH658	AAV8	subretinal	Chengdu Origen Biotechnology Co., Ltd.	1/2	not yet recruitung	06458595
nAMD	–	LX102	AAV	subretinal	Innostellar Biotherapeutics Co.,Ltd	1	recruiting	06198413
nAMD	–	LX102	AAV	subretinal | intravitreal	Innostellar Biotherapeutics Co.,Ltd	2	recruiting	06196840
nAMD	–	NG101	AAV	subretinal	Neuracles, Inc	1/2	recruiting	05984927
nAMD	–	rAAV.sFlt-1	AAV	subretinal	Lions Eye Institute, Perth, Western Australia	1/2	completed	01494805
nAMD	–	RGX-314	AAV8	subretinal	Novartis Pharmaceuticals	4	completed	01972789
nAMD	–	RGX-314	AAV8	subretinal	Oregon Health and Science University	3	completed	00469352
nAMD	–	RGX-314	AAV8	subretinal	AbbVie	2	recruiting	04514653
nAMD	–	RetinoStat	LV	subretinal	Oxford BioMedica	1	active - not recruiting	01678872
nAMD	–	RGX-314	AAV8	subretinal	AbbVie	2	completed	04832724
nAMD	–	RGX-314	AAV8	subretinal	REGENXBIO Inc.	1/2	completed	03066258
nAMD	–	RGX-314	AAV8	subretinal	AbbVie	2	enrolling by invitation	03999801
nAMD	–	RGX-314	AAV8	subretinal	AbbVie	3	recruiting	05407636
nAMD	–	RGX-314	AAV8	subretinal	AbbVie	2/3	recruiting	04704921
nAMD	–	RRG001	AAV	subretinal	Shanghai Refreshgene Technology Co., Ltd.	1/2	recruiting	06141460
nAMD	–	SKG0106	AAV	intravitreal	Skyline Therapeutics (US) Inc.	1/2	recruiting	05986864
nAMD	–	SKG0106	AAV	intravitreal	Peking Union Medical College Hospital	1	recruiting	06213038
nAMD	–	RetinoStat	LV	subretinal	Oxford BioMedica	1	completed	01301443
nAMD|Diabetic Macular Edema|Retinal Vein Occlusion	–	BD311	LV	suprachoroidal	Shanghai BDgene Co., Ltd.	1	unknown	05099094
Neuronal Ceroid Lipofuscinosis	CLN5	NGN-101	AAV	subretinal	Neurogene Inc.	1/2	active - not recruiting	05228145
Neuronal Ceroid Lipofuscinosis	CLN2	RGX-381	AAV8	subretinal	REGENXBIO Inc.	1/2	active - not recruiting	05791864
OPA1 Gene Mutation	OPA1	PYC-001	ASO	intravitreal	PYC Therapeutics	1	not yet recruitung	06461286
Retinal Degeneration	RPE65	tgAAG76 (rAAV 2/2. hRPE65p.hRPE65)	AAV2	subretinal	University College, London	1/2	completed	00643747
Retinitis Pigmentosa	ChR2	RST-001	AAV	intravitreal	AbbVie	1/2	active - not recruiting	02556736
Retinitis Pigmentosa	RHO	QR-1123	ASO	intravitreal	ProQR Therapeutics	1/2	active - not recruiting	04123626
Retinitis Pigmentosa	CNGA1	VG901	vgAAV	intravitreal	ViGeneron GmbH	1b	recruiting	06291935
Retinitis Pigmentosa	PDE6B	AAV2/5-hPDE6B	AAV2/5	subretinal	eyeDNA Therapeutics	1/2	recruiting	03328130
Retinitis Pigmentosa	RLBP1	CPK850	AAV8	subretinal	Novartis Pharmaceuticals	1/2	active - not recruiting	03374657
Retinitis Pigmentosa	–	MCO-010	AAV2	intravitreal	Nanoscope Therapeutics Inc.	2	completed	04945772

(*continued on next page*)

Table 1 (*continued*)

Conditions	Target gene	Drug name	Vector type	Injection method	Sponsor	Phase	Study status	NCT number
Retinitis Pigmentosa	–	MCO-I	AAV2	intravitreal	Nanoscope Therapeutics Inc.	1/2	completed	04919473
Retinitis Pigmentosa	FLVCR1	nL-FLVC-001	ASO	intravitreal	University of Colorado, Denver	1	enrolling by invitation	06565572
Retinitis Pigmentosa	–	RTx-015	AAV	intravitreal	Ray Therapeutics, Inc.	1	recruiting	06460844
Retinitis Pigmentosa	–	SPVN06	AAV	subretinal	SparingVision	1/2	recruiting	05748873
Retinitis Pigmentosa	RHO	OCU400	AAV	subretinal	Ocugen	3	recruiting	06388200
Retinitis Pigmentosa	hMERTK	rAAV2-VMD2-hMERTK	AAV	subretinal	King Khaled Eye Specialist Hospital	1	completed	01482195
Retinitis Pigmentosa	PDE6A	rAAV.hPDE6A	AAV	subretinal	STZ eyetrial	1/2	active - not recruiting	04611503
Retinitis Pigmentosa	–	ZM-02	AAV	intravitreal	Zhongmou Therapeutics	1	recruiting	06292650
Retinitis Pigmentosa	RHO	ZVS203e	AAV	subretinal	Peking University Third Hospital	1	recruiting	05805007
Retinitis Pigmentosa	–	GS030-DP | GS030-MD	AAV	intravitreal	GenSight Biologics	1/2	recruiting	03326336
Retinitis Pigmentosa|LCA	RHO	OCU400	AAV	subretinal	Ocugen	1/2	recruiting	05203939
Retinitis Pigmentosa|Usher Syndrome Type 2	USH2A	QR-421a	ASO	intravitreal	Laboratoires Thea	2/3	terminated	05176717
Retinitis Pigmentosa|Usher Syndrome Type 2	USH2A	QR-421a	ASO	intravitreal	ProQR Therapeutics	1/2	completed	03780257
Retinitis Pigmentosa|Usher Syndrome Type 2	USH2A	QR-421a	ASO	subretinal	Laboratoires Thea	2	terminated	05085964
Retinitis Pigmentosa|Usher Syndrome Type 2	USH2A	Ultevursen (QR 421a)	ASO	intravitreal	Laboratoires Thea	2/3	terminated	05158296
Stargardt Disease	ABCA4	ACDN-01	AAV	subretinal	Ascidian Therapeutics, Inc	1/2	recruiting	06467344
Stargardt Disease	ABCA4, ELOVL4, PROM1	MCO-010	AAV2	intravitreal	Nanoscope Therapeutics Inc.	2	completed	05417126
Stargardt Disease	ABCA4	JWK006	AAV8	subretinal	West China Hospital	1/2	active - not recruiting	06300476
nAMD	–	AAVCAGsCD59	AAV	intravitreal	Janssen Research & Development, LLC	1	completed	03585556
X-Linked Retinitis Pigmentosa	RPGR	4D-125	AAV (R100)	intravitreal	4D Molecular Therapeutics	1/2	active - not recruiting	04517149
X-Linked Retinitis Pigmentosa	RPGR	AAV2/5-RPGR	AAV2/5	subretinal	MeiraGTx UK II Ltd	1/2	completed	03252847
X-Linked Retinitis Pigmentosa	RPGR	AAV5-hRKp.RPGR	AAV5	subretinal	Janssen Research & Development, LLC	3	active - not recruiting	04794101
X-Linked Retinitis Pigmentosa	RPGR	AAV5-hRKp.RPGR	AAV5	subretinal	Janssen Research & Development, LLC	3	active - not recruiting	04671433
X-Linked Retinitis Pigmentosa	RPGR	AGTC-501	AAV2	subretinal	Beacon Therapeutics	2	enrolling by invitation	06275620
X-Linked Retinitis Pigmentosa	RPGR	BIIB112	AAV8	subretinal	Biogen	1/2	completed	03116113
X-Linked Retinitis Pigmentosa	RPGR	FT-002	AAV	subretinal	Frontera Therapeutics	1	recruiting	05874310
X-Linked Retinitis Pigmentosa	RPGR	rAAV2tYF-GRK1-hRPGRco| Control	AAV2	intravitreal	Beacon Therapeutics	2/3	recruiting	04850118
X-Linked Retinitis Pigmentosa	RPGR	rAAV2tYF-GRK1-RPGR	AAV2	subretinal	Beacon Therapeutics	1/2	active - not recruiting	03316560
X-Linked Retinitis Pigmentosa	RPGR	rAAV2tYF-GRK1-RPGR	AAV2	subretinal	Beacon Therapeutics	2	active - not recruiting	06333249
X-Linked Retinitis Pigmentosa	RPGR	FT-002	AAV	subretinal	Frontera Therapeutics	1/2	recruiting	06492850
X-linked Retinoschisis	RS1	IVB102	AAV	intravitreal	InnoVec Biotherapeutics Inc.	1	recruiting	06289452
X-linked Retinoschisis	RS1	RS1	AAV8	intravitreal	VegaVect, Inc.	1/2	active - not recruiting	02317887
X-linked Retinoschisis	RS1	JWK002	AAV8	subretinal	West China Hospital	1	recruiting	06345898
X-linked Retinoschisis	RS1	ATSN-201	AAV	subretinal	Atsena Therapeutics Inc.	1/2	recruiting	05878860
X-linked Retinoschisis	RS1	rAAV2tYF-CB-hRS1	AAV2	intravitreal	Applied Technologies Corp	1/2	completed	02416622
X-linked Retinoschisis	RS1	ZM-01	AAV	intravitreal	Zhongmou Therapeutics	1	recruiting	06066008

Abbreviations: RP retinitis pigmentosa, AMD age-related macular degeneration, IVT intravitreal injection, GA geographic atrophy, LCA Leber congenital amaurosis, LHON Leber hereditary optic neuropathy, NCT ClinicalTrials.gov identifier.

research aims to optimize AAV vectors for reduced immunogenicity, improved targeting, and enhanced delivery efficiency.

2.2. Promoters for AAV mediated retinal gene therapy

In retinal gene therapy, the selection of appropriate promoters is crucial for achieving targeted and efficient transgene expression. Ubiquitous promoters, such as the cytomegalovirus immediate-early (CMV) promoter and the hybrid CMV early enhancer/chicken β-actin promoter, are commonly employed due to their robust expression levels across many retinal cell types (Boshart et al., 1985; Höring, Neef, & von Gaisberg, 1990; Niwa, Yamamura, & Miyazaki, 1991; Radhakrishnan, Basma, Klinkebiel, Christman, & Cheng, 2008).

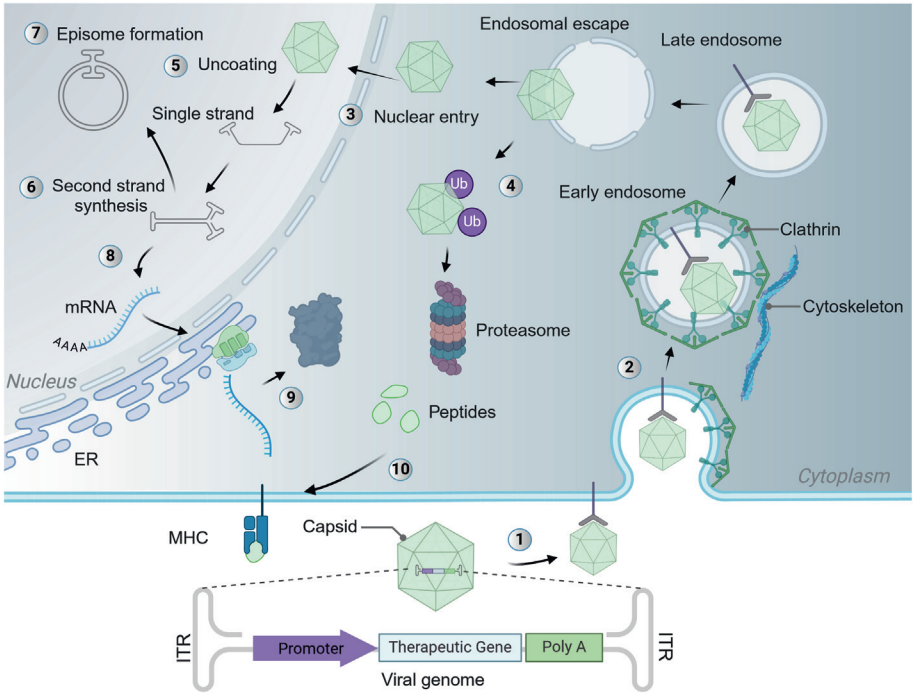

Fig. 2. Schematic representation of AAV cell entry and trafficking. 1. Binding to receptors: AAV binds to specific cell surface receptors, initiating the cell entry process. 2. Endosome formation: After receptor binding, AAV is internalized through endocytosis, forming an endosome. 3. Nuclear entry: A fraction of AAV particles escapes from the endosome and enter the nucleus of the host cell. 4. Ubiquitination and proteasome degradation: Some AAV particles undergo ubiquitination, leading to partial degradation by the proteasome. 5. Uncoating: In the nucleus, the AAV capsid uncoates and releases its single-stranded DNA genome. 6. Second strand synthesis: The single-strand DNA vector genome is converted into double-stranded DNA by the host cell machinery. 7. Episome formation: Double-stranded AAV DNA forms an episome, existing independently of the host genome. 8. Transcription: The episomal DNA is transcribed into mRNA. 9. Translation: The mRNA is translated into therapeutic proteins in the cytoplasm. 10. Presentation of peptides to the immune system: AAV capsid-derived (or vector-born) peptides are processed and presented on the cell surface by major histocompatibility complex molecules, which may lead to an immune response.

The use of cell specific promoters is, like the AAV capsid tropism, another way of limiting transgene expression to distinct cell types and avoiding off-target effects. For example, the human rhodopsin kinase (hGRK1) promoter and interphotoreceptor retinoid binding protein are utilized to drive gene expression in both rod and cone photoreceptors (Boye et al., 2012). For cone specific expression, promoters derived from human opsin genes (L opsin PR2.1, blue opsin, red/green opsin) or the human cone arrestin ARR3) promoter are commonly used (Alexander et al., 2007; Chen et al., 1994; Li, Timmers, Guy, Pang, & Hauswirth, 2008). To drive expression in RPE cells, the *RPE65* or *VMD2* promoters have been explored (Esumi, Oshima, Li, Campochiaro, & Zack, 2004; Nicoletti, Kawase, & Thompson, 1998). Specific promoters for almost all retinal cell types, including bipolar-, Müller-, ganglion-, amacrine cells as well as astrocytes have been established so far (Brenner, Kisseberth, Su, Besnard, & Messing, 1994; Buck & Wijnholds, 2020; Hulliger, Hostettler, & Kleinlogel, 2020; Jüttner et al., 2019; Khabou et al., 2023). The AAV capacity limit and a need for cell, disease and treatment specific promoters has also led to the engineering of synthetic promoters like NA65p, an optimized version of the human *RPE65* promoter, PR1.7, a smaller version of the L-cone promoter or hRHO, a truncation of the human rhodopsin promoter (Georgiadis et al., 2016; Wagner et al., 2021; Ye et al., 2016b).

With most gene therapies developed in animal models, translatability to clinical applications is a critical consideration in optimizing therapeutic efficacy and safety of promoters. The expression of the endogenous gene also dictates the specificity and strength of transgene expression, which can be impacted by differences in retinal cell types, gene regulation, and immune responses between species (Aguirre, 2017; Beltran et al., 2010; Guziewicz et al., 2013; Nicoud et al., 2007). While commonly used viral and tissue-specific promoters have shown robust expression in preclinical models, their performance in human retinal cells may differ due to variations in chromatin accessibility, transcription factor binding, and potential immunogenicity (Bergman et al., 2022; Jüttner et al., 2019). Emerging strategies, including the use of humanized promoters and regulatory elements tailored to the human retina aim to bridge this gap (Bergman et al., 2022; Weiss et al., 2001). Thus, careful validation in relevant human retinal models, including organoids and post-mortem tissue, is essential to ensure reliable promoter performance before clinical application (Simpson et al., 2019; Weiss et al., 2001).

3. Administration techniques for retinal gene therapy

Retinal gene therapy relies on several established and innovative injection methods that play crucial roles in the effectiveness and specificity of treatments. The three primary delivery methods currently used in clinical trials (Table 1) are intravitreal, subretinal, and suprachoroidal injections (Fig. 3), each with unique advantages and limitations. Subretinal injection is widely regarded as the gold standard for delivering AAV vectors for targeting photoreceptors and RPE cells (Lebherz, Maguire, Tang, Bennett, & Wilson, 2008; Mühlfriedel, Michalakis, Garcia Garrido, Biel, & Seeliger, 2013; Reichel et al., 2021). This technique involves the injection of AAVs into the subretinal space between the photoreceptors and RPE, creating a temporary retinal detachment and a cavity referred to as subretinal bleb that allows for precise targeting of the outer retina (Fig. 3). Due to high affinity to the extracellular matrix and receptors, conventional AAV serotypes allow only for local transduction of the photoreceptors and RPE cells lining the subretinal bleb area. Retinal cells outside the subretinal bleb are typically not reached because of insufficient spreading of the vectors. To overcome this limitation, AAV capsids were engineered to spread laterally and diffuse into more distant areas of the retina (Frederick et al., 2020). This concept is already explored in a clinical trial that aims to treat X-linked retinoschisis with a vector based on a laterally spreading

AAV capsid variant (NCT05878860). Despite its efficacy, transvitreal subretinal injection is surgically invasive and requires vitrectomy and retinotomy. It can therefore only be performed in specialized vitreoretinal centers (Mühlfriedel et al., 2019; Xue, Groppe, Salvetti, & MacLaren, 2017).

Intravitreal injection, on the other hand, is less invasive and involves delivering the viral vector into the vitreous cavity of the eye (Fig. 3). It is a routine injection procedure that can be performed in an outpatient setting. Vectors based on conventional AAV serotypes allow for targeting inner retinal cells, such as ganglion cells and Müller glia with this technique, as the viral vectors are not able to penetrate the inner retinal layers to reach the photoreceptors and RPE cells (Seitz & Tamm, 2013). While newer capsids and vector modifications have improved the efficiency of intravitreal injections for targeting the outer retina, their transduction efficiency remains lower than subretinal delivery, especially for RPE.

Suprachoroidal injection represents an emerging technique where the vector is delivered into the space between the choroid and sclera (Fig. 3). This method offers a balance between the invasiveness of subretinal injections and the accessibility of intravitreal injections, potentially allowing for widespread retinal transduction with less surgical complexity (Kansara, Muya, Wan, & Ciulla, 2020). However, further research is needed to assess whether the benefits of this technique outweigh its challenges, including its complexity and risks such as suprachoroidal hemorrhage and endophthalmitis. (Kansara et al., 2020).

Beyond these established methods, innovative techniques such as transscleral microneedle injections and electrotransfection of the ocular ciliary muscle are gaining attention (Yiu et al., 2020). Microneedle injections through the sclera rather than the retina offer a minimally invasive approach that does not require surgical or imaging equipment (Yiu et al., 2020). The avoidance of the retinotomy reduces the risks like retinal tears or inflammation associated with conventional methods. Electrotransfer on the other hand can be used for local delivery of genetic material in the form of naked plasmids into the easily accessible ciliary muscle (Bloquel et al., 2006). Ciliary muscle electrotransfer was applied in animal studies to deliver plasmids encoding various therapeutic proteins, such as TNF-alpha receptor type I, soluble VEGF receptor-1, and transferrin. Therapeutic levels were reached for each protein, leading to preservation of retinal structure in animal models of uveitis, retinitis pigmentosa (RP), macular edema, and retinal degeneration (Bordet & Behar-Cohen, 2019; El Sanharawi et al., 2013; Picard et al., 2015; Touchard et al., 2012). These approaches are still at the experimental stage, but are promising alternatives to expand the toolkits for gene transfer in ocular gene therapy.

4. Immunogenicity of current retinal gene therapy approaches

There is evidence of innate, humoral, and cellular immune responses towards the vector and vector-encoded products (Hamilton & Wright, 2021; Ronzitti, Gross, & Mingozzi, 2020; Weber, 2021). Managing immune responses is one of the significant challenges associated with gene therapy. An immune response can neutralize the viral vector, reducing its availability to transduce the target cells, thereby diminishing the overall effectiveness of the treatment. Pre-existing immunity, such as antibodies from previous exposure to naturally occurring AAVs, poses additional challenges, potentially rendering the therapy less effective. Immune reactions can also pose safety risks, with the potential for adverse events that could jeopardize patient health. Finally, managing immunogenicity is essential for the practical application of gene therapy, particularly in terms of a potential repeated dosing, which may be necessary for long-term treatment efficacy. Several factors may influence the inflammatory potential, including AAV serotype, dose, route of administration, baseline neutralizing antibody levels, disease-specific factors (e.g., blood-retinal barrier breakdown or preexisting disease-related inflammation), and transgene-triggered inflammation (Herzog et al., 2019; Kruzik et al., 2019; Li et al., 2008; Mingozzi et al., 2009; Rogers et al., 2017). Managing the immunogenicity of AAVs involves strategies like immune suppression protocols, lowering the dosage, selecting appropriate patient populations and vector engineering (Bainbridge et al., 2015; Cukras et al., 2018; Ye et al., 2016a). This consideration helps maximize the therapeutic benefits while minimizing potential risks. Conveniently, the eye's immune-privileged nature minimizes systemic side effects and enhances the stability of transgene expression, making this organ one of the most attractive targets for gene-therapeutic interventions (Taylor, 2016; Xiong et al., 2019).

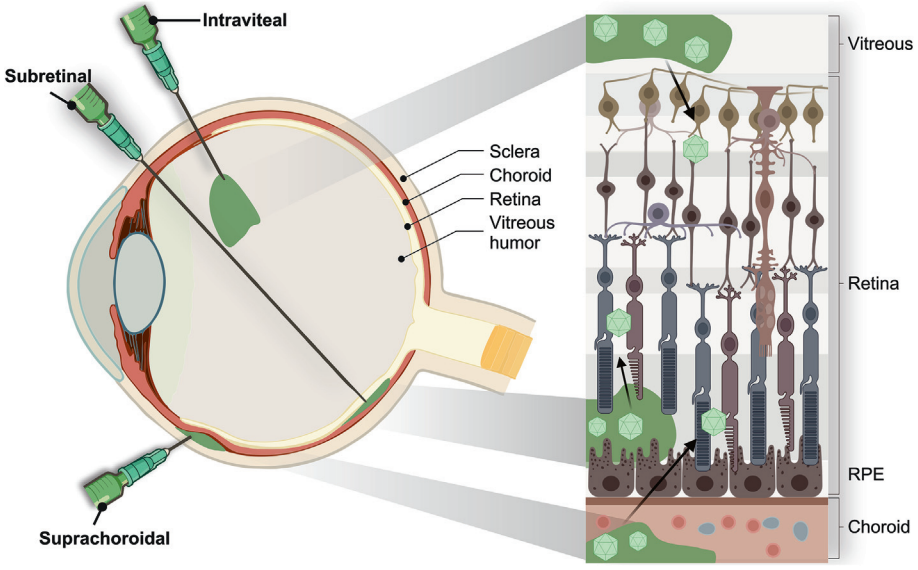

Fig. 3. Main injection methods used in retinal gene therapy. Intravitreal injections deliver the drug into the vitreous cavity from where it enters the retina through the inner limiting membrane. Subretinal injection deposits the drug closest to the photoreceptors, between the retina and the retinal pigment epithelium (RPE). Suprachoroidal injections place the drug between the choroid and the sclera, targeting the outer retina.

4.1. Immune reactions observed in recent clinical trials

Although AAV vectors generally have a lower immunogenic potential than other viral vectors (e.g., adenovirus), high systemic doses of AAV-based gene therapies are known to cause inflammation and liver toxicity (Shieh et al., 2020). When applied to the eye, only mild and transient immune responses to AAV-based vectors are typically observed in preclinical and clinical studies. This is because, compared to systemic AAV delivery, much lower doses are required for intraocular applications. The anatomy of the eye also favors ocular gene therapy with AAV vectors, as most of the AAV vectors hardly pass into the bloodstream after treatment (Mehta, Robbins, & Yiu, 2021). In addition, the (partially) immune privileged nature of the eye further reduce the risks for adverse effects (Willett & Bennett, 2013). Immune responses to retinal AAV-mediated gene therapy have been observed in clinical trials and may cause localized ocular inflammation (Bulcha et al., 2021; Gregory, Nazir, & Metcalf, 2011). However, these effects remain localized, are generally comparatively mild and can be managed with standard steroid treatment. In clinical trials, innate and adaptive immune responses to AAVs are prophylactically or reactively addressed with immunosuppressant medication (Kistner et al., 2024; Shen, Liu, & Ou, 2022). Specifically, prophylactic immunosuppression was administered in 74 % of AAV gene therapy studies, typically starting 24–72 h before or on the day of treatment (Vrellaku et al., 2024). Corticosteroids, including prednisolone, prednisone, and methylprednisolone, were the most commonly used immunosuppressants, either alone or with additional agents like tacrolimus and mycophenolate mofetil (Vrellaku et al., 2024). In about 25 % of recent clinical trial studies, in response to adverse events immunosuppressive treatments had to be adjusted by prolonging their administration and raising the dosage (Vrellaku et al., 2024).

While the adverse effects following AAV-based ocular gene therapy were mild in the vast majority of ongoing clinical trials, individual studies reported noticeable complications. A patient with diabetic macular edema (DME) treated with a high intravitreal dose of a AAV2.7 m8-vectorized VEGF drug aflibercept lost vision in the treated eye 30 weeks after treatment due to severe inflammation (Cheng & Punzo, 2021). Four additional patients suffered vision loss in this clinical trial, which the company has since discontinued. The exact reason for the vision loss in these five patients remains unclear. However, due to the late onset (>30 weeks after treatment), it is thought to be caused by inflammation associated with the gene product rather than by AAV vectors themselves (Cheng & Punzo, 2021). Mild to moderate inflammation was observed in 29 of 39 patients with Leber hereditary optic neuropathy (LHON) treated with intravitreal AAV2-ND4 (lenadogene nolparvovec) (Newman et al., 2021). Intravitreal administration of an AAV8-RS1 gene therapy in patients with X-linked retinoschisis resulted in the induction of various inflammatory markers. The ocular inflammation correlated with an elevated baseline CD4/CD8 ratio. This indicates that the pro-inflammatory immune phenotype, commonly observed in untreated retinoschisis patients, may play a role in amplifying the inflammatory response following treatment (Mishra et al., 2021). For voretigene neparvovec, chorioretinal atrophy inside and outside the subretinal injection area has been described (Gange et al., 2022; Kiraly et al., 2023; Reichel et al., 2023; Seitz & Tamm, 2013). Mechanical (i.e., surgery-related) damage and immune-mediated reactions to treatment were discussed as the main causes of these adverse events. A recent study reported acute subretinal deposits (which later improved or resolved) in young patients that received voretigene neparvovec treatment which they speculated could be an immune response to the AAV capsid (Lopez, Borchert, Lee, & Nagiel, 2023). Other adverse effects such as cataract formation, increase in intraocular pressure, iatrogenic retinal tears and intraocular inflammation appear to be dependent on the injection method, dosage and vector type and have occurred regularly in recent clinical trials (Gange et al., 2022; Kiraly et al., 2023; Verdera, Kuranda, & Mingozzi, 2020; von Krusenstiern et al., 2023).

After endocytosis, the free single-stranded AAV genome can be detected by toll like receptor 9 in the endosomal compartments which facilitates innate immune activation and pro-inflammatory cytokine expression (Faust et al., 2013; Martino et al., 2011; Zhu, Huang, & Yang, 2009). The receptor binds unmethylated cytosine-phosphate-guanine (CpG) motifs in the transgenes as well as the ITRs, the only AAV-born sequences usually present in the AAV vector genomes. Codon optimization to avoid CpGs or to integrate Toll-Like Receptor 9 (TLR9) antagonizing sequences when designing vectors might help mitigating immune responses (Chan et al., 2021; Wright, 2020). In the retina, this signaling probably plays a minor role as long as the immune privilege is intact and TLR9 expressing cells are absence (Chan et al., 2021). While ITRs also contain 16 CpGs, reactions observed in AAV-based gene therapies are more likely caused by pattern recognition of AAV capsid proteins, the transgene, its product(s) after translation or the injection procedure (Li & Samulski, 2020; Salabarria et al., 2024). The influence of capsid and injection procedure was recently shown in a study on rats, where injection methods and serotypes were compared for their immunogenicity. The study found that suprachoroidal injection of AAV2 and AAV6 caused the highest immune cell activation and migration of T cells and B-cells into the neural retina (Wiley et al., 2023). AAV8 and AAV9 intravitreal injections on the other hand showed lower immune responses, highlighting the influence of the capsid and the injection procedure on the level response (Wiley et al., 2023).

4.2. Preexisting immunity to AAVs

Inactivation of AAVs by the complement system or through neutralizing antibodies by the adaptive immune system can impede successful gene transfer and render a treatment ineffective (Kotterman et al., 2015). Preexisting titers of neutralizing antibodies can stem from previous infections with wildtype AAVs or from a repeated application of AAVs as part of a treatment. Overall, ~40–80 % of the population carry neutralizing antibodies against AAV due to previous infections with naturally occurring AAVs (Calcedo et al., 2011). The percentage of pre-existing AAV-neutralizing antibodies is serotype-dependent, with up to 80 % of the population having titers against AAV1 and AAV2, while antibodies against AAV8 are found in approximately 38 % of people (Calcedo et al., 2011; Calcedo, Vandenberghe, Gao, Lin, & Wilson, 2009). While neutralizing antibodies, particularly in the vitreous humor, can neutralize AAVs and impact transgene expression—potentially diminishing the therapeutic effect—the extent of their influence in the otherwise immune-privileged retina remains debated. (Kotterman et al., 2015; Sobh et al., 2023). In principle, neutralizing antibodies can block the therapeutic AAVs from reaching their target cells. Consequently, patients participating in AAV-based clinical trials are routinely screened for the presence of neutralizing antibodies with enzyme-linked immunosorbent assays or total antibody-based assays (Mendell et al., 2022). Additionally, several innovative approaches are being pursued to make AAV-based gene therapy available for patients with pre-existing neutralizing antibodies against these vectors. For instance, AAVs packaged in extracellular vehicles (EVs) may partially evade neutralizing antibodies (Cheng et al., 2021). However, since the AAVs inside the vesicle don't interact with cell surfaces this method may alter tissue targeting, requiring additional modifications on EV surfaces to establish desired tropisms. To prevent AAV vector neutralization, two groups suggested clearing of IgM and IgG through bacterial endopeptidases (Elmore, Oh, Simon, Fanous, & Asokan, 2020). These enzymes cleave circulating human antibodies, thus creating an antibody-free window that enables efficient AAV intervention. Also, various antigenic epitopes were identified on the surface of AAV capsids, which opens up the possibility of using capsid engineering to develop variants that escape neutralization by antibodies (Emmanuel et al., 2022).

4.3. Engineered AAVs that are less immunogenic and more efficient

Recently developed engineered AAV capsids showed higher transduction efficiency, requiring lower titers and even allowed for less invasive intravitreal injection (Arjomandnejad, Dasgupta, Flotte, & Keeler, 2023; Ghauri & Ou, 2023; Wang et al., 2024). A powerful approach to create capsids with desired properties is directed evolution. In this process, researchers simulate natural selection in the lab to create AAV capsid variants with desired characteristics, such as improved tissue targeting, immune evasion, or higher gene delivery efficiency. Initially, a diverse library of AAV capsids is created through, e.g. error-prone polymerase chain reaction, structurally guided recombination, DNA shuffling or phylogenetic reconstruction (Barnes, Scheideler, & Schaffer, 2019; Büning, Bolyard, Hallek, & Bartlett, 2011; Gonzalez et al., 2023; Kotterman & Schaffer, 2014; Li & Samulski, 2020; Wang, Tai, & Gao, 2019; Zolotukhin & Vandenberghe, 2022). These variants are then subjected to a specific selection pressure, such as the ability to infect specific cell types or evade neutralizing antibodies. The variants that perform best under these conditions are isolated and further refined through additional rounds of selection. A widely used and proven approach for creating capsid variations is peptide insertion, which introduces additional amino acids at specific surface-exposed sites of the wild-type capsid monomers (Börner et al., 2020; Cronin et al., 2014; Dalkara et al., 2013; Deverman et al., 2016; Müller et al., 2003; Naumer et al., 2012; Perabo et al., 2003; Raupp et al., 2012; Sallach et al., 2014; Varadi et al., 2012). These peptide insertions can alter the properties of the AAV capsid, such as its ability to bind to specific cell receptors, evade the immune system, or improve tissue targeting.

Directed evolution approaches commonly rely on animal models (e.g. mice, dogs or non-human primates) for the selection of efficient AAVs from a library (Dalkara et al., 2013; Pavlou et al., 2021). For validation experiments, novel AAV capsid variants are often tested in large animal models, such as dogs, pigs or non-human primate models. Compared to rodents, these larger animal models offer a significant advantage, as their eye physiology and anatomy is much more similar to that of humans. This includes a more similar eye size, the presence of a cone-only fovea or area centralis, the consistency of the vitreous body and the structure of the internal limiting membrane (Beltran et al., 2014; Heegaard, Jensen, & Prause, 1986). The latter make them viable models for screening for AAVs that can transduce retinal cells efficiently after intravitreal injection (Byrne et al., 2020; Öztürk et al., 2021). In another recent study, genetically engineered AAV capsids were developed and validated in primary human retinal explants, enabling high-throughput screening in retinal cells of human origin (Westhaus et al., 2023).

In other approaches, bioinformatics methods were used to define the parameters for the construction of AAV libraries with specific properties. The integration of machine learning (ML) in AAV capsid development has shown promising advancements in enhancing viral assembly and gene delivery efficacy. ML-guided approaches can streamline the AAV capsid design and the development process, reduce costs, and thus overcome some key limitations of conventional methods (Fu, Suo, Zhang, & Chen, 2024; Zhu et al., 2021). Together, these advancements highlight the ongoing research efforts to optimize AAV vectors for safer and more effective retinal gene therapies. Some of the engineered AAV capsid variants have already been tested in clinical trials for retinal gene therapy. A prominent example is AAV.7 m8, which has been used in several clinical trials for intravitreal delivery of the VEGF inhibitor aflibercept for the treatment of neovascular AMD (nAMD) (e.g. NCT03748784, NCT05197270 NCT04645212). Further examples of intravitreally applied, engineered capsids are AAV2.NN (Pavlou et al., 2021) and R100, which are currently being investigated in phase I/II clinical trials for the treatment of forms of RP associated with *CNGA1* (NCT06291935) and *RPGR* (NCT04517149), respectively (Kotterman et al., 2021).

4.4. Alternative vectors for ocular gene therapy

Other viral vectors such as those based on lentiviruses (LV) and adenoviruses (AV) are also suitable as carriers of genetic material and are therefore used in preclinical and clinical studies for ocular and non-ocular applications. Compared to AAVs, both LV and AV exhibit significantly greater packaging capacities (LV: 8–10 kb, AV: 8–37 kb) (Ricobaraza, Gonzalez-Aparicio, Mora-Jimenez, Lumbreras, & Hernandez-Alcoceba, 2020). However, they generally demonstrate lower in vivo gene transfer efficiency, increased immunogenicity, and, in the case of LVs, pose risks such as insertional mutagenesis and potential germline transmission (Milligan et al., 2016; Poletti & Mavilio, 2021). Non-viral transduction methods like macrovesicles, liposomes, lipid nanoparticles (LNP) and membrane penetrating peptides on the other hand only cause very little vector attributed immune responses (Inglut et al., 2020; Lee, Jeong, Park, Jung, & Lee, 2023; Zhu et al., 2017). However, the main drawbacks are the short-term expression of the delivered genes and the limited gene transfer efficiency. Recently, subretinal delivery of LNP gene delivery was shown to facilitate expression of transgenes in the outer retina (Patel, Ryals, Weller, Pennesi, & Sahay, 2019). The long-term expression of transgenes, which is achieved with the help of viral vectors, can also be a disadvantage for certain applications. This applies, for example, to CRISPR/Cas (clustered regularly interspaced short palindromic repeats / CRISPR associated protein) applications, which are aimed at a one-off modification of the genome and therefore only need to be active for a short time. In such cases, gene transfer via non-viral vectors would be advantageous, provided that these vectors can achieve a certain level of efficacy in the target cells required to induce clinically relevant therapeutic effects (Gautam et al., 2023).

In an approach to combine respective advantages, LNPs containing the Cas9 mRNA and sgRNAs were combined with AAVs harboring the template DNA for homolgy directed gene knock-in. The aim of these experiments was to achieve short-term expression of Cas9 using LNPs and simultaneously enable efficient and target cell-specific expression of template DNA using AAVs (Lee et al., 2023). Although this approach has not yet been verified in the eye, it has already led to clinical trials for the treatment of hemophilia (Wang et al., 2024). However, a number of challenges still need to be overcome before such hybrid approaches can be applied more widely. These include demonstrating sufficient effectiveness of LNPs in other tissues, optimizing production processes and analyzing the biodistribution of LNPs, including the off-targets of the transgenes packaged therein (Lee & Han, 2024).

In another study aimed to combine benefits of viral and non-viral delivery systems, engineered virus-like particles have been used to deliver a prime editor (An et al., 2024; Banskota et al., 2022; Hamilton et al., 2021; Mangeot et al., 2019). These particles are spontaneous assemblies of retroviral polyproteins that do not have a genome but are able to transduce cells and release cargo. Therapeutic cargo, e.g. proteins or mRNAs, are fused to the capsid proteins and packaged to the inside of a particle. Such non-viral particles have recently been used to deliver a prime editing (PE) system to murine models of retinal degeneration where they were able correct a deletion in the *Mfrp* gene with 15 % efficiency (An et al., 2024).

5. Overcoming the limited cargo capacity of AAV vectors

Most of the current gene therapy clinical trials in patients with inherited retinal dystrophies utilize classical AAV-mediated supplementation of genes that do not exceed the genome packaging capacity of AAVs (e.g. *GUCY2D* (NCT03920007), *CHM* (NCT04483440), *PDE6B* (NCT03328130) and *RPGR* (NCT04671433, NCT04850118, NCT03584165, NCT04517149)). However, the coding sequence of more than 30 IRD-related genes (https://retnet.org), such as *ABCA4* (~6.8 kb), *MYO7A* (~7.5 kb), or *USH2 A* (~15.7 kb), is beyond this packaging limit so that alternative approaches are required for the transfer

of these genes. In early studies focusing on the supplementation of *ABCA4* or *MYO7A*, the packaging capacity of AAVs was effectively ignored and it was tested whether meaningful delivery of these large genes was possible within a single AAV vector approach (Allocca et al., 2008; Lopes et al., 2013). Although the full-length protein could be detected in these studies, it is assumed that this is due to the packaging of fragmented genomes that can be repaired, e.g. by homologous recombination in transduced cells (Dong et al., 2010; Hirsch et al., 2013; Wu, Yang, & Colosi, 2010). Undefined genome fragmentation would not only impair the efficacy of a vector but would also represent a regulatory hurdle for the release of the corresponding gene therapy product. For this reason, alternative approaches for the gene transfer of large genes, such as dual AAV vector approaches described below, seem more appropriate.

5.1. Dual AAV vector approaches

Dual AAV systems have evolved as an effective and frequently used strategy for the delivery of large genes into different tissues. In this method, the coding sequence of the gene of interest is split into separate AAV vectors containing elements essential for seamless reconstitution of the split fragments. Upon co-delivery to the target tissue, the gene fragments can be reassembled at the DNA, mRNA, or protein level, depending on the specific split approach used (Wang et al., 2024).

5.2. Reconstitution at the DNA level

One of the technologies that enables the reconstitution of split genes at the DNA level is based on the inherent ability of AAV genomes to be concatemerized through intermolecular recombination of the ITRs (Fig. 4A) (Duan et al., 1998; Yang et al., 1999). Upon co-transduction, the two ssDNA AAV genomes, each containing a gene cassette encoding half of the gene of interest, can recombine in various orientations, such as head-to-tail, head-to-head, or tail-to-tail heterodimers, with the head-to-tail configuration being the predominant one (Ghosh, Yue, Lai, & Duan, 2008). Subsequently, the rejoined split gene, interrupted by AAV ITR elements, can be seamlessly reconstituted using eukaryotic mRNA splicing signals (splicing donor and acceptor sites) to remove the ITR sequence during mRNA maturation. Several studies have shown that this approach (often referred to as trans-splicing) can precisely restore the split coding sequence, resulting in a functional protein of interest (Duan, Yue, Yan, & Engelhardt, 2000; Nakai, Storm, & Kay, 2000; Sun, Li, & Xiao, 2000).

Another strategy that has been developed for reassembly of split genes at the DNA level are the overlapping AAV vectors. In this approach, the two split gene fragments share a common recombinogenic region (overlapping sequence) at the 3′ end of the first half and at the 5′ end of the second half of the coding sequence (Fig. 4B). Upon co-transduction, these overlapping sequences undergo homologous recombination, enabling the recovery of the full-length gene. Although trans-splicing proved to be more effective than the overlapping AAV vectors for certain genes (Duan, Yue, & Engelhardt, 2001), other studies have shown that the latter strategy achieves higher gene expression levels (Duan et al., 2001; Pryadkina et al., 2015). Studies with trans-splicing AAV vectors in skeletal muscle suggest that transcription and splicing via the inverted terminal repeat (ITR) junction in the reconstituted genome have a decisive influence on the efficiency of this method (Xu et al., 2004). Additionally, the gene-splitting sites and intron sequences strongly influence the efficiency of trans-splicing AAV vectors (Lai et al., 2005). In comparison, the efficiency of reconstitution in overlapping AAV vectors is highly dependent on the presence of a strongly recombinogenic domain within the target gene and the joining of vector genomes in the head-to-tail orientation (Ghosh, Yue, & Duan, 2006).

In an attempt to further improve the efficiency of DNA trans-splicing dual AAV vectors, the hybrid vector system was developed which combines the features of both trans-splicing (Fig. 4A) and overlapping (Fig. 4B) strategies (Ghosh et al., 2008). In hybrid vectors, a highly recombinogenic alkaline phosphatase sequence was inserted between the splice signal and the ITR of trans-splicing vectors. Transgene reconstitution can occur through homologous recombination via the recombinogenic sequence or through ITR-mediated recombination, followed by mRNA cis-splicing to remove the recombinogenic sequences and recover the full-length transcript. The hybrid dual system has been successfully applied to a variety of genes and resulted in some cases in therapeutically meaningful levels of full-length gene expression (Carvalho et al., 2017; Dyka, Molday, Chiodo, Molday, & Hauswirth, 2019; Ghosh et al., 2008). *ABCA4* and *MYO7A* gene expression was evaluated after subretinal delivery of trans-splicing or hybrid dual AAV vectors with a F1 phage recombinogenic region in the pig retina (Colella et al., 2014). The study showed full-length gene expression in photoreceptors and in the retinal pigment epithelium. In another study the hybrid system was used to express ABCA4 in a *Abca4*$^{-/-}$ mouse model of Stargardt disease (STGD) and demonstrated reduction of A2E, a byproduct of the visual cycle that accumulates in STGD patients (Dyka et al., 2019). In this context, the hybrid vector system showed the highest efficiency and outperformed trans-splicing and overlapping AAV vectors. Another example of a successful application of hybrid dual AAVs is the reconstitution of *PCDH15*, another Usher syndrome type 1 (USH1F)-associated gene. In this study, robust PCDH15 expression in the retina of *Pcdh15KI* mice was observed, sustained electroretinogram recovery, improved light-driven phototransduction protein translocation, and increased levels of key retinoid oxime and retinal pigment epithelium enzymes (Riaz et al., 2023). Two studies testing dual AAV vector strategies that reconstitute at the DNA level reported positive preliminary results in pediatric patients with *OTOF*-related deafness (Lv et al., 2024) (https://investor.regeneron.com, https://www.technologyreview.com). In 2024, the LUCE-1 Phase 1/2 clinical study treated the first patient with *MYO7A*-related Usher syndrome with a hybrid dual AAV vector strategy (https://www.aavantgarde.com).

5.3. Reconstitution at the mRNA level

Another dual AAV system aims at the reconstitution of split genes at the mRNA level. In its basic molecular structure, this method is similar to hybrid AAV vectors (Fig. 4C). An important difference is the presence of a complementary binding domain instead of the recombinogenic sequence. This binding domain brings the two mRNA molecules together, before they can be joined with the help of strong splice sites. In contrast to DNA trans-splicing, both vectors contain a promoter so that the respective mRNAs can be expressed independently. An initial version of this dual AAV vector technology was tested in cell culture with the aim of reconstituting the *CTFR* gene (Song et al., 2009). However, this version was not subsequently evaluated in vivo or in the context of therapeutic applications. Recently, another study presented a dual AAV vector technology based on reconstitution via mRNA trans-splicing (REVeRT). In this system, strong splice donor and acceptor sites were used in combination with an optimized binding domain derived from the bacterial *LacZ* gene. REVeRT enabled the reconstitution of full-length *ABCA4* after intravitreal injection in a Stargardt disease mouse model and the reassembly of CRISPR/Cas modules for gene knockout and/or activation in wild type mice and in a mouse model for inherited retinal dystrophy caused by a gain-of-function mutation in rhodopsin (*RhoP23H*) (Riedmayr et al., 2023). Despite the promising results of this study in mouse models, it remains to be seen how REVeRT will perform in clinical trials in terms of efficacy and safety.

5.4. Reconstitution at the protein level

Reconstitution of dual AAV vector-derived transgenes at the protein level can be achieved by intein-mediated protein trans-splicing (Fig. 4D) (Levy et al., 2020; Tornabene et al., 2019). Inteins (internal

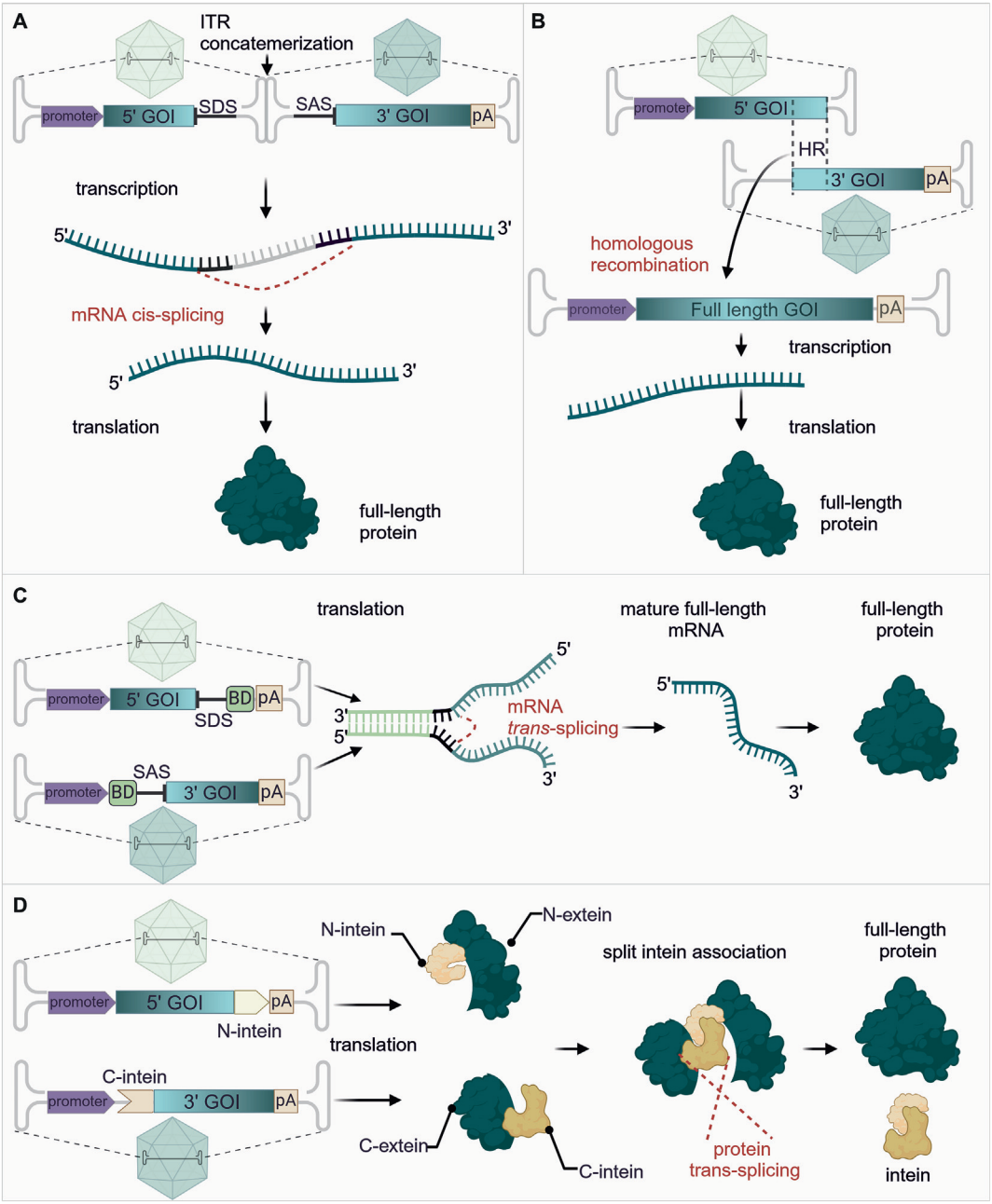

Fig. 4. Molecular mechanisms for reconstitution of split genes in dual AAV vector approaches. (A) Concatemerization of two vectors at their ITR sites leads to the transcription of an mRNA containing splice donor and acceptor sites, along with the combined ITR sequences. These sequences are removed post-transcriptionally via splicing, resulting in a full-length gene of interest mRNA. (B) The gene is divided into two parts that share a homologous region. This region enables the reconstitution of the gene through homologous recombination. (C) Two segments of a large gene are combined at the mRNA level via trans-splicing. (D) Protein trans-splicing mediated by the split intein sequences results in the reconstitution of the full-length protein. BD, binding domain; GOI, gene of interest; HR, homologous region; SDS, splice donor site; SAS, splice acceptor site; pA, polyadenylation signal.

proteins) are small, naturally occurring proteins that can autocatalytically excise themselves from a larger precursor polypeptide. This leads to the formation of a mature protein by assembling the flanking exteins (external proteins) (Anastassov, Filo, & Khammash, 2024; Lennon & Belfort, 2017; Mills, Johnson, & Perler, 2014; Shah & Muir, 2014). Although most naturally occurring inteins are expressed within a single polypeptide chain (protein cis-splicing), there are also natural and artificial split-inteins that pair through a zipper-like interface, forming a peptide bond between two separate exteins (protein trans-splicing) (Novikova, Topilina, & Belfort, 2014). The split-inteins remain inactive until they meet their complementary partner, at which point they excise themselves and catalyze the ligation of the exteins. Intein-mediated reconstitution activity relies on specific peptide sequences around the ligation junction, particularly requiring a thiol or hydroxyl-containing amino acid (Cys, Ser, or Thr) as the first residue in the C-extein for efficient trans-splicing (Shah, Eryilmaz, Cowburn, & Muir, 2013). This process has been harnessed in various biotechnological applications, including the reconstitution of large proteins via dual AAV vectors. By splitting a gene into two AAVs and incorporating split-inteins at the junction, the protein can be reassembled after translation in the target cells. High reconstitution efficiencies have been demonstrated for various large genes in vitro and in vivo (Davis et al., 2023; Peters et al., 2023; Tasfaout et al., 2024; Tornabene et al., 2019). An effective reconstitution of *ABCA4* and *CEP290* was reported in animal

models and in human retinal organoids using split-intein dual AAV vectors. In a subsequent study, the impact of different split inteins (Ssp^{intein}, Npu^{intein}, Rma^{intein}, Cfa^{intein}) and peptide split sites (1140, 1150, 1177) on ABCA4 protein expression levels were tested, with Rma^{intein} and split site 1150 showing the best performance (Li et al., 2023). The optimized split AAV8-*ABCA4* vector produced full-length ABCA4 protein with high efficiency and minimal by-products in *Abca4*-KO mouse retinas. This study nicely demonstrated the importance of the split site selection for the effectiveness of split inteins. A deeper understanding of target protein structure and function, combined with advanced computational algorithms, could facilitate the prediction of optimal split sites for dual or multi-vector strategies (Ho et al., 2021; Upmeier zu Belzen et al., 2019). Another challenge is that non-mammalian inteins remain as by-products of trans-splicing, potentially leading to immune or toxic reactions in target cells and raising regulatory issues. It has to be emphasized that these intein byproducts are produced at equimolar levels. The production of the desired therapeutic full-length protein thus comes at the expense of 100 % undesired byproduct. However, incorporating degrons, shortened *E. coli* dihydrofolate reductases, into the individual split portions of the transgene may mediate rapid ubiquitination and facilitate proteasomal degradation of the inteins to mitigate the risk of potential immune responses against the intein sequence (Tornabene et al., 2021).

While dual AAV systems are promising solutions for some IRD genes, the transduction efficiency of multiple AAV vectors is still significantly lower than that of single or dual AAVs (Akil, 2020; Maddalena et al., 2017). Such strategies are particularly relevant for very large genes that exceed even the packaging capacity of dual AAVs, as is the case for most genes associated with Usher syndrome. Despite ongoing improvements and recent successes, such as the successful reconstitution of full-length dystrophin using three AAVs (Tasfaout et al., 2024), this strategy may not be suitable for all genes. Therefore, there is still an unmet need for alternative therapeutic approaches that are independent of gene size.

5.5. Engineering of size reduced therapeutics

The engineering of so-called minigenes is another strategy for treating diseases caused by mutations in large genes using AAVs. In this process, the gene is specifically reduced to a size below the AAV capacity limit without impairing the essential functions of the corresponding protein. The key to design minigenes lies in protein engineering and structural bioinformatics using protein and gene databases. Well-defined 3D structures of target proteins and molecular dynamics simulations help filter out unsuitable variants, streamlining the experimental process. Several minigenes compatible with the capacity of AAV vectors have been tested for the treatment of Usher syndrome. In the case of the *USH2A* gene associated with Usher syndrome type 2, redundant motifs were deleted to create a smaller, functional variant. This variant was able to partially restore gene function in *Oc-k1* (*USH2A* −/−) cell cultures (Wang & Vandenberghe, 2021). A smaller variant of the *Pcdh15* gene delivered with AAV to the cochlea has rescued hearing in a mouse model of Usher syndrome type 1F (USH1F) and could also be a viable therapeutic option for the treatment of blindness in USH1F patients (Ivanchenko et al., 2023). Another example is the treatment of Leber Congenital Amaurosis with a miniaturized version of the *CEP290* gene (mini*CEP290*). Subretinal injection of AAVs carrying mini*CEP290* into neonatal *Cep290rd16* mice showed a delay in retinal degeneration and improved photoreceptor survival, morphology, and function (Zhang, Li, Su, Gao, & Khanna, 2018). While these strategies offer promising solutions, they also come with significant challenges. The development of minigenes requires extensive in silico and experimental validation to ensure that they retain essential functional properties without causing significant side effects. In addition, a drastic reduction in gene size is hardly possible with very large genes.

6. Gene size-independent approaches

Suitable treatment strategies also exist for very large genes that cannot be reduced or supplemented in their entirety. Technologies that can target and influence specified sites in the DNA of living cells have driven innovation in biotechnology and show promise for the treatment of genetic disorders. Among these technologies, the groundbreaking discovery of RNA-programmable CRISPR/Cas systems has paved the way for creating various methods capable of editing human cells at both genomic and epigenomic levels across a diverse range of target sites and within a wide array of cell types.

6.1. CRISPR/Cas nucleases

In December 2023, the U.S. Food and Drug Administration approved Casgevy, the first CRISPR/Cas9-based gene therapy for the treatment of sickle cell disease (SCD). The journey from its initial discovery to the approval of a CRISPR therapy in just 11 years marks a significant milestone in gene therapy, paving the way for many more treatments.

The CRISPR/Cas system, derived from the prokaryotic adaptive immune response, consists of an RNA-guided Cas nuclease and a programmable single-guide RNA (sgRNA) with a target-specific sequence (Jinek et al., 2012; Ran et al., 2013). Upon binding to the target DNA sequence, the Cas nuclease induces a double-strand break which is primarily repaired by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Fig. 5).

NHEJ is an error-prone process that results in a mixture of insertions and deletions (indels), making Cas nucleases a powerful tool for disrupting genes. Specific suppression of the mutated allele while preserving the wild-type functional allele would be a suitable therapy for autosomal dominant diseases and, particularly, gain-of-function mutations. CRISPR-Cas nucleases can achieve allele-specific genome modification by targeting sequence differences in the disease-causing allele that are absent in the healthy allele (Fig. 5). Several studies provided proof-of-concept for an allele-specific disruption and delayed retinal degeneration in the mouse model of the most common gain-of-function mutation (Pro23His) in rhodopsin (Giannelli et al., 2018; Latella et al., 2016; Li et al., 2018). In another study CRISPR/Cas9 was used to selectively target the second most prevalent mutation in the rhodopsin gene (Pro347Ser) in the respective mouse model (Patrizi et al., 2021).

CRISPR-Cas nucleases have been successfully used to remove an deep intronic aberrant splice donor site mutation (IVS26; c.2991 + 1655 A > G) in the humanized *CEP290* mouse model for Leber congenital amaurosis type 10 (Maeder et al., 2019). This strategy (EDIT-101) is currently tested in a phase 1/2 clinical trial in 12 patients with Leber congenital amaurosis type 10 (NCT03872479). As of 2024, the initial results showed no treatment-related serious adverse events, and nine participants exhibited improvements in photoreceptor function (Pierce et al., 2024).

The promising results from this study could facilitate the translation of further CRISPR/Cas-based therapies for IRDs into the clinic. However, the strategy of cutting out disease-associated mutations from the genome is very limited in its range of applications and can typically only be applied to mutations that are not located in coding or other important gene regulatory regions. In comparison, strategies aimed at correcting the pathogenic mutations in the genome of the target cells appear to be more suitable. One way to achieve this goal is the co-application of Cas enzymes with a template donor DNA flanked by target site-specific homologous regions to replace the defective allele. This strategy relies on HDR and has been successfully applied for many different genes and a variety of cell types (Zheng et al., 2023). Nevertheless, since HDR primarily occurs in the late S-G2 phase of the cell cycle, it is less efficient in non-dividing cells like photoreceptors or RPE. Accordingly, attempts to correct *Rpe65*, *Rpgr*, and *Pde6B* mutations in mice using CRISPR-Cas9 with donor DNA showed very low HDR

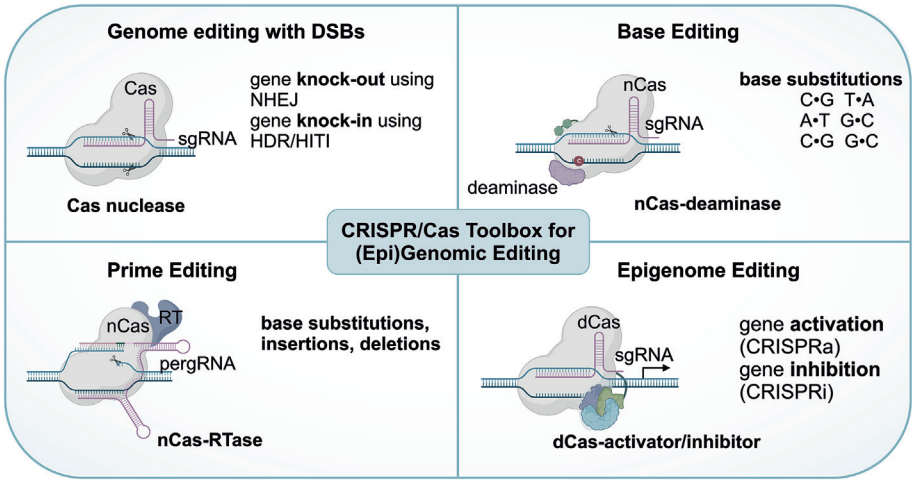

Fig. 5. Schematic representation of different (epi)genome editing tools. *Upper left:* Genome editing using Cas nucleases can be utilized to introduce double-strand breaks (DSBs) at the target locus, enabling both gene knock-out (through error-prone non-homologous end joining) and gene knock-in (via precise homology-directed repair). *Upper right:* Base editing employs a Cas nickase (nCas) fused to a deaminase, allowing for direct base substitutions without causing double-strand breaks. *Lower left:* Prime editors consist of a Cas nickase (nCas) fused to a reverse transcriptase (RTase), allowing them to facilitate more diverse and precise genomic alterations such as base substitutions, insertions, and deletions, and combinations. *Lower right*: Dead Cas (dCas) proteins in combination with effector domains can modulate gene expression by either activating or repressing transcription without altering the DNA sequence itself.

levels, but still resulted in some preservation of photoreceptor (Cai et al., 2019; Hu et al., 2020; Jo et al., 2019). Several strategies have been developed to increase the knock-in efficiency by blocking the NHEJ pathway or stimulating the HDR Pathway (Leal, Herreno-Pachón, Benincore-Flórez, Karunathilaka, & Tomatsu, 2024). However, due to low efficiency in non-dividing cells, off-target risks, and the generation of unwanted indel byproducts from double strand breaks, CRISPR-Cas9-mediated replacement of target sites via HDR plays a minor role in therapy of inherited retinal dystrophies.

Another innovative strategy to overcome the limitations of both HDR and traditional NHEJ involves NHEJ-based site-specific transgene integration (Suzuki et al., 2016). This homology-independent targeted insertion (HITI) approach leverages the CRISPR/Cas9 system to generate double-strand breaks in both the genome and the circular donor DNA (Suzuki et al., 2016). The NHEJ pathway can then repair the double strand break by either connecting the blunt ends of the genomic DNA and repairing it to its original state, or integrating the donor DNA into the genome in a random orientation. The donor sequence is designed to create Cas9 cleavage sites at its ends if the template DNA is integrated in reverse orientation. Since the repaired genomic DNA without integration restores the Cas9 cleavage sites, the excision and double strand break creation is repeated until the insert is integrated in the correct orientation. HITI has proven to be very effective in creating gene knock-ins in non-dividing cells (Suzuki et al., 2016). By delivering the required Cas9, guide RNA, and donor DNA via AAVs to a *Mertk*-deficient rat model for RP, the disease-causing mutation in the *Mertk* gene was corrected, resulting in improved vision in the treated animals (Meng et al., 2024; Suzuki et al., 2016). HITI has also been successfully applied to treat Bietti crystalline corneoretinal dystrophy by restoring gene function in both patient-derived cells and in a humanized mouse model for this disease (Esposito et al., 2024). In order to enable the translation of this technology into clinical trials, efforts are currently being made to improve its efficacy and confirm its safety (Meng et al., 2024; Tornabene et al., 2022).

6.1.1. Base editing

In recent years, alternative CRISPR/Cas technologies have been developed capable of achieving targeted conversion of a single base pair without creating double strand breaks. The base editing technology relies on a Cas nickase (nCas) fused to a nucleobase deaminase (adenine or cytosine deaminase), and can install C•G-toT•A (cytidine base editors), A•T-to-G•C (adenine base editors) or C•G-to-G•C (CG base editors) point mutations (Fig. 5) (Gaudelli et al., 2017; Koblan et al., 2021; Komor, Kim, Packer, Zuris, & Liu, 2016). This technology has extended the applicability of CRISPR/Cas-based gene correction to non-dividing cells, minimizing the generation of unintended byproducts like indels. Base editors show high levels of editing in vitro and in vivo and are already being used in early clinical trials for familial hypercholesterolemia (NCT05398029), T cell leukemia and lymphoma (NCT05885464), and severe sickle cell disease (NCT05456880) (Chiesa et al., 2023; Ledford, 2023; Naddaf, 2023). In addition, base editors have already demonstrated promising in vitro and in vivo results for various types of inherited retinal dystrophies, including Leber congenital amaurosis, RP, and Stargardt disease. For retinal gene therapy applications, the problem of delivery must be considered, as Cas9-based editors are too large to fit into a single AAV. Using split intein dual AAV vectors for the transfer of the adenosine base editors to retinal pigment epithelium cells, one of these studies corrected the c.130C > T (p.R44X) mutation of the *Rpe65* gene in *rd12* mice, an animal model for Leber congenital amaurosis (Jo et al., 2023). This resulted in ~10 % of editing efficiency at the DNA level as well as functional improvements of the retinal phenotype. Another study focused on correcting the c.1678C > T (p.R560C) missense mutation in the *Pde6b* gene of *rd10* mice, a commonly used model of RP. By subretinal delivery of an intein-based dual AAV system encoding a PAM-less adenosine base editor (SpRY-ABE8e), the base editing efficiency in photoreceptor cells reached up to 17.5 % at the DNA level and 49.1 % at the cDNA level. This correction led to a delay in retinal degeneration as well as an improvement in retinal function and vision-guided behavior (Wu et al., 2023).

Another recently developed strategy involves the application of two or more distinct base editors to correct different mutations simultaneously. The corresponding system termed multiplexed orthogonal base editor uses RNA aptamers to recruit cytidine or adenosine deaminase directly to the guide RNA, allowing for the concurrent introduction of two separate point mutations at different loci in human cells (Cowan et al., 2025). Although this approach is not yet proven for applications in the retina, it remains an attractive technology for the correction of various types of conditions, such as compound heterozygosity or digenic inheritance.

In summary, it can be concluded that base editing has proven to be a valid tool for retinal gene therapy. However, bystander base edits may occur within a window of 4–10 nucleotides and only a fraction of

point mutations can be corrected via this approach, also excluding insertions, deletions, and most transversions.

6.1.2. Prime editing

To further expand the scope of precise gene correction, the search-and-replace tool prime editing was developed (Fig. 5) (Anzalone et al., 2019). Prime editors allow the programmable and precise installation of all point mutations, insertions, and deletions as well as combinations of them, without creating double strand breaks. Special prime editor systems like twin prime editors or other related dual prime editor constructs enable large insertions and deletions of >100 bp or even insertions and deletions of >5000 bp DNA fragments (Anzalone et al., 2022; Yarnall et al., 2023).

The minimal working unit consists of a prime editing guide RNA (pegRNA) which specifies the target site and type of the desired edit, and a prime editor protein which is composed of a Cas nickase (nCas) fused to a reverse transcriptase (RT) (Anzalone et al., 2019). The pegRNA comprises a spacer sequence designed to target a specific position within the genome, an RNA scaffold binding to the nickase, a primer-binding site complementary to the opposite strand, and a reverse transcriptase template encoding the desired edit (Anzalone et al., 2019). pegRNAs that additionally include a structured 3′ motif, enhancing stability and preventing 3′ degradation to improve editing efficiencies, are referred to as engineered pegRNAs (epegRNAs).

Since its inception, prime editing technology has already seen significant advancements and improvements in a remarkably short time, showcasing its rapid evolution and potential for various applications. The original prime editor system (PE1) refers to a Cas9(H840A) nickase fused to a Moloney murine leukemia virus reverse transcriptase variant in combination with a pegRNA (Anzalone et al., 2019). The introduction of five mutations into the reverse transcriptase resulted in the more efficient prime editor 2 (PE2). This version was further improved by optimization of the nuclear localization signals, codon usage and linkers, as well as by the introduction of two Cas9 mutations (Chen et al., 2021). In the third generation of prime editors (PE3), an additional sgRNA is used to nick the complementary strand, promoting the eukaryotic mismatch repair system to favor the edit and increase editing efficiency. The fourth generation of prime editors (PE4) requires transient co-expression of *MLH1dn*, a dominant-negative variant of the protein MLH1 involved in the mismatch repair. This protein modulates the cellular mismatch repair to favor the installation of the edit. PE5 systems combine the additional nicking sgRNA (ngRNA) with the transient MLH1dn expression.

Phage-assisted continuous evolution in combination with protein engineering has led to more compact and efficient prime editors (PE6a-g) that could facilitate in vivo application (Doman et al., 2023). In a newer version of prime editors (PE7), the prime editor protein is fused to the N-terminal domain of the RNA-binding exonuclease protection factor La. This system demonstrated increased prime editing efficiency in combination with pegRNAs, engineered pegRNAs, and synthetic pegRNAs optimized for La binding (Yan et al., 2024).

After initially observing rather low editing efficiencies, particularly in induced pluripotent stem cells (iPSC) and in vivo studies, continuous improvements have led to a variety of promising results in preclinical studies with prime editors (Davis et al., 2023; Doman et al., 2023; Qin et al., 2023; She et al., 2023). A dual split-intein AAV approach with increased PE expression demonstrated therapeutically relevant prime editing efficiencies in mouse brain (42 %), liver (46 %) and heart (11 %) using different delivery routes (Davis et al., 2023). By subretinal injection of an intein-based dual-AAV PE3 system in rd12 mice, the pathogenic c.130C > T (p.R44X) mutation was corrected with up to 16 % efficiency. This correction restored RPE65 expression, preserved photoreceptor cells, and improved visual function in ERG and behavioral tests (She et al., 2023). In a separate study, a prime editing system was used to correct the c.1678C > T *Pde6b* mutation in the retina of rd10 mice. A SpCas9 variant with relaxed PAM constraints (SpRY) was utilized to create a prime editing system capable of targeting non-NGG PAMs. Subretinal injections of intein-based dual AAV vectors delivering PE^{SpRY} achieved editing efficiencies of up to 78 % in preselected transduced retinal cells. This correction significantly preserved photoreceptor cells, restored PDE6 phosphodiesterase activity, and improved outcomes in ERG and behavioral tests (Qin et al., 2023).

In view of the promising results, it is only a matter of time before the first prime or base editors are evaluated in clinical trials. Despite their great potential, however, it remains to be seen how the long-term expression of these modules will affect the target tissues. Delivery approaches that enable efficient transient expression of these modules would be of great importance to ensure therapeutic benefit without the risk of off-target toxicity due to long-term activity of the editor. However, the most important disadvantage of prime and base editors remains the fact that they are currently mutation- or, at best, gene-specific, which makes their broader application (e.g., the development of gene-independent approaches) for genetic diseases difficult.

6.1.3. CRISPR/Cas-mediated transcriptional modulation

Beyond the direct alteration of the DNA sequence, CRISPR/Cas-based technologies can also be used to modify gene expression via CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa) (Becirovic, 2022; Bendixen, Jensen, & Bak, 2023). The first transient transcriptional modulation utilized a nuclease-deficient Cas (dCas) protein targeting a region downstream of the transcriptional start site, inducing temporary gene inhibition through steric hindrance (Fig. 5) (Qi et al., 2013). Further optimization fusing dCas to transcriptional repressor domains like the Krüppel-associated box (KRAB) significantly enhanced the inhibitory effect of this CRISPRi system (Alerasool, Segal, Lee, & Taipale, 2020). This approach was utilized in a gene-agnostic approach to repress the *Nrl* gene in a mouse model of RP (Moreno et al., 2018). *Nrl* is a key regulator of rod photoreceptor determination, and its downregulation reprograms rod photoreceptors into cone-like cells, which appear more resistant to RP-specific mutations (Moore, Skowronska-Krawczyk, & Chao, 2020). However, although the suppression of transcription using CRISPRi works in principle in vivo, effective inhibition of gene expression can also be achieved by other methods (e.g., RNA interference, antisense oligonucleotides and microRNA) (Gemayel, Bhatwadekar, & Ciulla, 2021).

In CRISPRa systems, a dCas is used in combination with transcriptional activators targeting sites known to regulate the expression of the gene of interest. The first transactivation module used for activating endogenous genes was VP64, a tetramer of the Herpes simplex virus activator VP16, fused to dCas9 (Maeder et al., 2013). Since then, various transactivation modules have been developed to boost gene activation efficiency. The three most widely used today are dCas9 fused to SunTag, synergistic activation mediator, and the tripartite complex VPR (a fusion of VP64, the NF-κB p65 subunit, and the R transactivator domain from Epstein-Barr virus) (Becirovic, 2022; Chavez et al., 2015; Konermann et al., 2015; Tanenbaum, Gilbert, Qi, Weissman, & Vale, 2014). All three CRISPRa systems demonstrated high gene activation efficiencies across different cell types and species (Chavez et al., 2016). In addition, newer and smaller activators such as ddCas12-[Active] also showed promising gene transactivation (Campa, Weisbach, Santinha, Incarnato, & Platt, 2019).

One application of CRISPRa systems is to activate genes which are structurally and/or functionally related to the mutated gene and could thus compensate for the structural or functional deficits in the target tissue. In the context of inherited retinal dystrophies, it has been postulated that many genes have functional counterparts that could serve as potential therapeutic targets (Becirovic, 2022). In a proof-of-principle study, dCas9-VPR delivered via split intein-based dual AAVs into the subretinal space was used to activate the cone-specific *Opn1mw* gene in a rhodopsin-deficient mouse model of RP (Böhm et al., 2020; Riedmayr et al., 2023). Activation of *Opn1mw* led to improved retinal function and a delay in retinal degeneration, with no apparent adverse effects up to one year after injection. Recently, another

approach using mRNA trans-splicing dual AAVs for subretinal delivery of dCas9-VPR into wild-type mice has revealed high activation of the murine *Myo7b* gene (Riedmayr et al., 2023). This approach has not yet been confirmed in a relevant animal or cellular model, but could represent an attractive alternative for the treatment of Usher syndrome type 1B.

Overall, CRISPRi and CRISPRa in particular represent attractive options for the mutation-independent treatment of genetic diseases. However, the main limitation of this method is the fact that the currently available efficient modules require long-term expression in the target tissue. This strategy would therefore benefit greatly from the development of new approaches that enable sustained gene activation at the target site after short-term expression of CRISPRa modules.

6.2. Antisense oligonucleotides

Antisense oligonucleotides are short single stranded DNA or RNA molecules designed to post-transcriptionally reduce gene expression. They can prevent the expression of disease-causing genes by binding to an RNA transcript via base pairing (Hammond et al., 2021). Current efforts focus on specific modification of ASOs to enhance their functionality, safety and stability (Lauffer, van Roon-Mom, & Aartsma-Rus, 2024). For example gapmer antisense oligonucleotides are used for to repress the expression and/or to modulate the pre-mRNA splicing (splice switching antisense oligonucleotides) of target transcripts (Hammond et al., 2021; Roberts, Langer, & Wood, 2020). Gapmer antisense oligonucleotides consist of three regions: a central “gap” of DNA nucleotides flanked by chemically modified RNA-like wings, which enhance stability and binding affinity. Once the gapmer antisense oligonucleotide binds to its target mRNA, it recruits the enzyme RNase H, which recognizes the DNA-RNA hybrid and degrades the mRNA, leading to the reduction or elimination of the corresponding protein. Gapmer ASOs are particularly effective for targeting long non-coding RNAs (Roberts et al., 2020).

nL-FLVC-001 is an antisense oligonucleotide-based drug designed to target diseases caused by mutations in the feline leukemia virus subgroup C receptor-related protein 1 gene. It is currently being studied for treating posterior column ataxia with RP, a rare genetic disorder causing sensory ataxia and vision loss (NCT06565572). Ultevursen (formerly QR-421a) is another antisense oligonucleotide drug that was investigated Phase 2/3 clinical trials. Ultevursen is a single-stranded, fully phosphorothioated 21-mer designed to treat Usher syndrome type 2A and non-syndromic RP caused by the most common mutations in exon 13 of the *USH2A* gene (NCT05085964) (Girach et al., 2022). Ultevursen works through an exon-skipping mechanism, allowing the production of a shortened, but functional usherin protein (Girach et al., 2022).

Antisense oligonucleotides can also be designed to selectively reduce the expression of the mutant mRNA. This strategy can be applied to treat autosomal dominant diseases, particularly those caused by gain-of-function mutations. One example is QR-1123, an antisense oligonucleotide that selectively knocks down the toxic c.68C > A; p.Pro23His substitution in the rhodopsin transcript (Daich Varela, Georgiadis, & Michaelides, 2023). Five weeks after a unilateral intravitreal injection promising improvements were observed in the treated eye, with a gain of 1.4 letters in best-corrected visual acuity score and enhanced retinal sensitivity (NCT04123626) (Daich Varela et al., 2023). A similar concept was employed for selective targeting of the c.2991-1655 A > G mutation in the *Cep290* transcript that causes Leber congenital amaurosis (Russell et al., 2022). To suppress the translation of the mutated transcript, sepofarsen, an RNA oligonucleotide, was intravitreally injected into one eye of patients in a phase 2b/3 clinical trial (NCT03913143) (Russell et al., 2022). At 12 months after the first treatment, positive signs like improved best corrected visual acuity were observed, but also adverse effects that included cataract formation (8 out of 11 treated eyes) with 6 of those requiring lens replacements. The visual improvements and the manageable adverse effects warranted further clinical development of the program, which is currently in phase 3 (NCT04855045, NCT03913143) (Russell et al., 2022). Overall, antisense oligonucleotides show promising safety and efficacy in clinical trials focusing on patients with inherited retinal dystrophies. However, one of the main drawbacks of these drugs is the need for repeated intravitreal injections, which increases the risk of adverse effects or complications from the injection procedure. In addition, antisense oligonucleotides are usually gene or mutation specific, which further limits their wider use for the treatment of inherited diseases.

siRNAs are double-stranded RNA molecules, typically 20–25 base pairs in length, that play a critical role in the RNA interference (RNAi) pathway by silencing specific genes (Dana et al., 2017). When siRNA is introduced into a cell, it is incorporated into a multi-protein complex called the RNA-induced silencing complex. Once bound, the siRNA strand guides this complex to a complementary mRNA target, leading to the degradation of the mRNA (Dana et al., 2017). An ongoing phase 2 clinical trial tests SYL1801 (NCT05637255), an siRNA therapeutic administered by eye drops in patients with neovascular age-related macular degeneration with the purpose of inhibiting Notch-Regulated Ankyrin Repeat Protein, a regulator of choroidal neovascularization. Just like for antisense oligonucleotides, sustained expression of siRNAs is required to achieve a sustained therapeutic effect, thus, necessitating repeated injections.

As an alternative to siRNAs, shRNAs or artificial miRNAs could be used and delivered with an AAV or other suitable vector to enable long-term expression. RNAi-based approaches are often mutation- or at least gene-specific and therefore less suitable for the development of gene-agnostic approaches. From today's perspective, it remains unclear what role RNAi approaches will play for the treatment of IRDs.

6.3. Gene-agnostic gene therapy approaches to treat retinal dystrophies

Various approaches for the gene-independent treatment of retinal diseases are currently in preclinical or clinical trials. The respective strategies usually depend on the stage of the disease. In the early stages of the disease, approaches aim to preserve the remaining photoreceptor cells by slowing or, in the best case, halting disease progression, e.g. through neuroprotection, cellular reprogramming and/or modulation of the metabolism. In later stages, attempts can be made to render certain disease-resistant retinal cells light-sensitive or to replace the missing photoreceptors with new cells or artificial retinal implants (Botto et al., 2022; Roska & Sahel, 2018; Zuzic et al., 2022).

Examples for gene-agnostic approaches in the early stage of the disease are gene therapies designed to treat RP/LCA and Stargardt disease, respectively (AAV-NR2E3 (OCU400) and AAV-RORA (OCU410)). OCU400 is currently being tested in a phase 3 clinical trial for RP caused by mutations in *RHO* or other RP related genes (NCT05203939, Table 1). In this approach, a healthy copy of the *NR2E3* gene, a transcription factor which plays a central role in the development, cellular homeostasis, and metabolism of photoreceptors, is supplied subretinally in an AAV vector (Li et al., 2021). A phase 1/2 trial showed relatively good safety and efficacy in autosomal recessive *NR2E3*-linked and in autosomal dominant *RHO*-linked inherited retinal dystrophies (Lam et al., 2024). OCU410ST utilizes AAVs to subretinally deliver the retinoic acid-related orphan receptor alpha (RORA), a nuclear hormone receptor that regulates inflammatory response pathways. This strategy is currently being tested in phase 1 / 2 (NCT05956626) for safety and efficacy in STGD patients. Another approach uses an AAV vector to deliver the nucleoredoxin-like 1 gene, which encodes for different isoforms of the rod-derived cone viability factor and was shown to protect cone and rod photoreceptors from retinal degeneration (Aït-Ali et al., 2015; Byrne et al., 2015). A phase 1 / 2 clinical trial is currently testing an AAV-NXNL1 candidate (SPVN06) for safety and tolerability in patients carrying mutations in *RHO*, *PDE6A*, or *PDE6B* genes.

Gene editing, supplementation, neuroprotection strategies shown to be effective in the treatment of early to mid-stage IRDs, but are not applicable to late stages when the target cell population (e.g. the photoreceptors) has already vanished (Yan et al., 2023). One option to address late-stage degeneration is optogenetics, which aims to restore vision by delivering light-sensitive proteins (e.g., opsins) to the remaining disease-resistant retinal cells, such as the ganglion or bipolar cells. The ectopic expression of optogenetic tools in bipolar cells and retinal ganglion cells can render them sensitive to light so that they can generate electrical signals which are transmitted to the visual cortex (Yan et al., 2023). Using this strategy, a certain degree of vision can be restored even in the absence of functional photoreceptors (Bi et al., 2006; Busskamp et al., 2010; Lagali et al., 2008; Sahel et al., 2021). There are various optogenetic tools based on proteins of the rhodopsin superfamily, such as channelrhodopsin-2 (ChR2), a cation channel from algae that can trigger depolarizing currents when exposed to light, or halorhodopsin (NpHR), a chloride pump from halobacteria that induces inhibitory currents through chloride influx (Bi et al., 2006; Gradinaru, Thompson, & Deisseroth, 2008; Nirenberg & Pandarinath, 2012; Tomita et al., 2007). Preclinical studies have shown that ChR2 delivered to ON bipolar cells or RGCs can improve visually guided behavior in animal models, and long-term expression has been demonstrated in both mice and marmosets (Doroudchi et al., 2011; Jendritza, Klein, & Fries, 2023). Other approaches target dormant cones, which have been shown to persist in advanced RP, with AAV vectors expressing NpHR to hyperpolarize cones upon light exposure (Busskamp et al., 2010).

Several recent clinical trials have tested optogenetic therapies in humans. The first AAV2-ChR2 gene therapy trial (NCT02556736) is underway in patients with advanced RP, though no results have been published yet (PMC8558540). To respond to a wider range of visible light, ChrimsonR-tdTomato and other engineered channelrhodopsin variants, are designed with enhanced spectral responsiveness across a broader wavelength range (Sahel et al., 2021; Tomita et al., 2014). An ongoing clinical trial (NCT03326336) is using a AAV.7 m8 capsid variant for delivery of ChrimsonR-tdTomato to ganglion cells (Martel et al., 2020). This approach was shown to improve the spectral sensitivity of treated cells and enhance light detection in patients with late stage inherited retinal dystrophies (Sahel et al., 2021). An AAV2-based gene therapy product that delivers a broadly light-sensitive multi-characteristic opsin MCO-010 is currently tested in a phase 2b/3 clinical trial and was granted orphan drug status by the FDA for RP and Stargardt disease (NCT04919473, NCT04945772). Preliminary safety and efficacy results have been reported by the sponsor (https://nanostherapeutics.com/), but no results have yet been published in peer-reviewed journals.

While these trials mark a crucial step towards making optogenetic therapies clinically viable, further innovations are currently being developed to optimize their clinical efficacy for patients. One of these innovations is the improvement of light sensitivity through special goggles that transfer a high intensity image to the retina. As the expressed opsins have only a specific action spectrum peak, the goggles capture an image and transform it into a monochromatic projection in the optimal wavelength for opsin excitation to the treated retina (Sahel et al., 2021).

The fact that an optogenetic approach has already provided a first proof-of-concept in clinical trials could accelerate the further development of this strategy for the group of patients with progressed retinal degeneration or complete blindness.

7. Approaches for the treatment of acquired retinal diseases with complex backgrounds

Non-hereditary retinal diseases, such as AMD and DR, are multifactorial in nature, involving a complex interplay of genetic predispositions, environmental factors, and intricate metabolic pathways (Fritsche et al., 2016). Unlike monogenic IRDs, the subtle and symptomless onset of these conditions, combined with the heterogeneity of their underlying mechanisms, complicates the identification of effective treatments (Wong et al., 2014).

7.1. Anti-VEGF AMD gene therapy

Although certain gene variants that increase the risk of developing AMD have been identified, AMD is not a monogenic disease, making classical gene therapy strategies—such as supplementation or repair of defective genes—difficult to apply (Fritsche et al., 2016). Current treatment approaches elucidated in the introduction are not curative; instead, they aim to alleviate specific symptoms, particularly those related to complement dysregulation or neovascularization in wet AMD, with the primary goal of delaying or halting disease progression (Table 1).

Gene therapy offers a promising alternative to conventional drugs, potentially providing long-term expression of anti-VEGF drugs in retinal cells. This could significantly reduce the burden of frequent injections on patients, caregivers, and medical facilities while improving treatment outcomes. Several gene therapeutic approaches targeting continuous anti-VEGF drug expression in the retina are currently in clinical trials. Recent comprehensive reviews by Rowe and Ciulla (2024) have highlighted various anti-VEGF and complement-based treatments for both wet and dry AMD, most of which utilize AAV vector systems (Table 1) (Rowe & Ciulla, 2024).

For example, RGX-314 employs an AAV8 vector to deliver a transgene encoding a ranibizumab-like anti-VEGF monoclonal antibody fragment (NCT05407636). This therapy is undergoing testing through both subretinal and suprachoroidal injections (Campochiaro et al., 2024; Wan, Muya, Kansara, & Ciulla, 2021). Positive safety and efficacy results have been demonstrated in a Phase 1/2a study, with stability observed in best-corrected visual acuity (BCVA) and central retinal thickness over two years, leading to ongoing Phase 2b/3 trials (Campochiaro et al., 2024).

Another notable example, 4D-150, is an AAV-based low-dose intravitreal gene therapy that expresses aflibercept and a VEGF-C inhibitory RNAi targeting multiple angiogenic factors (Table 1). A Phase 2 randomized dose expansion trial revealed a significant reduction in the need for supplemental aflibercept injections while maintaining stable BCVA and improved retinal control (Khanani et al., 2024). Additionally, EXG102–031, which relies on subretinal injections of a recombinant AAV-based gene therapy, induces the expression of an angiopoietin domain and VEGF receptor fusion protein (NCT05903794). A Phase 1/2a study is currently ongoing to assess its safety and efficacy (NCT05903794). Further, FT-003 is a novel gene therapy that utilizes an AAV gene expression system for intraocular delivery of therapeutic levels of an unspecified anti-VEGF protein. It is currently being investigated in open-label dose-escalation trials for both DR and AMD (NCT06492863, NCT05611424, NCT06492876). Another therapy, KH631, based on an AAV8 vector, aims to produce a VEGF receptor fusion protein to inhibit VEGF-A, VEGF-B, and PlGF (Ke et al., 2023).

7.2. Complement based AMD gene therapies

The *CFH* gene polymorphism, characterized by a histidine substitution at position 402 (Y402H), represents the strongest genetic risk factor for AMD (Klein et al., 2005). Beyond this complement regulator, several other complement gene polymorphisms have been linked to AMD, including C2, CFB, C3, and CFI (Bailey, Fossum, Fimbel, Montgomery, & Hyde, 2010; Choe et al., 2013; Horodecki, Oppenheim, & Winter, 2005). The complement system, a crucial component of the immune response, identifies and eliminates pathogens and damaged cells through a cascade of proteinases. When improperly activated, this system can lead to inflammation and degeneration of healthy cells, as observed in AMD. The efficacy of complement inhibitors for AMD treatment was recently validated by the FDA approval of C3 (pegcetacoplan) and C5 (avacincaptad pegol) inhibitors, which reduced

geographic atrophy (GA) progression by 21.2 % and 27.4 %, respectively (Patel et al., 2023). However, these treatments necessitate lifelong intravitreal injections and do not offer a cure. Additionally, the European Medicines Agency recently deemed the effects for both treatments as not clinically relevant and refused approval with referral to the risks of repeated intraocular injections outweighing the benefits (https://www.ema.europa.eu/en/medicines/human/EPAR/syfovre, https://www.ema.europa.eu/en/medicines/human/EPAR/izelvay).

Other approaches targeting the complement system aim for long-term interference with this molecular pathway. For example, JNJ-1887 (formerly HMR-59) uses an AAV2-based vector to express a soluble form of CD59, an inhibitor of the membrane attack complex associated with AMD progression (NCT03585556). Although gene therapy is striving to treat the detrimental aspects of multifactorial diseases and slow or halt their progression, JNJ-1887 is being tested as a treatment for GA, the advanced form of dry AMD. A 24-month open-label study with 17 GA patients suggested that the high-dose group exhibited slower GA progression by 23 % compared to historical controls, although some cases of inflammation led to increased growth. Notably, no conversion to neovascular AMD was observed (Dreismann et al., 2023; Heier et al., 2024). JNJ-1887 is advancing to a Phase IIb trial, evaluating its efficacy under different dosing regimens combined with corticosteroid prophylaxis.

Efforts are also being made to deliver the CFH gene in AMD patients to inhibit complement pathway activity (Table 1).

Another gene therapy approach currently in the clinical phase is AAV5-hRORA (OCU410) that targets dry AMD by inducing local expression of a nuclear hormone receptor that regulates inflammatory cytokines and complement-related proteins (NCT06018558). OCU410 has shown efficacy in reducing oxidative stress and inflammation in GA patients (Singh et al., 2023). Additionally, a recent study reported reduced drusen formation and increased expression of complement regulator CD59 in the *Abca4*−/− mouse model, which simulates early onset Stargardt disease and late-onset dry AMD (Akula et al., 2024).

Another approach to treating GA involves gene addition of the Elongation of Very Long Chain Fatty Acids-Like 2 (ELOVL2) gene (Siddiqui et al., 2023). This strategy is based on the observation that levels of long and very long-chain polyunsaturated fatty acids decline with age. Although the exact mechanism remains unclear, subretinal injection of a vector that facilitates ELOVL2 expression could offer a promising way to halt or slow geographic atrophy progression (Skowronska-Krawczyk & Chao, 2019).

Gene therapy promises the ability to address underlying disease causes and halt progression even in early stages of AMD. However, the complexity of this endeavor is evident in setbacks from recent clinical trials. Gene addition of *CFI* in patients without risk factor mutations also faced challenges, with Gyroscope Therapeutics' GT005 trials being terminated due to lack of efficacy (Ellis, Buchberger, Holder, Orhan, & Hughes, 2020). The reasons for the lack of efficacy remain unclear, but may be due to the complex biology of the complement system and its multiple functions in tissue processes such as cell turnover, stress response and return to homeostasis after inflammation, which may require a delicate balance when interfering with the system. In line with this there were also drawbacks with anti-complement therapeutic approaches. For example, the Phase II trial for NGM621, a humanized monoclonal antibody targeting C3 activity, failed to meet its primary endpoint of reducing GA lesion area, leaving secondary endpoints and post-hoc analyses under review (Wykoff et al., 2022). Similarly, the Phase II trial for ANX007, which targets C1q to inhibit the classical complement pathway, showed preservation of visual function but did not significantly reduce lesion growth (Boyer, 2024).

Innovative advances in complement-based therapies are particularly relevant for retinal gene therapy of AMD. One example is Mini-FH, a truncated version of Factor H that combines domains 1–4 and 19–20. This modified protein effectively regulates complement activation on cell surfaces while avoiding the regulatory domains that could lead to side effects (Harder et al., 2016). Recently, an AAV-delivered expression system was developed that produces Mini-FH only in Müller cells when they become gliotic (Biber et al., 2024). By linking Mini-FH expression to the stress-induced promoter of glial fibrillary acidic protein, Müller cells are turned to producer cells for the therapeutic gene in response to inflammation or degeneration, thereby avoiding interference with complement homeostasis and only dampening the immune response when necessary. Intravitreally injected AAVs with capsids showing tropism for Müller cells have been shown to facilitate stable expression in models of acute retinal degeneration.

7.3. Current gene therapy approaches for the treatment of diabetic retinopathy

Given the similarities in neovascularization pathobiology, the same medications used to treat AMD are often applied to DR (Campochiaro, 2015). In particular, the proliferative form of DR is also characterized by aberrant outgrowth of leaky blood vessels, which can be alleviated by anti-VEGF treatments. However, again none of the available treatments can halt the progression of the disease and require repeated intravitreal dosing. Again, no clear genetic etiology exists for DR but the concept of gene therapy may also be applied for the development of local ocular one-time treatments of DR. Recent clinical trials for DR have highlighted both promising advances and significant challenges for gene therapeutic approaches. ADVM-022, an intravitreal gene therapy designed to deliver a VEGF inhibitor, initially demonstrated potential for reducing the need for frequent anti-VEGF injections (NCT04418427). However, in the INFINITY trial, which focused on diabetic macular edema, serious adverse events were reported in 6 out of 12 patients receiving high doses (https://investors.adverum.com). These included cases of hypotony (dangerously low intraocular pressure) and inflammation, leading to significant vision loss in some cases. The adverse effects did not occur in the low dose group or in the parallel trial on AMD patients. As a result, the sponsors decided to halt further development of ADVM-022 for DME due to safety concerns and the unpredictable nature of these adverse events (https://www.modernretina.com). RGX-314 gene therapy is not only applied to AMD, as mentioned above, but also to treat DR. The Phase 1/2 clinical trial revealed a positive safety profile. Early results suggest that RGX-314 can reduce the burden of frequent injections while maintaining efficacy, with no severe adverse events reported thus far (NCT04567550). Following in-office suprachoroidal delivery, a one-time treatment was able to halt or improve the disease state with no worsening observed in any of the 50 patients. Also, vision threatening events were reduced by 89 % of the treated patients compared to the control group. Another example for a drug that is being developed for nAMD and DR is 4D-150. The intravitreal gene therapy utilizes the R100 vector to deliver aflibercept and a VEGF-C inhibitory RNAi. An ongoing Phase 2 trial is assessing its safety and efficacy in adults with diabetic macular edema (NCT05930561). This trial involves two stages: a dose confirmation phase where participants receive different doses of 4D-150 or aflibercept, and a dose expansion phase based on earlier results. Enrollment for the first phase has been completed, and interim results are expected in 2024 (https://retinalphysician.com). Together, trials like these reflect the ongoing shift towards gene therapies that could offer longer-term solutions for patients, though safety remains a critical consideration moving forward.

8. Conclusion

Gene therapy represents a promising new treatment option for retinal diseases, offering the potential to provide a clinical benefit for both inherited and acquired retinopathies. While recent advances, such as the approval of voretigene neparvovec, mark significant milestones, numerous challenges remain. The complexity of some IRDs, the limited capacity of current gene delivery systems, and the need for targeted and

safe delivery methods underscore the necessity for continued innovation. Emerging techniques like dual-vector systems, genome editing, and hybrid delivery platforms offer potential solutions to these challenges. Furthermore, extending gene therapy applications to more common conditions like AMD and DR could revolutionize treatment paradigms, providing long-term, possibly one-off treatment options. As research progresses, overcoming these obstacles will be crucial in translating these therapies from experimental stages to widespread clinical practice, ultimately improving outcomes for patients with retinal diseases.

CRediT authorship contribution statement

Josef Biber: Writing – review & editing, Writing – original draft, Visualization. **Catharina Gandor:** Writing – review & editing, Writing – original draft, Visualization, Validation, Conceptualization. **Elvir Becirovic:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization. **Stylianos Michalakis:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

S.M. and E.B. are inventors on patents and/or patent applications covering different AAV technologies. S.M. is co-founder, shareholder and board member of ViGeneron GmbH. E.B. is a member of the scientific advisory board of ViGeneron GmbH. The remaining authors declare no competing interests.

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Data availability

No data was used for the research described in the article.

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