CRB1 gene therapy coming of age: mechanistic insight and rAAV assays on mouse & human retinal organoid models

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Chapter 1

1.1 Introduction: A short history on the apical polarity protein CRB1

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1.2 Recombinant Adeno-Associated Viral Vectors(rAAV)-Vector Elements in Ocular Gene Therapy Clinical Trials and Transgene Expression and Bioactivity Assays

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1.3 Aim and outline of this thesis

Inherited retinal dystrophies (IRDs) are disabling disorders of the visual system with so far, few to no treatment options available. More than 279 genes have been associated with IRDs including the Crumbs homolog-1 (*CRB1*) gene. *CRB1*-associated retinal dystrophies (RDs) have an autosomal recessive inheritance pattern requiring individuals to have two pathogenic variant alleles as either homozygous or compound heterozygote alleles. Clinical phenotypes range from retinitis pigmentosa (RP; 3-9% of all RP cases), Leber congenital amaurosis (LCA; 7-17% of all LCA cases), cone-rod dystrophies to sporadically foveal retinoschisis and macular dystrophy [1–6]. *CRB1*-associated RD patients in ophthalmic clinics may present with progressive visual loss, peripheral macular retinal thickening (but thinning over time), difficult to discern retinal layers, hyperopia, disruption of the normal blood-retinal barrier shown by cystoid fluid collections, reticular pseudo-drusenoid white spots or optic nerve disc drusen (i.e., acellular calcified deposits in ganglion cells), and preservation of para-arteria retinal pigment epithelium (RPE) [1,6,7]. Early phenotypes are found above the RPE at the outer limiting membrane (OLM) in the neuroretina where CRB1 protein and other apical polarity regulators localize, pointing towards a primarily cell adhesion-related phenotype [8,9]. The OLM is established in the fetal neuroretina and hold in place by Müller glial cells (MGCs) and photoreceptors (PRs), establishing adherens junctions between PRs-PRs, MGCs-MGCs, and PRs-MGCs. Many RD-*CRB1*-like animal models, RP-*CRB1*-like patient-derived retinal organoids as well as the observation in RD-associated *CRB1* patients establishes a disease mechanism related to a loss of adhesion between the OLM participating cell types and a dysregulation of the apical-polarity signaling pathways [8–12].

In 1984 and further worked out in 1990, the *crb* gene was discovered in the apical membrane of epithelia cells in *Drosophila melanogaster* [13,14]. The first phenotype in *crb*-null embryos was described as "*many small holes in cuticle*" like a crumbling morphology giving it its name *crumbs*. Other *Crb* orthologues have since been found in insects and vertebrates alike. In vertebrates, the *CRB* gene has undergone several gene duplication events during evolution (ENSG00000134376 gene tree). Yet, the prototypic (human, mouse, rat) CRB1/CRB2 as well as the CRB (fruit fly) protein is composed of a large extracellular domain containing many EGF-like domains and 3x LamG-like domains, a single-pass transmembrane domain, and a canonical short PDZ/ERLI intracellular domain [9,15,16]. The 37-aa intracellular C-terminal domain contains a FERM domain next to the transmembrane domain and a PDZ domain which form intracellular protein complexes interacting with the cell cytoskeleton. The human *CRB1* gene consists of 12 exons coding for 1406 amino acids (aa; 1405 aa in mice). In humans we also find the *CRB1* orthologues *CRB2*, *CRB3A*, and *CRB3B* with all being expressed in the retina. CRB2 (1285 aa) is highly similar to CRB1 but having less EGF-like domains and CRB3 (~120 aa) lacks the extracellular domains. However, all apart from CRB3B, are able to form CRB or PAR apical polarity complexes by recruiting PALS1 or PAR6 [17–20]. Interestingly, *Crb2* and *Crb3* gene ablation in mice is lethal while *Crb1* is not [8,21,22].

What is more, mouse CRB2 protein (while *CRB3* expression is actively repressed) promotes cell ingressions in the epithelial-to-mesenchymal transition (EMT) during gastrulation – a key developmental stage needed to establish the ectoderm, mesoderm, and endoderm [23,24]. Also, human CRB2 protein is found earlier than CRB1 during retinal neurogenesis [9]. However, the function and cellular expression of CRB's (including CRB1) diverged between species evolution making inter-species comparisons complex. For example, a hallmark of human or mouse CRB1 function is to participate in the neuroretinal polarity complex but zebrafish CRB1 does not [25]. Further, several *CRB1*-like mouse models and human CRB1/CRB2 protein localization studies suggests that CRB1 and CRB2 have at least overlapping roles and being able to compensate the loss of the other in the neuroretina [12,15,26–32]. Interestingly, CRB1 has a more dominant role in humans compared to mice being expressed in both photoreceptors and Müller glial cells at the subapical region while in mice CRB1 is only found in early retinal progenitor cells and later in Müller glial cells (see Figure 1). Further complicating, a 1003-aa CRB1-B isoform (delineating it from the classical 1406 aa CRB1-A protein) was detected having a shorter extracellular domain and lacking the ERLI domain for binding to the polarity complex members. However its significance is still under dispute as the mouse *Crb1-B* knockout model had no evident retinal phenotype and the protein is by large expressed in the photoreceptor outer segments instead of at the subapical region where CRB1-A takes part in apico-polarity protein complexes CRUMBS and PAR [33] (**Figure 1**).

In 1999, the human *CRB1* gene was first associated as a retinal disease causing gene [35]. Over the years, more than 300 pathogenic *CRB1* variants have been found with the most frequently found on exon 2, 7 and 9 [6]. Common variants are p.(Glu222Lys), p.(Cys250Trp), p.(Cys948Tyr), and p.(Met1041Thr) (https://databases.lovd.nl/shared/variants/CRB1). The pathogenic variants are almost exclusively found in exons (94%) & splice regions (4%) and covering the whole *CRB1* exome. Interestingly, RD-associated *CRB1* variants display a low genotype-phenotype correlation, yet variants ablating CRB1 proteins have been more clearly associated with early retinal phenotypes [36].

With the advent of gene therapy showing safety and long-lasting expression by recombinant adeno-associated viral vectors (rAAVs) in many studies [37], several options were explored for developing candidate vectors for clinical trials. Different promoters (CAG, CMV, CMVmin, GFAP, RLBP1, CD44), different rAAV serotypes (rAAV2/2, rAAV2/5, rAAV2/6, rAAV2/6-derived ShH10^{Y445F}, rAAV2/9), and different *CRB1* isoforms were tested [28,38,39]. An early challenge was that the size of the *CRB1* transcript (4218 bp) coding for the large 1406-aa protein being close to the maximum packaging size of an rAAV (~4800 bp). And additional room is needed for a promoter and a polyadenylation sequence. It was approached in three directions: (a) expressing a short native *CRB1* (*sCRB1*) lacking

exon 3 and 4, (b) replacing *CRB1* with *CRB2* (3855 bp), (c) developing a short Müller & photoreceptor cell promoter. The s*CRB1* caused retinal degeneration when injected subretinal [39].

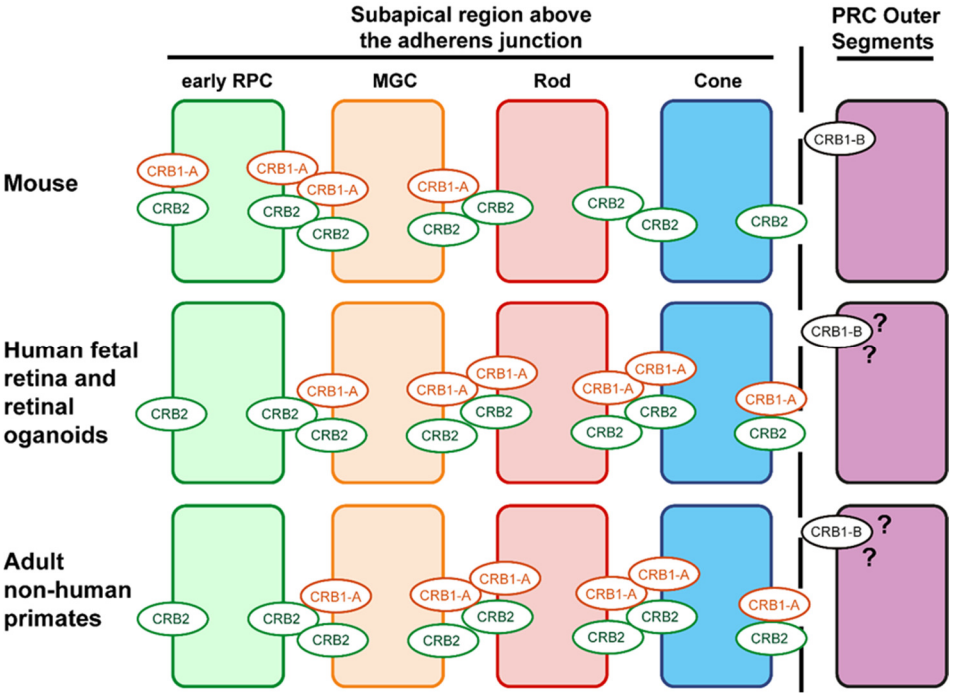

Figure 1. Cartoon representation of CRB1-A, CRB1-B and CRB2 protein location at the subapical region above the adherens junction (forming the outer limiting membrane) and in photoreceptor outer segments. The subcellular localization of CRB1-B in human photoreceptor outer segments is not known, therefore indicated in red with question mark. Figure adapted from [34]. MGC, Müller glial cells; PRC, photoreceptor; RPC, retinal progenitor cells.

Replacing *CRB1* with *CRB2* turned out be a highly efficient vector showing repeatedly rescue or protection in *in vivo* mouse studies [12,28]. And the generation of a CMVmin promoter provided robust *CRB1* expression to Müller and photoreceptor cells also being able to protect the retinal morphological phenotype but not visual function [12,28]. Then, a different AAV serotypes were screened on potency in human retinal explants and human retinal organoids showing that rAAV5 and rAAV6-derived ShH10^{Y445F} can efficiently infect both cell types. Recently, also a natural occurring *Crb1* rat model became available which we had difficulties in showing efficient rAAV-mediated neuroretinal infection [40]. rAAV gene therapy studies in retinal organoids are also under way. It will be exciting to see what other research will define therapies for patients with *CRB1*-associated retinitis pigmentosa.

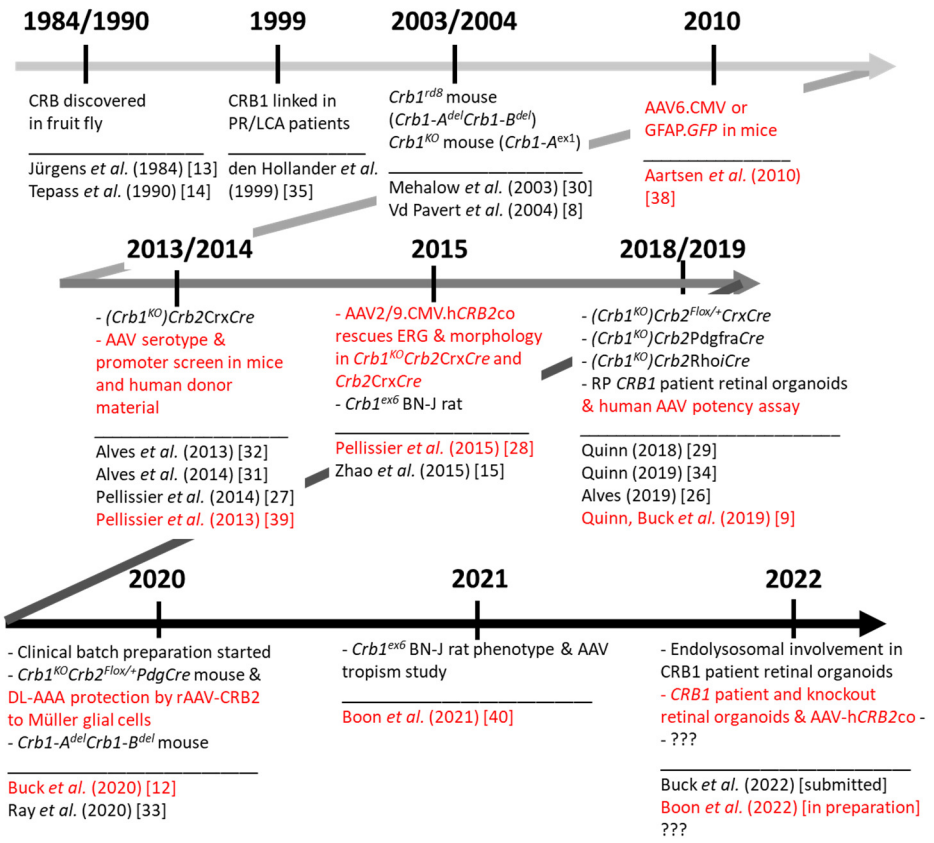

Figure 2. An incomplete short *CRB1* history. Focus: *CRB1*-like mouse models, CRB1-associated retinitis pigmentosa patient-derived retinal organoids and relevant rAAV gene therapy-related studies (in red).

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1.2 Recombinant Adeno-Associated Viral Vectors(rAAV)-Vector Elements in Ocular Gene Therapy Clinical Trials and Transgene Expression and Bioactivity Assays

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Abstract

Inherited retinal dystrophies and optic neuropathies cause chronic disabling loss of visual function. The development of recombinant adeno-associated viral vectors (rAAV) gene therapies in all disease fields have been promising, but the translation to the clinic has been slow. The safety and efficacy profiles of rAAV are linked to the dose of applied vectors. DNA changes in the rAAV gene cassette affect potency, the expression pattern (cell-specificity), and the production yield. Here, we present a library of rAAV vectors and elements that provide an intuitive workflow to design novel vectors. We first performed a meta-analysis on recombinant rAAV elements in clinical trials (2007-2019) for ocular gene therapies. We analyzed 32 unique rAAV gene cassettes used in 56 ocular clinical trials. The rAAV gene therapy vectors used six unique capsid variants, 16 different promoters, and six unique polyadenylation sequences. Further, we compiled a list of promoters, enhancers, and other sequences used in current rAAV gene cassettes in preclinical studies. Then, we give an update on pro-viral plasmid backbones used to produce the gene therapy vectors, inverted terminal repeats, production yield, and rAAV safety considerations. Finally, we assess rAAV transgene and bioactivity assays applied to cells or organoids *in vitro*, explants *ex vivo,* and clinical studies.

Introduction

Many reviews have been written on recombinant adeno-associated virus vector (rAAV) tropism in ocular tissue, rAAV host cell infection, and potential rAAV-treatable inherited retinal diseases [1–9]. Here, we review the ocular gene therapies developed over the past 20 years focused on the diversity of elements incorporated in rAAV vectors. Further, we discuss how the vectors were generated, tested, and further modified to increase the potency and safety of the gene expression vector. A library of validated elements allows researchers to streamline the modification of their vectors. Novel medical therapies, such as gene therapies, need to be carefully optimized to demonstrate efficacy and safety for human trials [10]. It is crucial to choose the most relevant biological model(s) (in vitro, in vivo, and ex vitro model) to test an optimized gene therapy vector in a transgene expression assay (TEA) and test the transgene activity in a biological activity assay (BAA). Novel developments in preclinical models, for example, human induced pluripotent stem cell (hiPSC) derived retinal organoid disease models, can result in FDA or EMA approval for phase I/II clinical trials. Such an approach, for example, reduced the need for further animal experimentations for the AAV2-hCHM (Sponsor: Spark Therapeutics) clinical trial preparation.

1.1. Why viral vector-based gene augmentation therapy for ocular diseases?

The environment of the eye offers a wide range of treatment possibilities because the blood-retinal barrier of the eye decreases viral vector diffusion to other organs and decreases systemic immune activation. The retina also consists of terminally differentiated cells reducing gene integration and chromosomal rearrangements. Also, many noninvasive techniques are available to monitor the treatment response. Treatments aim to slow the progression of the inherited retinopathies by reducing retinal cell death, augmenting retinal function, replacing cells, or creating an artificial retina (retinal prosthesis). Prevention of retinal cell death might be achieved by gene therapy, cell therapy, other drug treatment, dietary adjustment, and even by changes in lifestyle. There are few scientifically proven preventive or protective actions available to patients with inherited retinal diseases.. Some of the preventing measures might potentially decrease the quality of life, such as the continuous use of eye protection, photochromic lenses, or restriction to light exposure. Cigarette smoke or high Vitamine E intake can worsen ocular disease progression [11].

The ocular gene therapy strategy targets the basis of inherited retinopathies: The gene. The variant (disease-causing) gene can be silenced, replaced, or repaired by expressing a gene cassette in the target cell. The primary gene cassette carrier systems for ocular diseases are recombinant viral vector-based. Other potential strategies are the use of exosomes/liposomes, antisense oligonucleotides (AONs), electroporation of naked DNA/RNA, or application of nanoparticles [12,13]. In most clinical retinal gene therapy studies, the vector of choice is the rAAV gene expression vector [14]. Two genes that do not fit in a conventional rAAV gene cassette (*MYO7A* linked to Usher syndrome Type 1B and *ABCA4* to Stargardt disease) have

been delivered to the retina by recombinant lentiviral expression vectors [15–18]. *CEP290*-mutations linked to LCA has been rescued in patients by AONs correcting the mRNA transcript (QR-110 for LCA with *CEP290*; QR-421a for LCA with *USH2A*) [19]. Finally, the therapeutic product is administered to the target cells by either subretinal or intravitreal injection. Subretinal injections can target a focal area (e.g., macula), favoring high vector delivery to the RPE and photoreceptors. Intravitreal injections efficiently target the ganglion cell layer and spread the rAAV to the whole retina in rodents but not in the primate retina due to the properties of the inner limiting membrane. Figure 1 describes the considerations for choosing a gene therapy strategy.

1.2. rAAV gene therapy for ocular diseases – Advantages & disadvantages

rAAV DNA carrier systems have been used successfully because (1) they express the transgene within days or weeks and might reach full level expression after 4-6 weeks *in vivo* [20,21]. (2) rAAV DNA carrier systems allow long-term treatment for at least ten years in large animals [22], (3) and primarily deliver their gene cassette in episomal concatemers into the nucleus [23]. (4) rAAV DNA carrier systems do spread well within tissues to target large retinal areas [24]. (5) The capsid composition can be adjusted to fit one's goals [25], to achieve (6) low serious adverse events (SAE) in clinical trials [26]. rAAVs, similar to other viral strategies, have limitations such as (1) a small gene cassette capacity (up to 4.5 kb + 2x145 bp ITRs), (2) instability of the inverted terminal repeats (ITRs), (3) the need for high viral load for transgene expression, (4) and the occurrence of humoral immune reactions such as neutralizing antibodies that reduce the number of capsids reaching the target cells, the innate immune pathways silencing the gene cassette within the host cell, and the cell-mediated T-cell immune response against foreign protein expression [27]. It is important to outweigh the advantages of using rAAVs over other attractive strategies (Figure 1). Here, we describe how successful gene cassettes (vectors) have been designed for AAV gene therapies that could be potentially also explored for other viral gene therapies (*vectorology*).

2. Ocular rAAV vector-based therapies in clinical trials

Inherited retinal dystrophies (IRDs) and inherited optic neuropathies (IONs) are chronic and disabling disorders of the visual function affecting 1/2000 to 1/4000 people worldwide. They display considerable genetic, symptomatic, and anatomical heterogeneity (Figure 2A; [28–30]). More than 250 genes can cause IRDs and IONs [31]. IRDs include pigmentary retinopathies, maculopathies, and stationary retinopathies. Patients with pigmentary retinopathies regularly suffer from night blindness, tunnel vision, and photophobia. Maculopathies affect color vision and accurate vision. Some IRDs are syndromic. The most common syndromic retinopathies are ciliary or mitochondrial retinopathies. Common ciliary retinopathies are Usher syndrome, Bardet-Biedl syndrome, and Senior–Løken syndrome [32–34]. Lastly, IONs affect the ganglion cells transmitting the visual signal from the retina

through the optic nerve to the cortex. IONs progressively degenerate the optic nerve leading to vision loss.

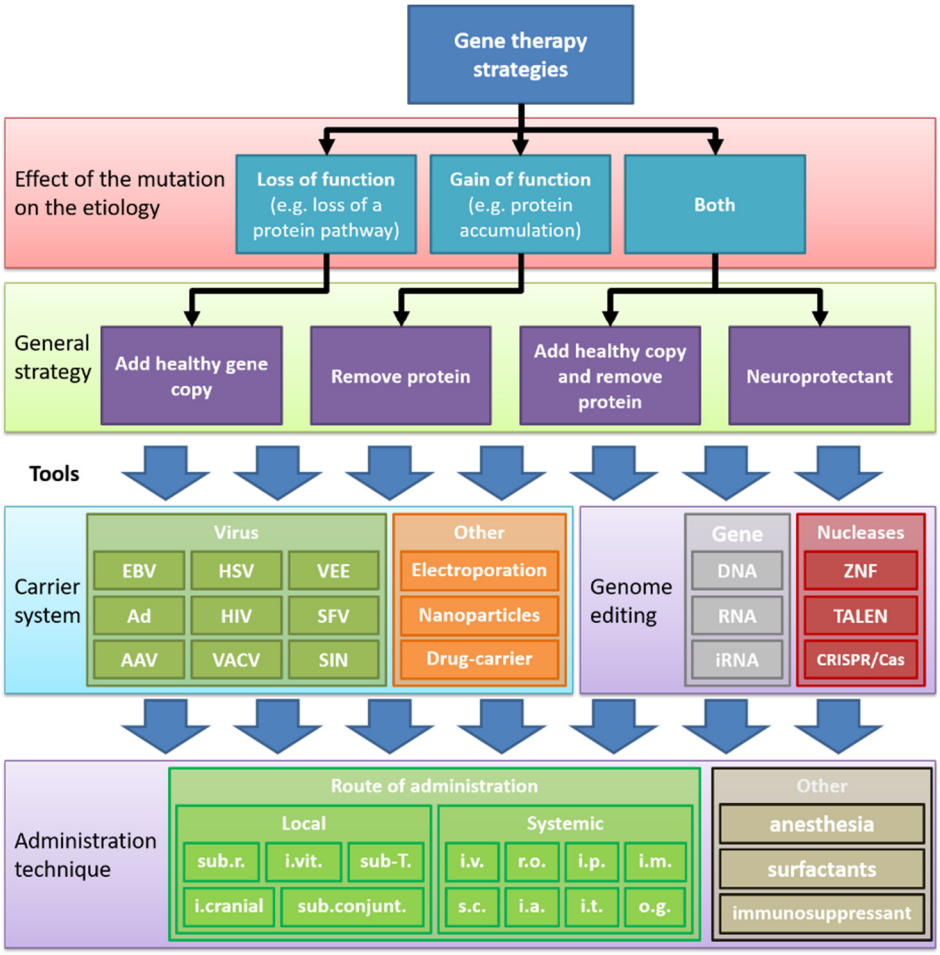

Figure 1. An overview of retinal gene therapy strategies. The effects of the gene variations determine the gene therapy rescue strategy to be applied. Physical DNA delivery includes electroporation, sonoporation, magnetofection, and bioballistic (gene gun) methods. Viruses: AAV, Adeno-associated virus; Ad, Adenovirus; alpha, alphavirus; Epstein-Barr virus (EBV); FV, Foamy virus; HSV, Herpes simplex virus; HIV, Human immunodeficiency virus; VACV, Vaccinia virus. Nucleases: ZNF, Zinc-finger nuclease; TALEN, transcription activator-like effector nuclease; CRISP/Cas, clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonuclease. Route of administration: i.cranial, intracranial; i.a., intraarterial; i.m.,, intramuscular; i.v., i.t., intrathecal; intravenous (e.g. tail vein or facial vein); i.vit, intravitreous; o.g., oral gavage; r.o., retro-orbital; sub.r, subretinal; sub-T, sub-Tenon; sub.conjunt, sub conjunctiva; s.c., subcutaneous.

rAAVs are one of the most promising gene augmentation tools for the treatment of inherited ocular diseases. The FDA approved in December 2017 the first rAAV-based retinal gene therapy (voretigene neparvovec-rzyl; Spark Therapeutics). More than 32 rAAV gene therapies have been delivered to clinical trials (Table 1). A wild-type copy of the gene is supplemented by rAAV delivery to RPE or photoreceptor cells for 13 genes causing inherited retinal diseases (Figure 2A). Two inverted terminal repeats of the AAV serotype 2 (ITR2) flank the gene cassette of conventional rAAVs. Many rAAV gene cassettes consist of a promoter, a wild-type copy of the cDNA of the gene-of-interest, other enhancers or transcript stabilizing elements, an intron, and a polyadenylation sequence. The promoter can be of viral origin, shortened-native, or synthetic. Many new promoters incorporate conserved transcription factor binding sites (TFBS), called enhancers, to boost transcription. Also, many additional sequences can be added to a gene cassette, including fluorescent probes, linkers, base editors, nuclear localization signals, or short-hairpin RNAs (See Figure 2B and section 3).

The first ocular rAAV clinical trial for *RPE65* was initiated in 2007. Over the years, five different AAV-*RPE65* products were tested in a total of 13 clinical trials by Applied Genetic Technologies Corporation (AGTC; Alachua, USA), Hadassah Medical Organization (Jerusalem, Israel), Spark Therapeutics (Philadelphia, USA), University of Pennsylvania (Philadelphia, USA), MeiraGTx (London, UK), Nantes University Hospital (Nantes, France), and University College London (London, UK). The clinical trial results lead to in the first and only retinal gene therapy (so far) approved by the FDA in December 2017 and EMA in November 2018 (AAV-*hRPE65v2*; voretigene neparvovec-rzyl, LUXTURNA; Spark Therapeutics). The five different strategies delivered the *RPE65* gene to RPE cells by subretinal administration of the rAAV2, rAAV4, and rAAV5 (Table 1). Administration of rAAV2/2-*hRPE65* at a high dose of 10^{12} viral genomes (vg) resulted in transient inflammation [35]. Switching to rAAV5 resulted in increased transduction of RPE cells. Shortening of the 1.6 kb long native RPE65 promoter to 750 bp (NA65p) and other modifications (SV40 intron; Kozak sequence; codon optimization) resulted in an even more potent and cell-specific expression of *hRPE65*. The resulting product (rAAV2/5-OPTIRPE65) seems to be at least as efficient as the AAV2/2-h*RPE65*v2 in RPE65-deficient mouse retinal pigment epithelium [36]. The search for the best product demonstrates the complexity of implementing native promoters ([long]RPE65, NA65) over ubiquitous strong promoters (CAG, CB-SB) in transcription regulation over different animal models and disease states (see also section 3.4 retina-specific promoters).

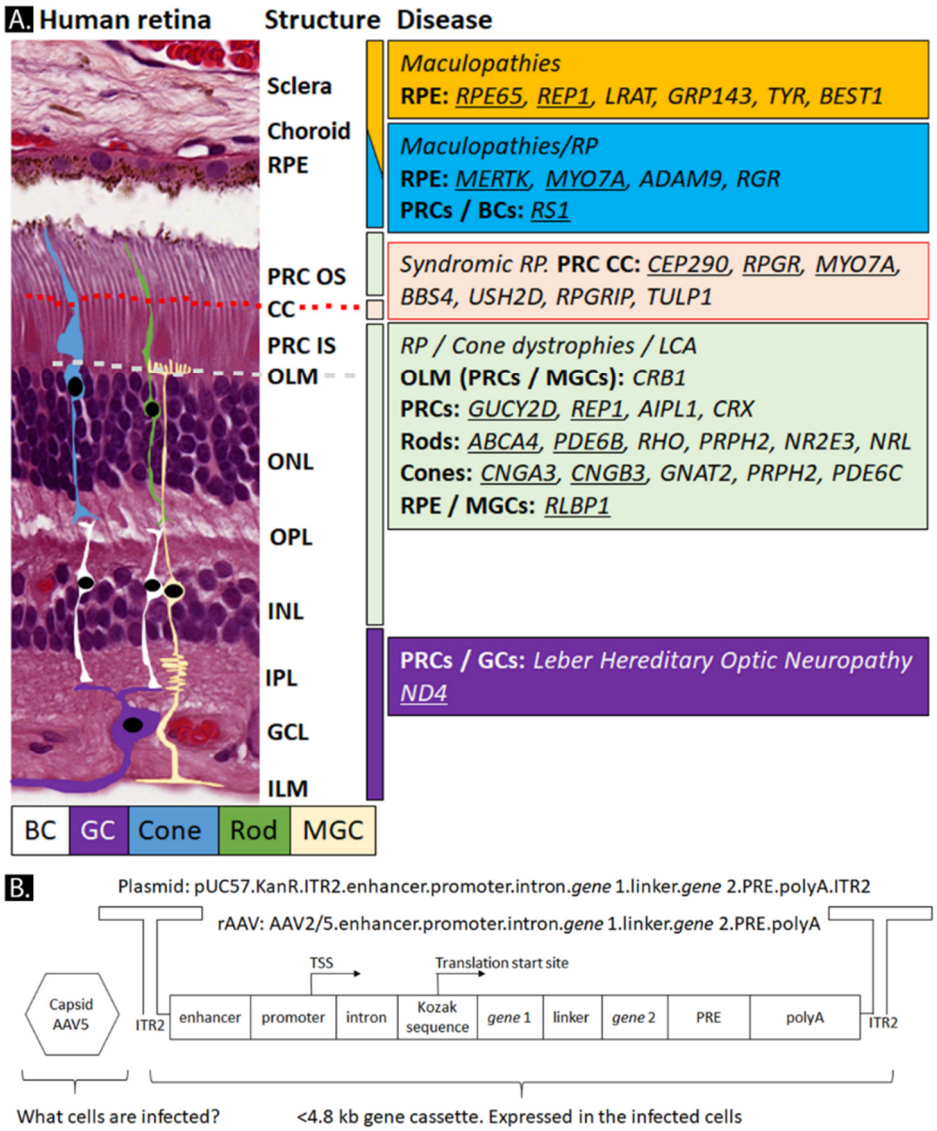

Figure 2. The development of recombinant AAV vectors targeting ocular diseases. (A) Main location (arrow) of frequent gene products (genes indicated) causing retinal diseases (color), and rAAV-gene supplementation therapy genes (in clinical trials; underlined genes). (B) Hypothetical rAAV gene cassette and the corresponding plasmid. AAV5, adeno-associated viral vector serotype 5; BC, bipolar cell; Cone, cone photoreceptor; CC, connecting cilium; GC, ganglion cell; GCL, Ganglion Cell Layer; ILM, Inner Limiting Membrane; IPL, Inner Plexiform Layer; ITR, inverted terminal repeat; KanR, kanamycin resistance gene; LCA, Leber congenital amaurosis; MGC, Müller glial cell; ONL, outer nuclear layer; OPL, Outer Plexiform Layer; PRC IS, photoreceptor inner segment; PRC OS, photoreceptor outer segment; polyA, polyadenylation sequence; PRE, post-transcriptional gene regulatory element; RP, retinitis pigmentosa; RPE, retinal pigment epithelium; rod, rod photoreceptor; TSS, transcription start site.

Many clinical trials are still far away from FDA / EMA approval. But many more clinical trials for hereditary ocular diseases, including *CRB1*-related retinitis pigmentosa, will be initiated. Numerous breakthrough clinical phase I/II trials were already initiated over the recent years (see Figure 2A and Table 1). ProQR was able to move from clinical trial phase I/II to II/III within one year (Product: AON QR-110 for *CEP290* mRNA). GenSight Biologics (GS010, rAAV2/2-*ND4*) was able to move to clinical trial phase III within four years for the treatment of Leber Hereditary Optic Neuropathy (LHON). NightstaRx Ltd initiated a clinical trial phase I/II with a linked clinical trial phase III for Usher's syndrome in which 200 patients in the XOLARIS study phase I/II might become included in the follow-up clinical trial phase III study (rAAV2/8-*RPGR*-ORF15). AMD might be treated either by monthly administration of aflibercept (Product ProCon consists of *sFLT01*, an antibody-like product; Regeneron Pharmaceuticals) or by potential long-lasting rAAV-*sFLT01* expression from transduced cells (Product: AAV2-*sFLT01*; Sanofi / Genzyme). Other exciting ocular therapies are displayed in Figure 2A and Table 1. All clinical trial identifiers, clinical trial start dates, and products can be found in Table S1.

Upon studying the rAAV capsids that have been administered to patients, we observed yet minimal clinical use of the various available serotypes. Only rAAV2 (and rAAV2 variants rAAV2-tYF and rAAV2-7m8), AAV5, and AAV8 have been injected into the eye compared to the vast and large number of novel capsids that have been developed over the past years. More clinical studies will follow evidently with the sharp increase in rAAV clinical trial initiations since 2017 and diversification of capsid use (Figure 3A). Also, rAAV products moved from the initial use of only the RPE65 promoter and ubiquitous promoter system to a more diverse pool of photoreceptor-specific promoters(Figure 3B). Large promoters, such as the CBA/CAG (1661 bp), are less common in recent clinical trial products. New products contain relatively small ubiquitous promoters such as CAG / CMV promoter versions of less than 1 kb (smCBA; CB-SB; CMV; CB7), or photoreceptor (PRC) / Müller glial cell (MGC) cell-specific promoters of less than 600 bp.

Apart from the promoter and capsid, we looked at a less documented area of described rAAV vectors, including enhancers, stabilizing elements (e.g., introns, splice donor/acceptors, WPRE), polyadenylation sequences, pro-viral plasmid backbones, and production platforms. The main enhancers used were the cytomegalovirus (CMV) enhancer that is present in CMV and the CBA/CAG promoters. The interphotoreceptor retinoid-binding protein (IRBP) enhancer was used in front of the hRS1 promoter (clinical trial NCT02317887). Common synthetic introns apart from native introns in the gene are rabbit β-globin intron with splice donor/splice acceptor (SD/SA; in CAG promoter), SV40 intron with SD/SA, human β-globin intron, and synthetic introns (e.g., 5'-splice donor of the first human β-globin intron and the 3'-splice acceptor of an intron of the immunoglobulin gene heavy chain variable region; Gene product: ADVM-022).

Table 1 rAAV gene therapy products registered on clinicaltrials.gov

Disease	Year	Product	Capsid	Promoter	Intron	Other	Gene	PolyA	Production
LCA	2007	h*RPE65*v2	AAV2	CAG	β-globin		*RPE65*	bGH	HEK293
LCA	2007	AAV-*RPE65*	AAV2	CB-SB			*RPE65*	SV40	HEK293
LCA	2008	tgAAG76	AAV2	hRPE65			*RPE65*	bGH	B50, helper adenovirus
LCA	2011	HORA-*RPE65*	AAV4	hRPE65			*RPE65*	bGH	HEK293
LCA	2016	OPTIRPE	AAV5	NA65	SV40		*RPE65*	SV40	HEK293
AMD	2009	*sFLT01*	AAV2	CAG	β-globin		*sFLT01*	bGH	HEK293
AMD	2011	OXB-201	EIAV	CMV		IRES + WPRE	*Endo+ Angio*	SIN-LTR	?
AMD	2011	AAV.*sFlt-1*	AAV2	CMV	Chimeric intron		*sFlt-1*	SV40	HEK293
AMD	2017	RGX-314	AAV8	CAG/CB7	β-globin		*aVEGFAfabH.F2A .aVEGFfabL*	rabbit β-globin	?
AMD	2018	HMR59	AAV2	CAG	β-globin$^{SD/SA}$		*sCD59*	bGH	?
AMD	2018	ADVM-022	AAV2-7m8	CMV	β-globin^{SD} Ig^{SA}	TLP-eMLP	*sFLT01co*		Sf9
AMD	2019	GT005	AAV2	CBA	β-globin	WPRE	*CFI*	bGH	?
LHON	2010	AAV2-*ND4*	AAV2	CMV	5'UTR COX10	3'UTR COX10(MTS)	*ND4*	bGH	HEK293, HSV1-rc/ΔUL2
LHON	2011	scAAV2-P1*ND4*v2	AAV2-tYF	smCBA		ATP1(MTS); WPRE	*ND4*	bGH	HEK293
LHON	2014	GSO10	AAV2	CMV	β-globin	COX10(MTS)	*ND4*	3'COX10	HEK293
Stargardt	2011	SAR422459	EIAV	CMV			*ABCA4*	SIN-LTR	HEK293
CHM	2011	AAV2.*REP1*	AAV2	CAG	β-globin$^{SD/SA}$	WPRE	*CHM*	bGH	HEK293
CHM	2015	AAV2.*REP1*	AAV2	CAG	β-globin		*CHM*	bGH	HEK293
RP	2011	AAV2.*MERTK*	AAV2	hVMD2	SV40$^{SD/SA}$		*MERTK*	SV40. bGH	HEK293
Usher	2012	UshStat	EIAV	CMV		WPRE	*MYO7A*	SIN-LTR	HEK293
Usher	2018	QR-421a				AON-USH2A			Synthetic
LCA	2019	EDIT-101	AAV5	U6; hGRK1	SV40$^{SD/SA}$	gRNA-CEP290	*SaCas9*	Synthetic	HEK293
LCA	2019	AAV5.*GUCY2D*	AAV5	hGRK1	SV40$^{SD/SA}$		*GUCY2D*	bGH	HeLaS3
XLR	2015	AAV2-tYF.*RS1*	AAV2-tYF	smCB	β-globin$^{SD/SA}$	WPRE	*RS1*	SV40	rHSV/sBHK
XLR	2017	scAAV8-*RS1*	AAV8	hRS1	RS1	IRBP enhancer	*RS1*	Human β-globin	HEK293
ACHM	2015	AAV2-tYF.*CNGB3*	AAV2-tYF	PR1.7	SV40$^{SD/SA}$		*CNGB3*	SV40	rHSV/sBHK
ACHM	2015	AAV.*CNGA3*	AAV8	hCAR		WPREm	*CNGA3*	bGH	?
ACHM	2016	AAV8.*CNGA3*	AAV8	hG1.7			*CNGA3*	SV40	HEK293
ACHM	2016	AAV8.*CNGB3*	AAV8	hCAR			*CNGB3*	SV40	HEK293
ACHM	2019	AGTC-402	AAV2-tYF	PR1.7	SV40$^{SD/SA}$		*CNGA3*	SV40	rHSV/sBHK
RP	2017	AAV8.*RPGR*	AAV8	hGRK1			*RPGRco-ORF15*	bGH	HEK293
RP	2017	AAV-*RPGR*	AAV5	hGRK1	SV40$^{SD/SA}$		*RPGRco-ORF15-Long*	SV40	HEK293
RP	2017	AGTC-501	AAV2-tYF	hGRK1	SV40$^{SD/SA}$		*RPGRco-ORF15*	SV40	rHSV/sBHK
RP	2017	RST-001	AAV2	CAG	β-globin$^{SD/SA}$	WPRE	*Chop2/ChR2*	bGH	HEK293
RP	2017	GS030	AAV2-7m8	CAG			*ChrimsonR-tdT*	bGH	?
RP	2020	BSO1	AAV?	?			*Chr90-FP*	?	?
RP	2017	AAV5.*PDE6B*	AAV5	hGRK1			*PED6B*	bGH	HEK293
RP	2017	CPK850	scAAV8	sRLBP1	mSV40$^{SD/SA}$		*RLBP1*	SV40	HEK293

Ordered on registration date (year) and disease. Full description, size (bp) of elements, and citations can be found in Table S1.

The regulatory element Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) was included in 8 products (RST-001; scAAV2-*P1ND4v2*; GT005; rAAV2-*REP1*; RetinoStat; rAAV.*hCNGA3*; UshStat; rAAV2tYF-CB-*hRS1*; See also section 3.6).

The main choice for polyadenylation (polyA) sequences are the effective bovine growth hormone (bGH) and the late SV40 polyA sequences (Figure 3C). New short (synthetic)

polyadenylation sequences are needed to allow CRISPR/*Cas9* constructs to fit in a single rAAV such as in the clinical trial product of Allergan / Editas Medicine Inc to correct the *CEP290* gene in patients (product: AGN-151587 / EDIT-101). We and others have employed a (modified) synthetic polyadenylation sequence [37–39]. Many clinical trial initiators exist today, with some companies acquiring efficient new potential therapies such as MeiraGTx and HORAMA (Figure 3D). A list of the pro-viral plasmids of clinical trials can be found in Table S1. Most rAAVs were produced in HEK293(T) cells without the use of helper viruses except for the products tgAAG76 (B50 cell line and helper adenovirus; [40]), rAAV2/2-*ND4* (HEK293 infected by HSV1-rc/ΔUL2; [41]), rAAV2tYF-CB-hRS1/rAAV2tYF-PR1.7-*hCNGB3*/rAAV2tYF-GRK1-*RPGR* (sBHK cells infected with rHSV; [42–44]), and ADVM-022 (Baculovirus Sf9; [42]). The choice of the production cell line might influence the tropism and potency of the rAAV vector. rAAV capsids have post-translational modifications such as glycosylations that depend on the species origin of the production cell [45]. Further, rAAVs produced in a human cell line (HEK293T) compared to baculovirus-*Sf9* produced rAAVs were more potent in transfecting the liver in mice *in vivo* and *in vitro* (HEK293T, Huh7, hiPSCs, primary human fibroblasts, mouse C2C12 cells). A more detailed description of pro-viral plasmids for the production of rAAVs for clinical trials is needed to move towards safer plasmids (more information in section 4.4.1: Production and Table S1).

3. Discovery of cell-specific promoters for ocular gene therapy

3.1. Core promoters and chromatin-association of rAAV-vectors

Eukaryotic RNA-polymerase II-dependent promoters consist of a core promoter and cis-acting regulatory elements that can include enhancers and silencer motifs [46]. In humans, the cis-acting regulatory domains and core promoters frequently contain cytosine-phosphate-guanosine islands (C:G ratio >60% for >200 bp). Recent studies indicated that the reduction of CpG islands in rAAV vectors increased transgene expression and reduced TLR9-mediated innate immune detection [47,48].

Minimal/core promoters require a transcription start site (TSS), a sequence motif for general transcription factors (e.g., TATA-binding protein or TFIIB) directing the binding of the RNA-polymerase II (e.g., ~35-bp upstream positioned TATA/CAAT/GC-box sequence) [46]. Many genes have more than only one TSS that are differentially active in tissue and at various developmental stages. Core promoters of strong ubiquitous promoters (e.g., core CMV [30 bp], SV40mini [106 bp], SCP3 [81 bp]. Table 2) can be linked with cell-specific enhancers and suppressors to generate strong cell-specific promoters [49–51].

Chromatin-modifying proteins have been extensively studied but are viewed as less relevant for rAAV vectors because rAAV vector DNA resides mainly in a concatemeric-episomal conformation in the nucleus, therefore potentially less regulated by epigenetics [23,52]. But chromatin immunoprecipitation did pull down rAAV concatemer vectors after treatment with

histone deacetylase inhibitor FR901228 of rAAV infected cells suggesting that rAAV might interact with histone-associated chromatin [53]. Also, viral DNA in minichromosomal confirmation has an identical density to chromosomal DNA indicative that chromatin-modifying factors might play a role for rAAV vectors [54]. Besides, the knockdown of chromatin assembly factor 1 increased rAAV transduction in HeLa cells [55]. Further studies need to address what kind of roles the chromatin-modifying factors have on naked DNA rAAV vectors in minichromosomal conformations in the nucleus. It is also interesting to point out that part of the infectious rAAV viral particles may redistribute to neighboring cells and may remain long term in the cytoplasm and nucleus [56].

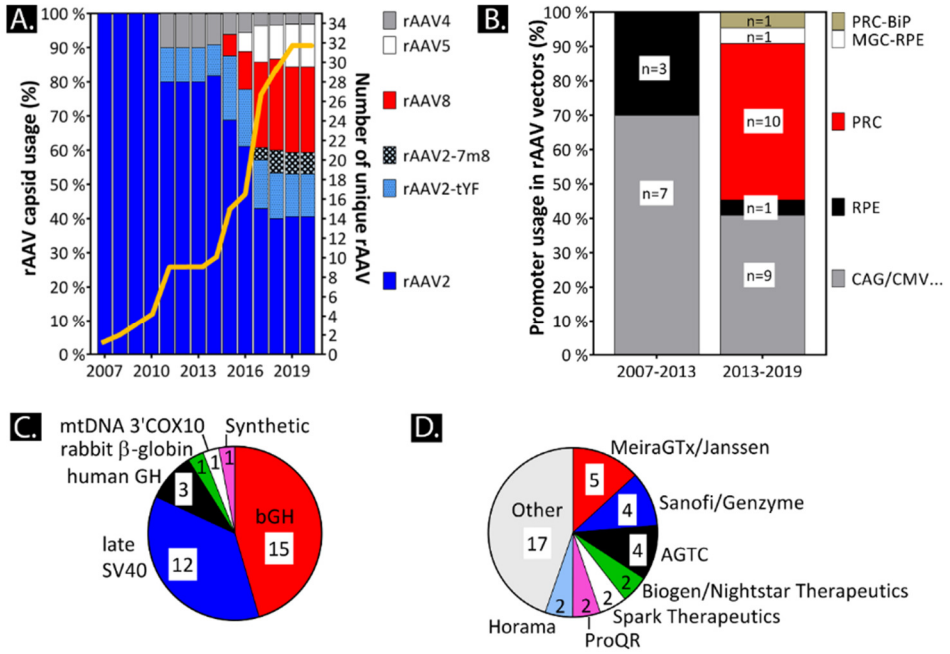

Figure 3. Development of rAAV therapies over the years. (A) Unique rAAV capsids usage over time (%; y-axis left) and unique rAAV treatments (genes) in clinical trials (number; y-axis right; Total=32 of 56 rAAV total clinical trials for the retina). (B) Unique rAAV treatments (genes) and their promoters for the retina (2007-2013 vs 2013-2019). (C) Unique rAAV treatments (genes) and their polyadenylation sequences in the retina (Total=28;). (D) Sponsors with unique rAAV, Lentiviral vector, and AON treatments in clinical trials for the retina/choroid (genes; %; Total=38). bGH, bovine growth hormone; CAG/CMV, ubiquitous promoters; late SV40, late Simian Virus; MG, Müller glial cell; RPE, retinal pigment epithelium; PRC-BiP, photoreceptor-bipolar-specific promoter; PRC, photoreceptor-specific promoter; rb β-globin, rabbit β-globin polyadenylation sequence.

3.2. Ubiquitous promoters in rAAV-vectors

Most of the promoters used in rAAV vectors are unidirectional ubiquitous promoters such as the cytomegalovirus early enhancer/cytomegalovirus promoter (CMV), the minimal CMV promoter (~300 bp), the cytomegalovirus early enhancer/chicken β-actin promoter (CBA aka CB7; 800 bp), the cytomegalovirus immediate-early enhancer/chicken β-actin

promoter/rabbit β-globin intron (CAG or CAGGS or CBA), the human phosphoglycerate kinase (PGK) promoter, and the elongation factor-1 alpha (EF-1α) promoter [57–61]. Ubiquitous promoters transcribe relatively stable expression of transgenes. The CAG, CMV, and CBA promoters outperform the EF-1α and PGK in total expression in the retina [60]. Smaller derivatives of the promoters have been developed with comparable expression patterns in some but not all tissues, such as the CMV/CBA-derivative CMV early enhancer with the chicken β-actin promoter with a chimeric chicken β-actin minute virus of mice (MVM) viral capsid protein (VP1) intron (CBh; ~800 bp), the CBA-derived CMV early enhancer with the chicken β-actin promoter and a truncated SV40 late 16S intron (CBA aka CB7, ~800 bp), and the minimal CMV promoter (~260 bp [24]; see Table 2). However, several ubiquitous promoters are silenced in specific cell types and tissues. For example, the CMV promoter had a sharp onset of expression but was silenced compared to the CBh promoter over ten weeks when expressed in the hippocampus, the spinal cord, or the substantia nigra. In contrast, the CMV promoter was not silenced in the striatum [60,62–64]. The role of CMV cis-regulatory silencing in the retina is less established. Administration of CMV.*eGFP* DNA incorporated into nanoparticles showed robust expression in the retina after two days, but the expression was not detectable after two weeks [65]. We have found protein expression (GFP, CRB1, or CRB2) in photoreceptors after one to three months of subretinal or intravitreal injection of rAAV9-CMV(min) or ShH10-Y445F.CMV(min) vectors in wild-type and *CRB1*-related retinitis pigmentosa mouse models [24,66]. *SpCas9* was also detected in retinal flat mounts in mice two weeks post-subretinal injection of the rAAV-CMV.*spCas9* [67]. Similarly, the expression of GFP was detected after two to four weeks in human iPSC-derived retinal organoids transduced with AAV5-, AAV9- or ShH10-Y445F.CMV.*eGFP* vector [68]. The studies indicate that the ubiquitous CBA and CMV promoters are most likely less affected by the retinal disease state or cellular differentiation status [36].

3.3. Bicistronic and tricistronic promoters in rAAV-vectors

Expression of two or more genes in a gene therapy vector can be achieved with an Internal Ribosomal Entry Sequence (IRES; non-read through linker), or otherwise, at least two different promoters could be used. The promoters could drive the expression of multiple genes with a fusion protein linker (e.g. $(Gly_4\ Ser)_2$ spacers (~30 bp; read through linker), or a sequence encoding self-cleaving peptides (T2A, P2A, E2A, F2A; ~30-75 bp; read-through linkers) between the gene sequences. Fusion proteins can, however, alter the function of some of the proteins. The cleavage efficiency of genes connected with self-cleaving peptide sequences varies. Also, self-cleaving peptide sequences add additional amino acids that stay attached to the protein product. Also, several genes from one promoter connected with a linker generally reduce the expression of each subsequent gene. Nevertheless, researchers demonstrated the feasibility to mediate expression by a single promoter of three different genes (for example, *Oct4*, *Sox2*, and *Klf4*) connected with self-cleaving peptide sequences in

a rAAV9 expression vector upon transduction of ganglion cells. The rAAV9-*Oct4*/*Sox2*/*Klf4* expression vector rescued ganglion cell survival in an optic nerve crush mouse model [77].

The interspersing IRES (572 bp) or minimum IRES (436 bp) allows efficient expression of independent genes into cap-independent RNA transcripts [78]. Yet, studies indicate a decrease in protein production of the protein-coding DNA located behind the IRES compared to the use of a conventional promoter. Adding a spacer (~30-90 bp) in the inter-cistronic sequence can enhance the IRES-dependent translation of the second gene [79,80]. Many different IRES exist that have been extracted from different viruses, such as in the family of the picoronaviridae. Placing two promoters in opposing directions next to each other also allows efficient bicistronic gene expression from one gene cassette [81]. Bi- or tricistronic rAAV gene cassettes are especially useful where the protein of interest (e.g., *Cre*-recombinase) is expressed in a specific cell type together with an internal marker (e.g., for reporter gene assays), or when studying retinal circuits (e.g., by calcium imaging), or when performing rAAV-retrograde labeling [58,82–85].

Bicistronic rAAV gene cassettes hold the key to supplement a wild-type transgene and removing disease-causing variant proteins in an all-in-one rAAV vector therapy. For example, in autosomal recessive retinal disease, ocular gene augmentation therapies express a functional gene in retinal cells that lack a functional copy of that same gene. However, in autosomal dominant (e.g., rhodopsin) or X-linked dominant (e.g., some variations in *RPGR*) retinal diseases, the allele bearing the dominant-negative variation needs to be specifically inactivated. Such inactivation can be achieved, for example, by gene editing or small interfering RNA to allow gene augmentation therapy to work. In the latter case, to prevent inhibition of the newly introduced gene, codon-optimization of the transgene might prevent inactivation by the gene-editing or siRNA tools used [86]. Here, an rAAV vector expressing a wild-type *RPGR* transgene and downregulation of the mutant *RPGR* transgene could benefit patients.

Similarly, many inherited retinal diseases benefit from the administration of cell survival factors [87–91]. The expression of cell survival factors, such as the basic fibroblast growth factor (*bFGF*; 470 bp), ciliary neurotrophic factor (*CNTF*; 600 bp), glial cell line-derived neurotrophic factor (*GDNF*; 511 bp), and brain-derived neurotrophic factor (*BDNF*; 750 bp) could be expressed concomitantly with the gene-of-interest boosting the treatment effect. Combining a gene supplementation therapy with a supporting factor expressed from one rAAV vector is very promising for future treatments.

3.4. Retina-specific promoters

The retina and surrounding tissue consist of several different cell types including bipolar cells, ganglion cells, horizontal cells, amacrine cells, cone photoreceptor cells, rod photoreceptor cells, Müller glial cells, RPE cells, vasculature cells (pericytes, endothelial

cells, smooth muscle cells, fibroblasts), immune-related cells (microglia, macrophages, dendritic cells), oligodendrocytes around the optic nerve, lens cells and the ciliary body (ciliary nerves, ciliary muscles, ciliary ganglion). Tissue-specific promoters restrict the expression to the specific cell type(s) and, therefore, potentially increase the safety of the product. Also, a native promoter of a gene of interest with all the essential promoter elements, enhancers, and silencers might allow for a more normalized expression. The use of these native occurring regulatory sequences may actively modulate transcription and thereby preventing overexpression. A native promoter could, therefore, potentially reduce toxicity due to overexpression of the transgene. Cellular toxicity can, for example, be observed in rAAV shRNA overexpression studies in which ubiquitous promoters were used that caused saturation of cellular miRNA pathways [92].

Table 2 Common ubiquitous promoters for rAAV-based ocular gene therapies.

Ubiquitous promoters	Size (bp)	Origin, cell expression, strength	References
CAGGS aka CBA or CAG	1,600	Ubiquitous, +++. Cytomegalovirus immediate-early enhancer, chicken β-actin promoter, chimera between introns from chicken β-actin and rabbit β-globin. pDRIVE CAG plasmid (Invivogen, San Diego, Calif.; having 100% sequence homology with the pCAGGS). The University of Pennsylvania considers CBA and CAGGS the same.	[69]
mini CAG (SV40 Intron)	800	Ubiquitous, +++	[70]
Mini CAG no intron	250	chicken β-actin promoter, Ubiquitous, +	[63]
CBA/CB7	800	Ubiquitous, ++	[71]
smCBA	953	Ubiquitous, ?	[72]
CBh	800	CBA.MVM Ubiquitous, ++	[60,73]
MeCP2	229	ubiquitous	[74]
CMV	800	Ubiquitous, ++, prone to silencing	[60]
shCMV	220	Ubiquitous, ++	[24]
CMVd2	52	Low basal activity. Ubiquitous, Promega, +	[75] Cat.: pFN23A Halo Tag CMV d2
core CMV	30	Not active without enhancers	[49]
SV40mini	106	SV40 minimal promoter	[49,50]
SCP3	81	Super core promoter. (TATA box, Inr, MTE and DPE)	[49]
EF1-α	2500	Ubiquitous, ++	[57,76]
PGK	426	Ubiquitous, ++	[59]
UbC	403	Ubiquitous, ++	[76]

The relative strength (+ being the weakest and +++ being the strongest). Adapted from [63].

Many retinal cell-type-specific promoters have been developed (Table 3). The selection and validation of tissue-specific promoters can be complicated and time-consuming. Many tissue-specific promoters in mice turned out to be less specific in human or non-human primates [51]. Further, many tissue-specific promoters drive much lower gene expression compared to the CBA/CMV/CAG ubiquitous promoters. Nevertheless, many tissue-specific promoters are very potent: NA65p (RPE cells), Nefh (ganglion cells), hGRK1 (rod and cone photoreceptor cells), hRLBP1 (Müller glial cells and RPE cells) and others [24,36,93–95]. For an extensive list, see Table 3. Codon-optimization, introns (e.g., MVM, SV40), and enhancers (e.g., CMVe, IRBPe, Grm6e) to tissue-specific promoters can substantially increase their potency. For example, the NA65p promoter is derived from the hRPE65p but now has a 150x higher potency than the CBA and 300x higher potency than the hRPE65 promoters (clinical trial NCT02946879) [36]. Many viral promoters evolved to maximize the survival of the virus in different cellular contexts. Several viral promoters exhibit ubiquitous expression in many cell types in various cell "states" (stressed, developmental state, cell cycling) or in animal tissues, indicating that the promoter activity is less affected by (temporal) cellular-specific transcriptional factor changes compared to native promoters. Adding viral enhancers to native promoters might decrease the susceptibility to gene silencing of the rAAV gene cassette. Nevertheless, the testing of synthetic and native promoters needs to be closely monitored on disease-related and species-related cell profiles.

Still, many tissue-specific promoters are too large to fit into rAAV vectors. Fitting depends on the size of the gene of interest, which is why many promoters are further shortened and optimized for cell-type-specific expression. We reduced the length of a Müller glial cell-specific CD44 promoter from 1775 bp to 363 bp but then abandoned the shortened CD44 promoter because of a substantial loss of expression *in vivo* [24,96]. The full-length glial fibrillary acidic protein (GFAP) promoter (2789 bp) showed excellent Müller glial specific gene expression in human retinal organoids and human retinal explants [24,68]. Furthermore, a shortened version of the GFAP promoter called gfaABC1D (686 bp) showed similar expression strength in neurons (brain), whereas the gfaABC1D promoter maintained Müller glial cell-specific expression in the retina [97,98].

3.5. Small nuclear RNA (snRNA) promoters

RNA polymerase (RNAP)-dependent regulatory promoters (U1, U2, U6, U7, H1; ~250 bp) can be used to drive short hairpin RNAs (shRNAs). The human U6 promoter is a potent promoter that has been widely used for the expression of shRNAs. However, the relatively large size, the requirement that the transcript starts with a G or A, the sometimes too active transcription, and the sensitivity to specific cellular profiles make the human U6 promoter a less versatile promoter [131–133]. RNA polymerase III promoters also have been re-engineered with CMV enhancers [134] or tissue-specific enhancers (heart, muscle) for siRNA expression [135]. The tissue-specific enhancers increased expression but were less

tissue-specific. Single guide RNAs (gRNA) are typically expressed by a U3 or U6 RNA promoter in rAAV gene cassettes. Relative tissue-specific expression of two gRNAs for CRISPR/Cas gene editing in myotubes was achieved by linking a muscle-specific MHCK7 promoter (pol II) with gRNA-linked self-cleaving ribozyme sequences derived of Hepatitis delta virus (HDV) and a Hammerhead (HH) sequence [135–138].

3.6. WPRE, introns, miRNAs and other elements in an rAAV-gene cassette

Post-transcriptional regulatory elements (PRE) can substantially increase gene expression. Woodchuck posttranscriptional regulatory element (WPRE; 600 bp) or Hepatitis B Virus Posttranscriptional Regulatory Element (HPRE; 533 bp) increase the transgene expression up to 6-9 times [63]. The addition of a WPRE also protects from silencing in human ES cells and the brain. To validate the use of WPRE for retinal gene therapy, rAAV2/2.CMV.*eGFP*.pA vectors with or without WPRE were applied to human retinal explants or injected in mouse eyes [139]. A shorter version of the WPRE (WPRE3; 247 bp) showed only a 15% drop in expression in hippocampal neuron cultures or GFP expression in rAAV infected hippocampal CA1 region in the mouse brain [140]. A modified WPRE version that removed any viral protein expression has been patented for retinal use [141]. WPRE might be redundant if used in combination with a promoter containing introns such as found in the CAG or EF-1α promoters [81,142]. Inclusion of natively occurring or synthetic introns can strongly boost protein expression, especially for vectors with low efficiency of gene splitting sites [143]. Many introns have been developed for rAAV gene cassettes that can enhance the gene expression (Table 4). Especially, the strong MVM intron-1 of the viral capsid protein (*VP-1*) of only 67-to-97 bp can increase the transcript expression by 10x [144]. Also, the development of minicircle rAAVs has contributed to novel introns that are placed in the backbone of pro-viral plasmids to boost production yield. This strategy will be discussed further under section production [145].

Adding microRNAs (miRNA, ~18-25 bp) can alternatively be used to prevent the ectopic expression of the transgene in ocular gene therapy. Adding 4x the complementary sequence of miRNA204 to a rAAV2/5.CMV.*eGFP*.WPRE.*4xmiRNA204T* significantly reduced eGFP expression in RPE cells after subretinal injection in mice and pigs. Similarly, adding 4x the complementary sequence of miR-124 removed the expression in photoreceptors [146]. Also, a dual-acting rAAV2/5 vector expressed the miRNA (5, B, 7), against Vascular endothelial growth factor A (VEGFA) and antiangiogenic protein pigment endothelial-derived factor (PEDF) driven by an RPE-specific Bestrophin 1 (VMD2) promoter, to suppress choroidal neovascularization in a wet-AMD mouse model [147]. However, the oversaturation of the cognate miRNA needs to be considered when using miRNAs, because they can decrease the function of native miRNAs in the cell. 4x miRNA placed in an rAAV-CMV expression cassette generally is sufficient for miRNA expression without inducing side-effects [146]. Others have used miRNAs to inhibit transgene expression in antigen-presenting cells (APCs)

with miR-142-3p [148]. Still, short hairpin DNA sequences need to be placed at least proximal to the second ITR and be tested for possible rAAV genome truncation for proper expression of the short hairpin RNA (shRNA). Short-hairpin DNA can effectively truncate rAAV genomes during production and produce non-intact shRNA expression cassettes [149]. A more detailed review of miRNAs can be found here [150]. Moreover a rAAV vector-based microRNA (miRNA) sensor array (*Asensor*) has been developed [151]. The *Asensor* is based on the principle that rAAV vectors are relatively stable at +4°C so that 96-well plates can be coated with live rAAV vectors. Then, the plate is loaded with the cells of interest that are infected by the rAAV vector-based microRNA (miRNA) sensor that starts to express luciferase. The miRNA activity is subsequently measured on a microplate luminometer for high-throughput microRNA profiling.

3.7. Polyadenylation sequences in rAAV-gene cassette

To allow for efficient pre-mRNA processing, an efficient polyadenylation sequence needs to be included behind the transgene to form a proper poly(A) tail at the RNA's 3' end. Polyadenylation sequences in rAAVs gene cassettes are for example SV40 late (135 bp; +++), bGHpolyA (250 bp; ++), synthetic polyadenylation (spA) + 2x SV40 late upstream elements (100 bp, ++), +), 2x sNRP1 (34 bp, +/++), synthetic polyA (spA; 49-60 bp, +), hGHpolyA (624 bp, +), 1x sNRP1 (17 bp, +), and adenovirus L3 (21 bp, +) polyadenylation sites [63,167] (Table 5).

Recent developments allow for shorter and more potent expression cassettes. The SV40 late polyadenylation signal upstream element and the SV40 late polyadenylation signal combined with the WPRE3 (420 bp), decrease the length to less than half compared to the commonly used WPRE-bGHpolyA gene cassette (919 bp) but maintain a similar expression profile [140]. The removal of a WPRE sequence reduced the expression by 80%. But using a synthetic polyadenylation sequence (49 bp) + 2x SV40 late upstream elements (50 bp), increased the GFP expression compared to the use of a robust bGHpolyA sequence. Interestingly, the interplay of the polyadenylation sequence with transcriptional regulation enhancers can increase transcript levels, such as paring a CMVβ enhancer with an SV40 polyA. But the effect was lost when the CMVβ enhancer was paired with a bGHpolyA [152]. Also, the rAAV gene cassette for hemophilia B was tested with different polyadenylation sequences. The bGHpolyA was the strongest for the *FIX* gene expression, outperforming the synthetic polyA, mouse β-globin pA, rabbit β-globin pA, and H4-based pA [144]. Studying polyadenylation sequences can be very valuable for rAAV gene cassette size reduction. Notably, a 17-bp soluble neuropilin-1 (sNRP-1) polyA sequence efficiently expressed transgenes on infection of an rAAV vector. When the sequence was used twice (2x sNRP-1 polyA), then the potency was as efficient as an SV40polyA sequence [167,168]. Yet, the 2x sNRP-1 polyA was less suitable for specific transcripts compared to bGHpolyA or spA [169]. The effects of polyadenylation sequences for specific transcripts are still less well

understood. For example, whereas polyA's increase transcript stability/expression, certain polyadenylation sequences can also reduce viral titers during rAAV particle production [170]. Thus, different polyadenylation sequences should be tested for optimal gene expression and virus production.

3.8. rAAV vector cassettes and inducible promoters

Many gene supplementation therapies rely on constant overexpression of the therapeutic gene. The constant active expression increases the risk that the rescue vector itself becomes toxic to the cell. Stress (GFAP promoter) or hypoxia-driven GFAP promoter (HRSE-6xHRE-GfaABC1D) have been generated that might be safer for cells that are sensitive to continuous overexpressed artificial gene vectors [97,98,100,101,103]. Other inducible On/Off gene expression systems have been described: Tetracycline (Ptet), dihydrofolate reductase (DHFR) protein destabilizing domains, riboswitches, metal activated promoters (metallothionine-Ia; MT-1), and hormone-activated promoters (dexamethasone, MMTV LTR. Table 6) [175–178]. All but the riboswitches require the expression of an exogenous

Table 3 Retina cell-specific promoters in rAAVs for ocular gene therapy

Müller glial cells	**Size (bp)**	**Origin, cell expression, strength**	**References**
CHX10	164	Retinal progenitor cells	[99]
GFAP	2600	Müller glial cells,	[100,101]
GFAP	2200	Müller glial cells (Novartis)	[102]
GfaABC1D	686	Müller glial cells	[97,98]
HRSE-6xHRE-GfaABC1D	~820	Hypoxia-induced reactive MGC promoter. HRE is (A/G)CGT(G/C)C. HRSE from metallothionein II promoter (90 bps)	[98,103]
RLBP1	2789	Müller glial cells	[24,93]
Short RLBP1	581	Müller glial cells	[102]
Murine CD44	1775	Müller glial cells	[24,96]
Murine shCD44	363	Müller glial cells	[24,104]
ProB2	592	Müller glial cells	[51]
Photoreceptor cells	**Size (bp)**	**Origin, cell expression, strength**	**References**
Mouse RHO	1400	Rod PRCs	[105]
Human RHO (rhodopsin)	800	Rod PRCs	[106]
Human RHO	520	Rod- PRCs	[24]
Mouse rod opsin mOp500	500	Rod- PRCs –385/+86	[107]
Mouse rod opsin	221	Rod- PRCs	[108]
Human Rhodopsin kinase (RHOK/GRK1)	294	Rod and cone PRCs. AY327580.1: bp 1,793–2,087 (-112 to +180). More efficient than IRBP in NHP for cone transduction	[24,95,109–111]
Human blue opsin HB570	570	S-cone and subset of M-cones PRCs	[112]
Human blue opsin HB569	569	blue cone opsin PRCs	[107,113]
PR0.5	496	Red cone PRCs	[107]
PR1.7	1700	Red cone PRCs	[107]
PR2.1	2,100	Red cone PRCs	[107]
3LCR-PR0.5	~600	Red cone PRCs	[107]
Mouse blue opsin (mBP500)	500	Mouse S opsin	[114]
Human interphotoreceptor retinoid binding protein (hIRBP)	235	Cone & rod PRCs X53044.1, bp 2,603–2,837	[115]

IRBPe/GNAT2	500	Cone PRCs	[116]
Mouse CAR / ARR3	500	Cone PRCs, some rods, and RPE	[116]
Human CAR / ARR3	500	Cone PRCs, some rods, and RPE cells	[116]
CAR / ARR3	215	Cone PRC	[117]
Human red opsin	2,100	Human red cone opsin	[118]
Human green red opsin (G1.7p)	1700	Cone PRCs. Core green opsin promoter including a mutation (0.5 kb) + Locus Control Region (LCR; 1.2 kb) upstream of the red opsin gene	[119–121]
Crx2kb	2000	Cone & rod PRCs	[122]
ProA1	2000	cone PRCs	[51]
ProA4	2000	cone PRCs	[51]
ProC1	731	Cone & rod PRCs	[51]
ProA6,ProB5,ProC22, ProC32,ProD2,ProD3, ProD4,ProD5,ProD6	1229,619 774, 814, 366, 691, 552, 321, 448	rod PRCs	[51]
Synp161	150	Mouse CD47 enhancer + SV40-mini promoter. Rod PRCs	[50]
Bipolar cells	**Size (bp)**	**Origin, cell expression, strength**	**References**
Mouse metabotropic glutamate receptor 6 (mGrm6)	200	On-bipolar cells	[99]
4x mGRM6e+SV40	1,000	On-bipolar cells. 203 bp SV40 minimal promoter	[123]
Grm6e-Chx10-Cabp5	809	200 bp Grm6 + 164 bps Chx10 enhancer + 445 bp Cabp5 promoter. Wide overlapping bipolar expression	[99]
Grm6-SV40	400	Grm6=mGluR6. 200 bp mGluR6 enhancer + SV40 promoter. On-Bipolar cells	[99]
Cabp5	445	Bipolar cells	[99]
Chx10-SV40	364	164 bp Chx10 enhancer + 200 bp SV40 promoter. Bipolar cells and Müller Glial cells	[99]
Grm6-mGluR500P	700	On-bipolar cells.	[124]
In4s-In3e- Grm6-mGluR500P	1997	690 bp shortend Intron 4s + 807 bp Intron 3 + 500 bp mGluR500P	[124]
ProB4	1317	Off-bipolar cells	[51]
Amacrine cells	**Size (bp)**	**Origin, cell expression, strength**	**References**
ProC2	964	All amacrine cells + few MGCs	[51]
ProB1	394	Amacrines with processes in one stratum	[51]
Horizontal cells	**Size (bp)**	**Origin, cell expression, strength**	**References**
ProC3	694	Some off-target in amacrine and ganglion cells	[51]
Retinal Ganglion cells	**Size (bp)**	**Origin, cell expression, strength**	**References**
Syn1	495	Off target amacrine, strength: ++	[125]
Nefh	2251	Strength: +++	[94]
hSNCGp	948	Human SNCG promoter (–785 to +163 region)	[126]
ProA3	2000	Synthetic	[51]
Ple344	801	Gene TUBB3. GCL & corneal nerves. ++	[127]
Ple345	2693	Gene NEFL. +++ (stronger than smCBA)	[127]
RPE	**Size (bp)**	**Origin, cell expression, strength**	**References**
hRPE65p	1383	Chr1.68449936-68451318. RPE+ some PRC infection	[128]
NA65p	1383	Codon optimized hRPE65p+SV40 intron+Kozak seq, 150x more efficient than CBA and 300x more efficient than hRPE65p	[36]
VMD2	646	NG_009033.1, bp 4,870–5,516	[126,129]
Synpiii	1317	+ SV40 mini promoter	[130]

The relative strength (+ being the weakest and +++ being the strongest). Adapted from [63].

Table 4 Other elements in rAAV vectors: A. Introns, PRE, and enhancers. B. Miscellaneous

Introns & PRE & enhancers	Size	Description, strength	References
CE (CMV early enhancer)	431	+++, 1.5-67x increase; -118/-522 TSS pCMVβ / 5'CMV enhancer	[152]
IRBPe	235	human interphotoreceptor retinoid-binding protein proximal enhancer. – 1619 to – 141 IRBP	[116]
metabotropic glutamate receptor 6 enhancer (Grm6e)	200	Grm6 proximal enhancer	[99]
Woodchuck Hepatitis Virus PRE (WPRE)	600	+++, 6-10x increase	[140,153]
Hepatitis B Virus PRE (HPRE)	533	+++, 6-10x increase	[153]
WPRE3	247	++, 6x increase	[140]
MVM	67-97	+++, minute virus of mice, 10x increase	[144]
chCMV.HBB2	~506	Chimeric CMV (146 bp) + human β-globulin intron 2 (340 bp) + exon 3 20 bp incl SA/SD	[154]
Hybrid adenovirus SD# / IgG Sa*	230	+++, pAdβ, 2x increase to synthetic polyA	[152]
SV40 late SD# / Sa* (19S/16S)	180	+, pCMVβ (Promega; 1.6x increase)	[152]
Modified SV40 SD# / Sa*	157	modSV40 SA/SD= modified SV40 splice acceptor/donor intron, 157 bp in length, nucleotides 502–561 and 1,410–1,497 of SV40 genomic sequence (NC_001669.1) + connecting sequence CGGATCCGG between two fragments.	[102,155]
Mini SV40 SD# / Sa*	100	Mini SV40 SD# / Sa* intron	[44,156,157]
Human β -globin intron 2 SD# / Sa*	875	0.5-86-fold increase. pZac2.1	[140,158–160]
F.IX truncated intron1	300	+, human factor IX (100x)	[144,161]

Miscellaneous	Size	Description	References
2A	75	Self-cleaving linker	[162]
internal ribosomal entry site (IRES)	600	Ubiquitous. Placed between two genes. The second gene is transcribed without a promoter (at a lower expression compared to the first gene)	[163]
SPTP	154	Synthetic polyA signal/transcriptional pause site frp, pGL4.25	[164]
PolII miR-155	~500	Block-iT PolII miR vector system based on miR-155 expressing artificial miRNAs engineered to a target sequence resulting in target cleavage	Cat.: K493600 ThermoFisher Scientific
shRNA-YB1	N/A	7-to-45 fold AAV production increase in physical titer	[165]
MIP backbone	N/A	mini-intronic plasmid (MIP) backbones for AAV production increased transgene expression by 40-100 fold *in vivo*	[145]
R6K	545	+ (~40x),pUC + prokaryotic RNA-OUT antibiotic-free, minicircle AAVs	[145]
OIPR	1300	+ (~40x),pUC + prokaryotic RNA-OUT antibiotic-free, minicircle AAVs	[145]
Shorter OIPR	500	+ (~5x),pUC + prokaryotic RNA-OUT antibiotic-free, minicircle AAVs	[145]

The relative strength (+ being the weakest and +++ being the strongest). Adapted from [7,63,166].

Table 5 Polyadenylation sequences

Polyadenylation	Size	Description, strength	References
SV40 late	135	+++	[140]
2x SV40 late	100	++/+++	[171]
bGHpolyA	250	++	[152]
2x sNRP1	34	+/++	[169]
Rabbit gbpA	56	Rabbit β-globin	[152]
spA	49	+/++ (7x lower than bGHpolyA, 3x lower than SV40 late)	[140,152]
hGHpolyA	624	+	[42,172,173]
1x sNRP1	17	+	[169]
HSV TK poly(A)	48	herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal. Generally used for NeoR and KanR genes	[174]
Adenovirus (L3) USE	21	+	[171]

The relative strength (+ being the weakest and +++ being the strongest). Modified from [7,63,166].

(bacterial) protein. The TET-system is activated by an antibiotic (tetracycline or doxycycline), making it suboptimal for human use. An example of an efficient TET-off rAAV system is an rAAV expression cassette that includes 6x the mutated tetracycline response elements (TRE; ~200 bp) placed in front of a minimal promoter (CMV; ~40 bp; total cistronic size: ~270 bp). The full rAAV plasmid (Addgene #35625) demonstrates the possibility of expressing it from rAAV gene cassettes. Upon rAAV infection of the cell, then the ubiquitous promoter UbC will drive the transactivator reverse tetracycline transactivator 3 (rtTA3), making it a Tet-on system. Upon Cre recombinase expression, the rtTA3 is floxed-out, rendering the plasmid to a Tet-off system. The rtTA3 binds to the TRE in the presence of doxycycline, starting the expression of the TurboRFP open reading frame (ORF) that allows tracking of the target mRNA knockdown because the miR-30 sequences induce the Drosha and Dicer processing of the expressed target sequence. The promoter drives the microRNA adapted short hairpin RNA. If the Drosha/Dicer degradation complex recognizes the target sequence specified by the shRNA, then the transcript of the target sequence and the TurboRFP transcript is degraded. The construct allows fast testing of the efficiency of shRNAs [179].

Riboswitches have gained considerable attention for rAAV ocular therapies because of their small size (100 bp cis-acting RNA sequence), adaptability to ligands, and the development of synthetic riboswitches [175,180,181]. The riboswitch is encoded downstream of the polyadenylation sequence in the rAAV-vector. It encodes for a ligand-sensing aptamer, a communication module (linker), and an effector domain (ribozyme) that depending on the presence of the ligand, cleaves the mRNA of the gene expression cassette. A *proof-of-concept-study* for anti-VEGF expression by the activating ligand tetracycline in a wet AMD mouse model demonstrated the feasibility of the riboswitch in ocular gene therapy [181].

Table 6 Inducible promoters

Inducible promoters	Size (bp)	Origin, cell expression, strength	References
MT-1	13200	Zinc, cadmium or copper-inducible sheep metallothionine-Ia promoter	[176]
MMTV LTR	792	dexamethasone (Dex)-inducible mouse mammary tumor virus. Active when glucocorticoids or progestins present	[177,178]
Ptet	270	tetracycline On or Off system promoters (Ptet). 6x mutated TRE (~200 bp) core CMV (~40 bps)	[179]
T7lac	42	T7 bacteriophage promoter (17 bp) requires T7 RNA polymerase and lac operator (25 bp). Induces expression by IPTG	[182]
Riboswitches	~100	ligand-sensing aptamer, a communication module (linker), and an effector domain (ribozyme)	[175,180,181]

Adapted from [63]

4. Optimizing genes for rAAV vector therapies (minigenes, dual/triple rAAV-vector, ITRs)

4.1. Intron removal, exon removal and finding surrogates for large genes

rAAV gene cassettes only allow expression of small genes because of the limited packaging size of around 4.5-4.6 kb (excluding the two ITRs). It has been challenging to fit large genes into rAAV vectors. Introns and sometimes exons are truncated to allow proper packaging. Thus, many large cDNAs in AAV gene cassettes do not contain any or contain only a few native introns (see Table 1, and Table S1). Introns can serve many functions, for example, to increase mRNA stability, modulate RNA synthesis rate, introduce alternative splicing, and decrease DNA damage on highly expressed genes. Also, an intron can dictate the mRNA export mechanism from the nucleus to the endoplasmic reticulum [183,184]. Removal of introns in rAAV gene cassettes might further alter the intrinsic gene regulation apart from artificial promoters and rAAV expression system. For example, the CRB1 protein (UniProtKB P82279-1) consists of 1406 amino acids and is encoded on 210,251 bp (GRCh37 197,237,334-197,447,585. ENSG00000134376). The exon-coding sequence alone is 4221 bp, including the 3' stop codon, whose size is close to the maximum packaging capacity of the rAAV. Nevertheless, we achieved an efficient expression of CRB1 in retinal cell types by making use of short promoters (<300 bp) and 50 bp synthetic polyadenylation sequences [66].

Shortened versions of proteins (exon truncation) are generally not advised because many shortened proteins lose their functionality. For example, many shortened versions of the Duchenne muscular dystrophy gene (*DMD*; 2.3 Mb; 79-exons; 3685 amino acids; 11,055 bp) do not rescue the Duchenne muscular dystrophy phenotype in muscle cells except for the micro-dystrophin for which was reported a milder clinical phenotype [185,186]. Nevertheless, the micro-dystrophin rescue in patients in clinical trials has been at least

suboptimal and needs further optimization [187]. We also tried a native occurring short version of the CRB1 protein encoded by a *CRB1* cDNA lacking exons 3 and 4, but whereas the short *CRB1* (*sCRB1*) was expressed, it also caused retinal degeneration upon subretinal injection of the AAV9-CMV-*sCRB1* or AAV9-hGRK1-*sCRB1* [24]. Instead of employing a shortened protein, one can also apply a surrogate protein such as utrophin for dystrophin [187,188]. We also developed a surrogate gene therapy for patients with *CRB1*-related retinal dystrophy by employing the Crumbs homolog 2 (*CRB2;* 3.8 kb). However, such a strategy can only be performed if the proteins of interest execute similar functions in cells, which is the case for CRB1 and CRB2 [68,189–202]. The rAAV-CMV-*CRB2* rescued the loss of CRB1 function in mouse Müller glial cells [66]. Interestingly, mice lacking CRB1 or mouse retinas lacking CRB2 develop the same phenotype as observed in human iPSC-derived retinal organoids lacking CRB1 [68]. Surrogate proteins might be less immunogenic than the native protein - like utrophin over dystrophin - because the surrogate protein is already expressed in the body. Surrogate proteins could have great potential for many rAAV gene therapies.

4.2. Lentiviral and dual/triple rAAV vectors

Retroviral 3rd generation lentivirus-based systems have a larger packaging size of ~8.5 kb compared to 4.5 kb in rAAV [203]. They can infect both dividing and nondividing cells and integrate into the genome. Equine infectious anemia virus (EIAV) 3rd generation lentivirus-based gene therapies for the *MYO7A* gene (6645 bp; 2215 amino acid) and the *ABCA4* gene (6819 bp; 2273 amino acids) delivered to photoreceptors are in clinical trials (NCT01367444; NCT01505062) since 2011/2012. Though the clinical trial ended (May/June 2019), no data has been published since the start of the trial.

Dual and triple rAAV vectors are another strategy to circumvent the small capacity of rAAVs. Nicked ITRs (ΔITRs) have been used that allow for annealing of two or three different rAAV gene cassettes. An update on dual and triple rAAV vectors can be found here [204]. A dual AAV vector system rescued the *ABCA4* gene in *Abca4* knockout mice [205,206]. The first generation of dual rAAV vector cassettes resulted in a high ratio of truncated gene expression. Adjustments, such as 200-300 bp of specific compatible overhangs, have resulted in normal concatemerization of independent gene cassettes such as the hybrid dual rAAV approach [204].

4.3. rAAV-vectors expressing CRISPR/Cas

Staphylococcus aureus CRISPR associated protein 9 (SaCas9) is an RNA-guided endonuclease enzyme associated with the CRISPR (type II prokaryotic Clustered Regularly Interspaced Short Palindromic Repeats) complex. Cas9 unwinds, checks, binds, and finally cuts in the DNA (causing a double-stranded DNA break [DBS]) complementary to the annealed 20-nucleotide genome-specific part of the single guide RNA (gRNA). The genome-specific part of the gRNA anneals proximal to the 3-bp protospacer adjacent motif (PAM). The guide RNA can be adjusted to target the whole genome as long as a PAM sequence is

found close by (for *S. aureus*: NGG). Many Cas protein homologs and orthologs have been described with the most significant ones for rAAV gene-editing cassettes being Cas9, Cas12a (Cpf1), Cpfl1, SpCas9, SaCas9 [207]. The large Cas9 (*SpCas9*, 4100 bp) or type-V Cas system (*AsCpf1*, 3921 bp; *LbCpf1*, 3684 bp) together with the gRNA cassette generally do not fit smoothly in a single rAAV gene cassette. The new generation of *SaCas9*, *CjCas9*, and *NmCas9* (2.9-3.3 bp) allows the packaging of both Cas9 and gRNA in a single AAV vector. CRISPR/Cas gene editing can inactivate the dominant-negative effect or can regulate positively or negatively the transcription of genes. However, if left active in cells, functional rAAV-CRISPR/Cas9 systems do increase the number of off-target integration events into the genome [208].

The large *SpCas9* (4100 bp) would require a dual rAAV system to incorporate all elements, including the gRNA cassette. A dual rAAVs system (rAAV.RKp.*SpCas9*; rAAV.U7.*gRNA-Nrl*) rescued vision in three mouse lines of rod retinal degeneration (*Crx-Nrl*$^{-/-}$; rd10 or *Pde6b*$^{-/-}$; *Rho*$^{-/-}$) by knocking out *Nrl* in one or both alleles [209].The *Nrl*-knockout pushed rod photoreceptors to a more cone-like state helping in the survival of the remaining photoreceptors. Similar results have been reported in a second independent study [210]. The mutant rhodopsin gene encoding a dominant-negative form of rhodopsin (*Rho*$^{P23H/P23H}$) was also silenced by gene editing in a mouse model of retinal degeneration (*Rho*$^{P23H/P23H}$) by a dual rAAV-vector administration (rAAV2/8(Y733F)-sCMV-*SpCas9*.spA and rAAV2/8(Y733F)- U6.*gRNA1gRNA2(mRho)*.mRho.h*RHO* .SV40-polyA) rescuing retinal degeneration [67].

A shorter Cas protein, *CjCas9* (2950 bp), allows expression from a single rAAV vector. Intravitreal injection of a single rAAV2/9-vector at P0 in mice efficiently downregulated angiogenesis genes (rAAV-gRNA against *Vegfa:Hif1a.CjCas9*-T2A-*GFP*) protected mice of visual loss when the mice were challenged by a laser inducing wet age-related macular degeneration at P42 causing choroidal neovascularization [211]. Their follow-up paper showed that 14-months post-injection, the *CjCas9* is still active but does not affect the retinal function as measured by electroretinogram (ERG), indicating that the therapy might be safe [212].

Cas proteins can also be altered and fused to other proteins. For example, the 3200 bp cDNA encoding a nonfunctional nuclease-activity-dead *S. aureus* ortholog Cas9 (dCas9) can be fused with a cDNA encoding a transactivation domain such as VP64 fused to the two transcription factors p65 and Rta (*dCas9-VPR*). The cDNAs encoding VP64, p65, and Rta are 150 bp, 357 bp, and 570 bp in length, respectively. Because of the relatively large size of 4277 bp of *dCas9-VPR* cDNA, the authors used a dual rAAV system to express the *dCas9-VPR* and gRNA expression cassettes [213,214]. Recently, a single rAAV expression vector has been developed driving the gRNA by a U6 promoter (360 bp) and a shortened but 3x less active *VPR* (500 bp) and *dSaCas9* (3200 bp) from an SCP1 promoter (80 bp) attached to 2x-

sNRP-1 polyA signal (34 bp). A modified rAAV-vector version with a full-length CMV promoter and the bGHpolyA efficiently upregulated a gene (*Actc1*, *Neurog2,* or *Hbb*) upon infection of N2A neuron derived cells by 50-150x *in vitro* [169]. A single rAAV vector expressing dCas9 fusion protein, as well as a sgRNA, shows excellent potential for positive or negative regulation of transcription in many genetic pathways involved in retinal diseases. Several other exciting *Cas9* gene cassettes in rAAVs will most definitely be developed. A recent review reported an rAAV-CRISPR vector that can self-inactivate its Cas9 protein by encoding an anti-Cas9 gRNA on the same construct that harbors the Cas9 itself [150]. Nevertheless, single guide RNAs (*sgRNA*) comprise short hairpin sequences that potentially cause truncation of the rAAV production similar to short-hairpin RNAs (shRNA). Placing the gRNAs close to the second ITR might increase the production yield and increase proper vector expression upon infection of the target cells [149].

4.4. Production and rAAV vector integration

4.4.1 Production: The backbones and bacterial resistance genes

Impurities in rAAV products hinder the release of pharmaceutical products but might also negatively impact the potency of the expression vector. During the rAAV production, the gene therapy vector is packaged in the rAAV capsids. However, under suboptimal conditions, the capsids do also package other sequences such as vector plasmid backbones (~3%), helper plasmids (~0.05%), and even human genome sequences (~0.15%) [215]. Increasing the size of the vector plasmid backbone to above 5 kb considerably reduced inappropriate packaging and also reduced the number of empty capsids. But larger plasmids give lower DNA plasmid yields in bacterial culture and are somewhat harder to transfect efficiently into cell lines. Jean Bennett's group enhanced safety and maximized the therapeutic effect by adding a stuffer sequence for rAAV product hRPE65v2 [40,216]. The global health agencies (FDA, EMA, WHO) also discourage the use of β-lactams (i.e., ampicillin, penicillin) and streptomycin resistance genes in plasmids for gene therapy [217]. Kanamycin and neomycin are both members of the aminoglycoside antibiotic class. These antibiotics are tolerated but switching to antibiotic-free systems or minimizing the use of antibiotics is preferred. Some researchers use, therefore, minicircle DNA vectors devoid of prokaryotic and antibiotic DNA sequences for their AAV production [218]. No differences were found for minicircle single-stranded rAAV2.*eGFP* production. For the self-complementary rAAV2.*GFP* vector, the plasmid backbone was packaged 30 times less into capsids. Also, the use of minicircle plasmids during rAAV production allowed for high transduction titers of rAAV vectors on HeLa cells [219].

Pro-viral plasmids can also include short-hairpin RNAs to downregulate host cell proteins that hinder rAAV capsid assembly during production. Downregulating the Y-box binding protein 1 (YB1) in HEK293T cells did increase the physical titer by 47x for rAAV2, yet it failed to improve the yield for rAAV5 [165].

4.4.2 Production: ITR stabilization

An ITR consists of an A-A', RBE-RBE', B-B', C-C', D, and terminal resolution site (trs) sequence (Figure 4A). The AAV Rep78 and Rep68 proteins expressed by the pHelper plasmid induce a nick on the trs site on the ITR [220]. The RBE-RBE' initiates the binding of Rep78 and Rep68 proteins, and the B-B' further stabilizes the proteins so the Rep78 and Rep68 can efficiently induce DNA replication during the rAAV production cycle [221]. The D sequence is the packaging signal, is important for the AAV replication, and can bind the double-stranded D sequence binding protein (ds-D-BP) [222–224]. When high copy numbers of a D sequence expression vector are present in HEK293 cells, then the the interferon-λ-mediated activation of the major histocompatibility complex class II (MHC-II) is dampened, potentially by the binding of the ds-D-BP protein with the D-sequence instead of the the X-box (RFX) regulator [225]. rAAV can be produced with only one ITR if the other ITR has large deletions. Also, ITR deletions can be recovered during production [223,226]. Also, replacing the 5' ITR with a U-shaped hairpin allows rAAV production and episomal concatemerization [149]. A systematical study into ITR mutations (deleting B-B and C-C' regions) indicated a reduced yield (4-8x fold) but a 4-fold increased transgene expression in HEK293 cells 72 hours post-infection [227]. The deletion of the BC region caused an ITR change from a T-shape to a U-shape hairpin (similar to short hairpin RNA). The authors postulated that the 2x34bp ITR deletions might allow larger packaging of rAAV gene cassettes.

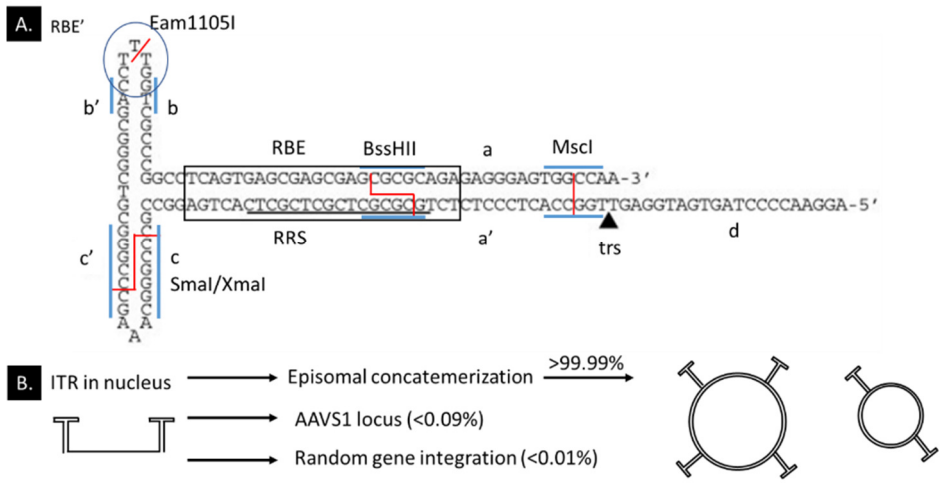

Figure 4. How to assess Inverted Terminal Repeats (ITRs) of rAAV and how they allow concatemerization. (A.) Restriction enzyme sites in the AAV serotype 2 ITR in the flop configuration. RBE'/RBE binds Rep68 (RBE, Rep-binding element) and initiates the Rep helicase. The Rep helicase nicks the trs (trs, terminal resolution site. Restriction enzyme recognition site indicated in blue and the actual cut in red. Figure adapted from [228]. (A) (B.) ITR structure in the nucleus after second-strand DNA synthesis in dividing cells favoring homologous recombination. Most rAAV-vectors form episomal concatemeric circular double-stranded DNA.

Recombinant AAV plasmids can lose/damage ITRs in many *E. coli* cells during plasmid production or rAAV production. We also noticed an almost complete loss of one ITR within one production cycle of a pro-viral plasmid in bacterial GeneHog cells (Invitrogen) (TMB & JW, unpublished data). We validated the loss in the pro-viral plasmids by restriction enzyme digestion with XmaI at the C-C', BssHII for the RBE, and Eam1105I for the RBE' region. ITR Sanger sequencing has been intricate on circular DNA. We sequenced the whole ITR by first linearizing the plasmid by Eam1105I digestion at the RBE', and then Sanger sequenced the pro-viral plasmid from both directions. The method allows us to use similar functional ITR ratios between batches (Figure 4A).

4.4.3 rAAV vector integration in the host genome

Integration of rAAV in the genome is unwanted because it might be genotoxic and lead to oncogenesis, especially in dividing cells and nontarget cells. The rAAV gene cassettes only harbor the palindromic inverted terminal repeats (ITR) of the original wild-type AAV. All other AAV wild-type sequences are lost during the rAAV production. The ITRs are part of the Long Terminal Repeats (LTRs) family. The LTRs are extensively exploited by retrotransposons or the pro-viral DNA of retroviral RNA. ITRs, similar to LTRs, are essential to allow AAV genome integration or episomal concatemerization. The ITR hairpin structures allow self-priming (primase independent synthesis of double-stranded DNA). The ITR-gene cassette stays as a monomeric episomal form in the nucleus at low multiplicity-of-infection (MOI). High MOIs, the ITRs form head-to-tail end-to-end joining, essentially making circularized DNA (>12kb). Further, the 5'LTR generally has a promoter function, and the 3'LTR can act as a termination sequence. Each of the ITRs of the AAV serotype 2 (ITR2) is only 145 bp long and lacks the promoter and termination functions (Figure 2B).

rAAVs lack viral proteins for efficient genome integration. Integration of foreign DNA (rAAV gene cassettes) into the mammalian genome is related to the amount of double-stranded DNA (dsDNA) breaks and the DNA repair pathway that is active in the cell. Integration events can be increased by increasing dsDNA breaks *in vitro* by adding intron-encoded endonuclease I-SceI, etoposide, or γ-irradiation [229]. Dividing cells favor the homologous recombination (HR) DNA repair pathway during the cell cycle S-phase that requires a DNA template to guide repair such as a viral gene cassette. However, quiescent cells, such as retinal and RPE cells, favor the nonhomologous end-joining (NHEJ) pathway ligating the ends directly without the insertion of a template.

The safety profile of the rAAV relies on that upon intravenous injection, more than 85-95% of rAAV vector genomes remain episomal in the dividing hepatocytes in the mouse liver (Figure 4B; [230]). The study might have overestimated integration events in hepatocytes. Others estimate the AAV integration events closer to 0.1-1% [231]. Yet, follow-up studies indicated that 53-62% of rAAV integrations in the liver fused into actively-transcribed genes, and 3-8 % into ribosomal DNA [232]. rAAV genome integration into mouse muscle tissue

DNA compared to hepatocyte DNA was hard to detect or not present, indicating that the integration frequency also depends on the cell type [23]. Integration of wtAAV compared to rAAV for human cardiomyocytes at high MOI (50,000 viral particles per cell) was 5.6x higher with both AAVs integrating into mitochondrial DNA [233]. A recent study looked at integration events in nonhuman primates and patient DNA in clinical trials (liver biopsy) that received the rAAV2/5-*cohPBGD* and found 10^{-3} to 10^{-5} integration events per cell or 0.04–9% integration events [234]. Very little information is available for rAAV vector genome integration events into retinal tissue. A recent CRISPR/Cas9 study indicated that Cas9 breaks caused >1-20% insertion events of the rAAV cassette (EDIT-101) into the dsDNA break in the CEP290 intron in human retinal explant DNA, not counting integrations in other regions of the genome [38]. The insertion of the rAAV was higher when more indels, deletions, and inversions were detected (over 25 independent samples). The results indicate, as expected, that rAAV integrations events in photoreceptor cells correlate to the rAAV dosage. rAAV integration studies in the retina is an underrepresented research field. Almost no rAAV study specifically investigated rAAV integration events.

The integration of rAAV vectors at ribosomal DNA (rDNA) can be exploited by adding 1 kb homology arms of the rDNA locus adjacent to the ITRs. The homology arms increased the integration frequency in dividing cells favoring homologous recombination by 10-30x from a baseline of 0.001-5% AAV vector integrations per 100 cells (depending on the vector dose). Further, many rAAV gene cassette integrations caused deletions in the genome [235].

The unique T-shape AAV serotype 2 ITR-DNA (ITR2) conformation enables even gene editing. A defective eGFP reporter plasmid in dividing cells was rescued (gene-edited) by adding only the ITR2-*eGFP*part(165 bp)-ITR2 with 40 bp *eGFP* homology arms adjacent to the ITR2 [236]. Nevertheless, the rAAV gene expression from integration events is generally silenced within eight passages. Also, wild-type AAV integrates preferentially at 94% at the AAVS1 locus on chromosome 19 (Chr19) because the ITR sequence is homologous to the AAVS1 locus, but this requires the AAV integrases Rep78 and Rep68 that have been removed in rAAVs. Thus, rAAVs do not integrate at the AAVS1 locus. Interestingly, very little to no rAAV integration has been found in the genome of CRISPR/Cas9 gene-edited quiescent cells suggesting that off-target editing requires cell division. Unmistakably, many successful AAV gene therapies in mice and >130 rAAV clinical trials in humans, have indirectly demonstrated that the genome integration / genotoxic events are of a lesser concern [234].

4.5. Codon optimization and self-complementary rAAVs

The final rAAV gene cassette could be codon-optimized to improve the optimal expression of the transgene in the target cell and organism. Essentially, codon-optimization is primarily looking at codon frequencies that might rate limit the transcription of the gene in the target cell, such as favoring the codon GUG over GUU for valine in humans. The codon

optimization of rAAVs should prevent the inclusion of potential hairpin structures, repeats, extreme GC content, alternative open reading frames (ORF), and cryptic splice sites. Different codon optimizations have been tested for the rAAV gene therapy for Crigler-Najjar syndrome that increased the *FIX* transgene expression by 4-10-fold [237]. Also, the codon-optimization (humanized; removal of cryptic splice sites; elimination of alternative ORFs) of rAAV2/5.hRPE65.*hRPE65* to rAAV2/5.OPTI*RPE65* could theoretically reduce the vector dose 300-fold in mice [36]. The authors claimed that the future use of codon optimization for human cells might further improve the potency to 1800-fold in humans. The final lower rAAV administration might reduce temporal capsid-mediated toxicity found in their earlier studies [36]. For the rAAV vector AGTC-501 expressing the human *RPGR-ORF15* gene, the Codon Adaptation Index (CAI) of human codon-usage frequency was increased from 0.73 to 0.87, and the Frequency of Optimal Codons (Fop) was increased from 32% to 57%. Further, the GC content was increased, and the maximum repeat size decreased. Such adaptations in the AGTC-501 vector resulted in reduced frequency of alternative splicing and increased mRNA stability [44,111]. Interestingly, the same stabilized RPGR sequence of the AGTC-501 produced a full-length RPGR-and a truncated form of RPGR-ORF15 in the retina of mice *in vivo*. But when applied to HEK293T cells in vitro, then the AGTC-501 vector produced only the full-length RPGR-ORF15 protein. The different products produced from the same *RPGR* expression cassette indicated species differences in the regulation of gene transcription or RNA splicing or differences between *in vitro* versus *in vivo* transgene expression systems. Transgene expression cassettes for clinical retina studies should, therefore, be tested in cultured human cells and preferentially in human retinal organoids or human RPE cells.

Recombinant self-complementary AAV (scAAV) vectors are more potent to express high levels of transgenes than recombinant single-stranded AAV (ssAAV) vectors. Once the cell is infected by the scAAV and the scAAV becomes decapsidated, the rate-limiting step to create double-stranded DNA is overcome more efficiently in scAAV than in single-stranded rAAV. A major disadvantage of scAAV is however the reduced packaging capacity of the gene expression cassette from up to 4.9 kb to a maximum of 2.5 kb, including the two ITRs. Transgene expression could be enhanced by 5 up to 140 fold *in vitro* [238]. Deleting the terminal resolution site sequence from one ITR (ITR2Δ) increased the yield of dimeric genomes by 90% [239]. When compared to ssDNA, lowered rAAV doses of scAAV gene therapy vector can be used to reach similar transgene expression levels in the retina *in vivo* [102,155,240,241]. ScAAVs can also have significant effects on the promoter choice. The rescue by subretinal injection of scAAV8-sRLBP1p.*RLBP1* rescued the rate of dark adaptation measured by electroretinography (ERG) in *Rlbp1* knockout mice [102,155]. The transcription of hRLBP1 by a short RLBP1 promoter increased 50-fold at a low dose ($1x10^8$ viral genome) and 6.4 fold at a high dose ($1x10^9$ viral genome) in cynomolgus monkey retina. The scAAV might be especially beneficial when weak promoters are required or if low viral

doses are desired to prevent capsid toxicity of specific target cells. A more specific review on scAAV can be found here [239].

5. Transgene & bioactivity assays in ocular tissue

Quality control is essential in rAAV production. Quality control includes testing the rAAV production on safety (sterility, viral contaminants, mycoplasma, endotoxins, bacterial & fungistatic activity), appearance, pH, osmolarity, potency (viral genome titer, infectivity, expression), purity, and vector genome identity [242]. Here, we focus on the rAAV potency: infectivity and *in vitro* & *in vivo* expression.

5.1. In vitro immortalized epithelial cell lines for transgene and bioactivity assays

The most straight forward, high-throughput, fast, cheap, robust, but less predictive transgene expression assays are still monoculture systems *in vitro*. The ideal system would allow a fast characterization of the transduction profile, including rAAV capsid-specific infection, promoter expression profiling (mRNA level), and protein-of-interest expression. Such an assay would allow fast screening of different gene cassettes in research development but also validating the gene therapy products and batches in the clinical-grade production cycle.

rAAV transfection efficiency (potency: infectivity) and transgene expression (potency: *in vitro* expression) has been tested on rAAV epithelial production cell lines (for example: HEK293T, HER911, HeLa-E1, Per.C6). One can measure the rAAV infection or functional titers measured in transducing units per mL (TU/mL) by two common assays: (1.) Median tissue culture infective dose (TCID50) based on rAAV-vector (MOI 20,000 viral genome/cell) infecting HeLaRC32 (HeLa AAV rep-cap expressing cell line) and concomitant adenovirus type 5 (Ad5; 500 IUs/cell) infection (Outcome: vector genome quantification). (2.) Infectious center assay (ICA) based on HeLaRC32 and Ad5 (500 IUs/cell) infection (Outcome measure: hybridization of a probe to the rAAV gene cassette). The HeLaRC32 cells allow rAAV replication if the rAAV gene cassette can enter the nucleus [243].

Spark Therapeutics also developed an *in vitro* potency assay for rAAV-*RPE65* vectors. Here, modified HEK293 cells constitutively expressing lecithin-retinol acyltransferase (LRAT) are transduced by the rAAV-*RPE65* at different MOIs. Then, 72 hours later, the cells are lysed by adding lysis buffer, and the lysate is incubated with all-trans-retinol and CRALBP for 2 hours in the dark to assess the enzymatic activity of RPE65 (an isomerohydrolase) to convert all-trans-retinol to 11-cis-retinol [244]. The rAAV-*REP1* for the treatment of choroideremia is assessed on *in vitro* prenylation of RAB6A in HEK293 cells [245]. The directly injectable dose can also be assayed. The potency of the residual diluted vector (rAAV2-*REP1*) from the syringe used to inject patients was applied to the REP1-deficient cell line HT1080 for REP1 expression [246]. An endothelial-like Human Trabecular Meshwork (HTM) immortalized

cell line is currently explored for glaucoma gene therapy. Still, the surrogate cell line might also be interesting for studying the off-target effects of rAAV vectors injected intravitreally [247,248]. However, it is not always possible to develop informative assays on epithelial cells, especially for large screens for novel retinal-specific capsids or retinal specific promoters. For example, rAAV5 vectors infect very poorly HEK293 cells, HeLa cells, and BJ fibroblasts [249] but infect RPE cells and human retinal organoids efficiently [68,250]. Consequently, if a rAAV5-CAG vector does not express a gene product in a HEK293 assay, then the vector might still express the gene in human RPE or human retinal organoids.

5.2. In vitro immortalized ocular cell lines for transgene and bioactivity assays

Researchers can also use various human retinal cell lines to achieve improved infection and expression of retinal specific capsids or promoters. Promising ocular cell lines are the 661W mouse photoreceptor cell line / retinal ganglion precursor-like cell line (a surrogate for cone photoreceptors), the Adult Retinal Pigment Epithelial cell line-19 (ARPE-19; a surrogate for RPE cells), hTERT RPE-1 (ATCC® CRL-4000™), human astrocytes and the MIO-M1 (surrogates for Müller glial cells).

661W cells express Opn1SW, Opn1mw, Rbpms, Brn3b, Brn3c, Thy1, γ-synuclein, nestin, NeuN, Map2C, Map2D, and β-III tubulin. GFAP is not expressed in the 661W cell line. The 661W cells are light-sensitive but do not have visible outer segments [251,252]. 661W and ARPE-19 cells allow for screening of RPE-specific (mCARpro, MOPS500, VMD2) and ubiquitous (smCBA) promoters for most rAAV serotypes [253]. The 661W cell line has been further modified to achieve improved transfection by overexpression of the universal adeno-associated virus receptor (AAVR) or more stably express key photoreceptor genes such as *GRK1* and *CAR* by rAAV-VPR-*dCas9* vector infection [254]. Two major drawbacks to immortalized ocular cell lines are that most tend to be very heterogeneous, some of the cell lines express multiple cell-type-specific markers such as for retinal ganglion cells (Brn3) as well as for cones (Opn1mw) in addition to neuronal cell markers (Nestin, NeuN). And none of the cell lines have mature photoreceptor outer segments. Another RPE cell line (hTERT RPE1; ATCC® CRL-4000™) was used for liposome co-flotation assays expressing a biological active truncated CEP290 (1-580 amino acid) protein (rAAV-*CEP290*$^{1\text{-}580aa}$) in the primary cilium [255]. A *CEP290* knockout hTERT RPE1 line was constructed, showing the cilia-related CEP290-phenotype [256].

Human primary astrocytes have been successfully employed to select for novel rAAV capsid variants that are specific for (Müller) glial cells. An example is the rAAV6 variant ShH10-Y445F that efficiently infects rat, mouse, and human Müller glial cells [66,68,257]. Human astrocytes also express common Müller glial cell markers such as SOX9, GFAP, GLAST, GS, Kir4.1, and S100β [258,259]. A human Müller glial-like cell line (MIO-M1) expresses the proteins GLUL, VIM, low GFAP (but found on mRNA level), RLBP1, GLAST, EGFR, SLCA1, AQP4, Kir4.1, THY1, NEFH, MAP2, NEUROD1, NEUN, Nestin,

SOX2, Chx10, PAX6, NOTCH1, βIII tubulin. But the Müller glial-like cell line also contains mRNA for the following opsins or visual cycle-related proteins: OPN1SW, OPN2, OPN3, OPN4, OPN5, RRH, GNAZ, GNAT1, and GNAT2 [260,261]. The MIO-M1 cell line has been successfully used to screen for rAAV infectivity, rAAV, and lentivirus cell-specific promoter expression [262]. The hypoxia-Müller glial specific promoter (scAAV2.HRSE.6xHRE.GfaABC1D.*luciferase*) is active in MIO-M1 cells under hypoxic conditions. The hypoxia-induced Müller glial specific promoter showed no luciferase expression in HEK293, C6, HT22, and ARPE19 cells [98].

All described cell lines hold great promise for further rAAV studies. One needs to be cautious of the results because (1.) changes in culture condition can strongly affect the "cell-specific" gene expression, (2.) cell contamination has been found in several lines such as the rat ganglion cell line 5 (RGC-5) being a subclone of the 661W cell line, (3) multiple cell-type-specific gene markers expressed for example in 661W, and (4) the overall lower biologically relevance compared to 2D and 3D cell or *in vivo* studies.

5.3. In vitro differentiation of human induced pluripotent stem cells (hiPSCs) to retinal pigment epithelium (RPE) cells

Human patient induced pluripotent stem cells (hiPSCs) can be differentiated to photoreceptors [263]. 2D differentiation of hiPSCs to photoreceptors peaks at 45 days of differentiation, but it declines fast, making rAAV studies difficult because of the short time window and the inherent instability of inner/outer segments.

Human patient induced pluripotent stem cells (hiPSCs) can also be differentiated to monolayers of RPE. rAAV-*REP1* vector transduction of patient hiPSC-derived *CHM*-RPE rescued the biochemical phenotype [264]. The patient hiPSC-derived RPE can be efficiently used for testing the AAV-*CHM* vector for rescuing prenylation, phagocytosis, and protein trafficking [265]. Also, a *proof-of-concept* for dominant retinitis pigmentosa due to haploinsufficiency rescued phagocytosis and cilia formation by AAV2/Anc80-*PRPF31* in hiPSC-derived *PRPF31*$^{+/-}$ RPE cells [266]. Spark Therapeutics filed a patent application for a potency assay of rAAV-CHM on hiPSC-derived RPE cells lacking CHM expression [267].

5.4. In vitro differentiation of human induced pluripotent stem cells (hiPSCs) to retinal organoids for transgene and bioactivity assays

We and others used human retinal organoids to study rAAV transduction and potency [68,250,268,269]. In summary, photoreceptors are transduced by rAAVs such as rAAV2, rAAV2-7m8, rAAV5, rShH10, rShH10-Y445F, rAAV8, rAAV8T(Y733F), and rAAV9 albeit at different transduction efficacies. The rAAV2-7m8, rAAV5, rShH10, and rShH10Y-445F capsids infect photoreceptors efficiently. Interestingly, (early) radial retinal progenitor cells in retinal organoids or common cell lines can be effiently infected by rAAV6, the rAAV6 variants (ShH10 and ShH10Y-445F), and the AAV2-7m8 [21,68,250,269]. For

example, rAAV6 and rAAV-derived vectors (ShH10; ShH10-Y445F) can efficiently infect hiPSCs and hiPSC-derived RPE cells [21,68,250]. The rAAV2-7m8 and rAAV5 also efficiently infected RPE cells [250,269].

Many challenges still lay ahead. For example, the quality of the starting material (hiPSCs) and the differentiation method can significantly affect the success in differentiation to retinal organoids [270,271]. Also, the medium composition altering the extracellular matrix can influence rAAV infection. For example, the fibroblast growth factor receptors (FGFRs) are important for the stabilization of the heparan sulfate proteoglycans (HSPGs) on the extracellular matrix of the cell [272]. FGF-2 binds with low affinity to the heparin sulfate chains of HSPGs and the FGFRs [273]. It can be found in many medium compositions as supplement or in fetal bovine serum (FBS; 8-45 pg/mL) [274]. Many AAV capsids require the HSPGs, the universal AAV receptor (AAVR), and the FGFRs for efficient cell entry. For example, rAAV2 requires FGFR1 receptor that can be blocked by FGF-2 supplementation [275]. But other rAAV serotypes, such as AAV4 derivates are less dependent on the HSPGs and FGRFs for rAAV capsid cell entry [272]. Thus, the FGF-2 concentration in the medium needs to be defined for rAAV potency assays. Not all co-receptors for rAAV entries have been discovered yet, adding to the uncertainty of rAAV potency assay data.

Other limitations are the loss of ganglion cells in long-term culture, improper lamination of the ganglion cell layer including astrocytes, no innervation of the optic nerve that is required for proper foveal development, no vascularization of retinal organoids (therefore no pericytes), no sclera & Bruch's-membrane (blood-brain-barrier), no immune cells (macrophages, microglia, dendritic cells), and no integration with other organs (brain, heart, liver, kidney). More sophisticated models are currently in development. For example, human iPSC-derived retinal organoids and RPE-sheets can be cultured in a microfluidic chip system that enhances photoreceptor maturation and stabilization *in vitro* [276].

5.5. Human ex vivo retinal culture for transgene and bioactivity assays

We and others have also demonstrated that rAAVs can be tested on *ex vivo* cadaveric human retinas [38,51,68,277–281]. *Ex vivo* studies have especially become more attractive since the advent of more efficient medium compositions with better inner-outer photoreceptor segment (IS/OS) quality and ganglion cell survival that allow for longer rAAV-transgene expression. rAAV1, rAAV2[MAX], rAAV2(quad Y-F), rAAV2-7m8, rAAV4, rAAV5, rAAV6 and to a lesser degree rAAV2 and rAAV9 can efficiently infect photoreceptors on *ex vivo* human cadaveric retinas. We have shown that rAAV5, rAAV6 variants ShH10 and ShH10-Y445F can efficiently infect both human photoreceptors and Müller glial cells *ex vivo* [66,68]. rAAV2(quad Y-F) and rAAV2-7m8 also infected Müller glial cells and some rod photoreceptor cells *ex vivo* [278,279]. rAAV2/8BP2 infected photoreceptors, Müller glial cells, amacrine cells, ganglion cells, and horizontal cells *ex vivo* [51]. Also, the hRLBP1 promoter (2.6 kb) can restrict expression to Müller glial cells and RPE [66]. Recently,

rAAV5.GRK1.*SaCas9* vector particles were added to retinal explants that were subsequently cultured for 28 days. The human photoreceptor-specific rhodopsin kinase (GRK1) promoter directed Cas9 expression specifically in human photoreceptors [38]. We have also shown that the GRK1 promoter can limit expression to photoreceptor cells in *ex vivo* cadaveric human retinas [282].

We have previously shown that mouse retinal explants at P0 can be cultured longer than adult retinal explants [283]. Likewise, human fetal retinae can be cultured for three weeks, with preservation of the general morphology preserved and only the loss of ganglion cells [284]. More astonishingly, fetal retinal tissue that maintained some photoreceptor morphology has been cultured for 293 days *in vitro* [284]. However, the access to and quality of the donor human fetal material limits the application for many researchers [68,285,286]. We observed that efficient transduction of photoreceptors by rAAVs required the presence of well-developed inner/outer segments of photoreceptors. Interestingly, rAAVs infected Müller glial cells more efficiently in explants that showed retinal degeneration [68,279].

5.6. In vivo studies for transgene and bioactivity assays

5.6.1 Developmental stage and rAAV infection

Many adult mouse rAAV potency studies have been performed and are reviewed elsewhere [2,7]. It is interesting to note that rAAV infection can differ depending on the developmental stage of the ocular tissue. Mouse fetal retina (embryonic day 13; subretinal injection) can be transduced well by rAAV5 but not by rAAV1 and rAAV2. But rAAV1, rAAV2, and rAAV5 transduce photoreceptors well at P30 [286]. Mouse photoreceptor cells at postnatal day 0 can be efficiently targeted by subretinal injection of rAAV1, rAAV5, rAAV9, and rAAV11 [122]. However, AAV1 transduces mainly RPE cells at the adult stage [2,7]. Also, rAAV8-CMV-*GFP* transduces photoreceptors and Müller glial cells at postnatal day six but only transduces photoreceptors at postnatal day 0 [287]. Here, the rAAV vector containing the CAG promoter compared to the CMV promoter showed expression in more cells (photoreceptors, horizontal cells, amacrine cells, and retinal ganglion cells vs. only photoreceptors) at postnatal day 0. The difference in the infection efficiency at early development compared to more mature retinas has been linked to rAAV receptors and co-receptors important for rAAV cell entry [269]. The main universal AAV receptor (AAVR) was present already in 44-day-old human retinal organoids. However, the poor transfection efficiency of rAAV9 of retinal organoids [250,288] was linked to the low abundance of N-linked-galactose at early retinal developmental stages [269]. It points to the importance of describing the receptor composition and medium composition accurately at the time of infection to make meaningful comparisons between the infectivity of different rAAV capsids.

5.6.2 rAAVs overcoming membranes in the retina and the retinal disease state

Delivery of rAAV gene supplementation to photoreceptors or RPE cells is generally done by subretinal injection because many rAAVs cannot penetrate through the inner limiting

membrane (ILM) when injected intravitreally. The ILM is close to the ganglion cells and the Müller glial endfeet with a thickness varying between 100 nm up to 2000 nm in nonhuman primates [289]. The rAAV subretinal injection creates a fluid bleb between the retina and the RPE layer causing temporal retinal detachment and infection of cells at foci. Intravitreal injections target a larger retinal area. Intravitreal injections of empty rAAV capsids can induce a temporary immune inflammation of the aqueous and the vitreous [290]. Enzymatic digestion (proteasome inhibitors) of the ILM or ILM/OLM or disruptions of the ILM/OLM by the disease can alter rAAV infection and allow rAAV infection deeper into the retina [100,279,291,292]. Applying a low trans-ocular electric current also allowed efficient transduction of RPE and photoreceptors by rAAV8 upon intravitreal injection in adult mice [293]. Finally, the application of tyrosine kinase inhibitors might improve the passage of rAAV through the ILM or OLM [294]. The novel methods might make the intravitreal injection more common for photoreceptor and Müller glial cell infection. Intravitreal injection of some rAAVs might result in the transduction of a larger pool of off-target cells, such as the ciliary body and iris epithelium [66].

Disease-induced changes to the retinal morphology do impact the rAAV infectivity. In many retinal diseases, for example in *Crb1* retinitis pigmentosa mouse models, we first find that Müller glial cells express stress markers (gliosis), the outer limiting membrane (OLM) which contains adherens junctions between photoreceptors and Müller glial cells disrupts at foci, the inner/outer segments of photoreceptors shrink, some of the photoreceptors die, macrophages and microglial cells are activated and assemble in the photoreceptor segment layers, the outer and inner nuclear layers mix and thin out, and neovascularization takes place [68,189–202]. Likewise, transgenic rats overexpressing rhodopsin variants causing autosomal dominant retinitis pigmentosa show an early (P20), intermediate (P30), and advanced stages of retinal degeneration (P60). rAAV1 or rAAV5 intravitreally injected in rats showed no accumulation of AAV particles at the ILM [291]. But under the disease condition or enzymatic digestion of the ILM, all rAAV (1, 2, 5, 8, and 9) traversed, most likely via Müller glial cells, to RPE cells through the retina [100,291,295]. rAAV2-7m8 vectors infected a wide range of cells in the degenerate retina of rd1 mice (*Pde6b*$^{rd1/rd1}$) with little differences found in tropism when the vector was injected subretinally or intravitreally [279]. The potency to infect the degenerate photoreceptors by subretinal injection of *Abca4*^{KO} mice compared to wild-type mice was lower for four different rAAVs (rAAV2, rAAV5, rAAV2rec2, rAAV2rec3) while the potency to infect INL cells was increased in *Pde6b*$^{rd1/rd1}$ mice at least for rAAV5, rAAV2/Rec2 [296]. Intravitreal injection of rAAVrh-10 shifted infection from mainly INL cells towards photoreceptor and RPE cells in *Rs1*^{KO} and *Rho*^{KO} mice (XLRS and RP models, respectively) compared to wild-type mice [297,298]. The studies indicate that disease models allow deeper penetration of rAAVs in disease state compared to healthy retinas with generally lower photoreceptor infection. How well changes

in the OLM and ILM impacts rAAV infection needs to be determined separately for each type of retinal disease (Figure 5).

The changes in rAAV vector transduction and expression can be linked to the differentiation stage of cells and cellular stress. Nondividing terminally differentiated cells allow efficient expression from rAAV gene therapy vectors because the cells downregulate proteins of the DNA damage response [299]. In AMD and retinitis pigmentosa, retinal

cells might show an increase in DNA damage response, including more double-stranded DNA breaks and impaired (decreased) autophagy leading to increased cell size, granularity, and protein accumulation [300–302]. How the increase in DNA-damage sensors or the decrease of terminal differentiation is linked to rAAV vector transgene expression needs to be further evaluated. Cell stress-induced expression was observed upon induction of gliosis, by light or application of ciliary neurotrophic factor (*CNTF*), in *Crb1*-deficient retina injected intravitreally with rAAV.GFAP.*eGFP* [100].

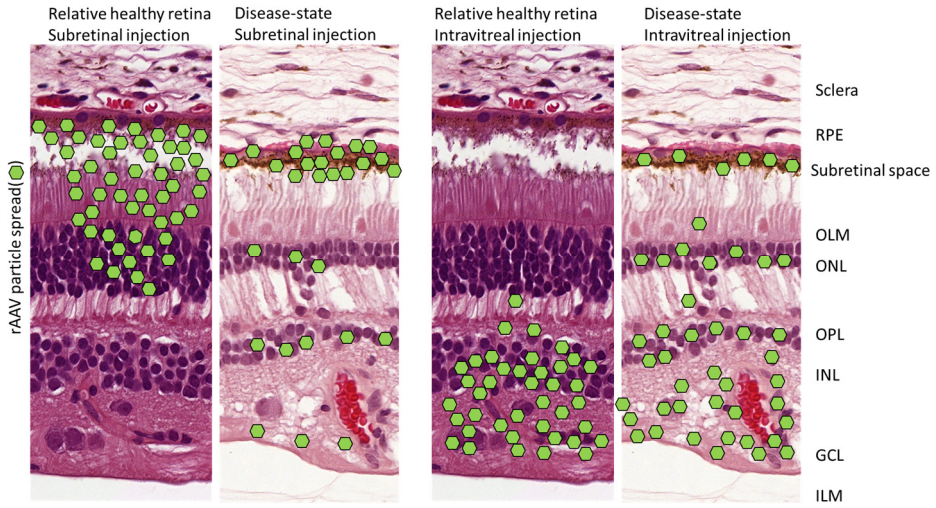

Figure 5. A hypothetical model of the spread of rAAV capsids (serotypes 1, 2, 5 8, and 9) after intravitreal or subretinal injection in disease or non-disease mouse retinas *in vivo* based on the studies [100,279,291,293,295,296]. RPE, retinal pigment epithelium; OLM, outer limiting membrane, ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; ILM, Inner Limiting Membrane.

5.6.3 Nonhuman primate studies and rAAV infection

Several rAAV serotypes are studied for safety/toxicological assessment in retinas of nonhuman primates (NHP) before entering clinical trials. Some information on tropism and promoter cell specificity has been acquired. Subretinal injection (ubiquitous promoter): rAAV1, rAAVrh64R1, rAAV2, rAAV2-7m8, rAAV5, rAAVrh8R, rAAV7, rAAV8, rAAV8BP2, rAAV9, rAnc80L65 infect rod photoreceptors and RPE cells [265,278,303–

307]. rAAV8BP2, rAAV9 and rAAV5 infect cone photoreceptors more efficiently than rAAV2 [305,306,308]. Novel AAV capsids, such as the rAAV7m8 and rAAV8BP2, also infected some INL cells and ganglion cells [51,306]. High titer rAnc80L65 and rAAV5 might infect some NHP Müller glial cells [309]. Intravitreal injection (ubiquitous promoter): rAAV2 infects Müller glial cells and ganglion cells [310]. rAAV2-7m8 infects well Müller glial cells and retinal ganglion cells, whereas rAAV8BP2 infects ganglion cells. Self-complementary rAAV2tYFinfects Müller glial cells, at least if the ILM is peeled off before injection [311]. rAAVrh-10 showed promise in transducing the whole rabbit retina and patches of RPE cells, including photoreceptors [297].

Different cell-type-specific promoters have been tested by subretinal or intravitreal injection in NHPs with surprising results. The hGRK1 promoter expressed GFP specifically in rod- and cone photoreceptors [308]. The strong cone-specific promoters in mice (mCAR, PR2.1, and PR1.7) all showed some rod expression and strong cone expression. However, the mouse cone arrestin promoter (mCAR) also expressed eGFP in rods, inner nuclear layer cells, and ganglion cells [278]. A large scale study compared novel synthetic cell-specific promoters using previously described rAAV serotypes (AAV8, AAV9, AAV8BP2) between mice, NHPs, and human retinal explants [51]. Unsurprisingly, NHP and human retinal explants matched closer (correlation r=0.66-0.67) compared to mouse/NHP or mouse/human (r=0.34-0.38; r=0.24-0.32). But a predictive value mean correlation of 0.3 demands that rAAVs and cell-specific promoters need to be tested in human systems. Nevertheless, if an rAAV vector expressed well in human retinal explants and NHP, then it is more likely (conditional probability = 0.12-0.14) that the rAAV vector works as well in the mouse retina. The results suggest, therefore, that mouse retina can be used to pre-screen rAAV vectors for cell-type specificity.

5.6.4 Cis-regulatory toxicity of rAAV vectors in vivo?

AAV retinal-specific promoters were compared by subretinal injection at P0 for cis-regulatory sequence toxicity in the CD-1 albino mouse line [312]. This CD-1 mouse line is prone to hearing and vision loss, whereas albino mice are more susceptible to light-induced retinal damage [313]. The encapsidated rAAV vectors containing cis-regulatory ubiquitous promoters (CMV, CAG) or the RPE-specific promoter (hBEST1) showed higher dose-dependent toxicity to the RPE and photoreceptors than photoreceptor-specific promoters (Rho; RedO, CAR, GRK1). The ubiquitous UbC promoter showed no toxicity to the mouse RPE [312]. Interestingly, C57BL/6J mouse retinas injected with AAV8- or AAV5-CMV-*GFP* at P0 and subsequently analyzed at P30 showed RPE aberrations by SD-OCT but no ERG or OKT differences at $3x10^9$ vector genome copies [312]. However, stronger rAAV vectors such as the ones that contain a CMV promoter and a WPRE element such as the vector rAAV2/8.CMV.*eGFP*.WPRE.bGHpolyA caused retinal degeneration in mice at $5x10^{10}$ vg (ONL reduction and ERG) [314].

Interestingly, photoreceptor cells that were infected by rAAVs carrying a non-coding gene cassette (rAAV-flox vector) that is floxed out in Cre recombinase expressing cells also caused toxicity $1x10^{11}$ vg or above, indicating that the rAAV-capsid can cause toxicity [314]. Further, the ubiquitous CAG promoter caused more toxicity than the rhodopsin specific promoter. Thus, further studies on how promoters might activate the innate immune system by TLR2 or TLR-9 activation, what sequence motifs are more prone to induce toxicity, or what time points are especially sensitive are of great importance. However, antigen-presenting cells can take up capsids and express the antigens on MHC class II receptors that can activate $CD4^+$ T-helper cells releasing cytokines that stimulate $CD8^+$ T-cells. This immune cascade model might explain why re-administration in patients could become difficult, and why the administration of rAAVs in patients with high neutralizing antibodies (nAbs) are generally not included in clinical trials [315]. However, the innate, humoral, and cell-mediated immune response might contribute to vector toxicity. For example, intravitreally injected empty rAAV capsids can induce a transient inflammation of the aqueous and the vitreous body [290]. Most importantly, cis-regulatory-sequence, rAAV-capsid, and transgene-related toxicity need to be investigated in the retinal degeneration model. Further, the more efficient gene expression cassettes might allow a rescue strategy at lower rAAV titers, thereby causing less toxicity and lower transient inflammation [290].

6. Concluding Remarks and Future Prospects

The tools for modulating rAAV gene therapy vectors are expanding rapidly. Novel rAAV capsids, production platforms, (short) promoters, stabilizing introns, and polyadenylation sequences are continuously published. However, many papers on rAAV ocular trials do not sufficiently describe the used pro-viral plasmids, especially on the backbone side (origin and bacterial selection marker). The vast expanse and the poor description make a fair comparison of rAAV vector elements very challenging because large scale comparative rAAV element studies are missing. Nevertheless, recent studies indicate that:

(1) The use of tyrosine-mutated rAAV2 capsids (AAV2-tYF; AAV2-7m8) increases retinal penetration and infection potentially replacing wild-type capsids (Section 2, 5.6)
(2) The strong viral promoter CAG expresses the transgenes in the RPE for many years without being silenced [22] (section 2, 3, 5.6)
(3) Native promoters are more prone to differ in expression in disease models, different species, and *in vivo/in vitro/ex vivo* models (section 3.4, 5)
(4) Inducible promoters (riboswitches and dead-Cas9) offer exciting opportunities to control protein expression (section 3.8)
(5) Surrogate (homolog/ortholog or synthetic) gene supplementation might circumvent cellular immunogenicity (Section 4.1)
(6) The rAAV production cell line might influence the transduction efficiency [45] (section 2 and 4.4)

(7) The inverted terminal repeats of rAAVs are essential for high production yields but less critical for the efficient transgene expression (section 4.4.2)
(8) Genome integrations of rAAV vectors and the potential cell-toxic effect of genome integrations are insufficiently studied in retinal tissue (section 4.4.3)
(9) Differences in medium composition, culturing techniques/protocols, and the developmental stage influence rAAV infection and transgene expression (section 5.6.1)
(10) The disease state strongly influences rAAV-vector penetration, potency, and tropism of the retina (section 5.6.2)

A large panel of models are available for studying retinal diseases. However, all models have inherent drawbacks. Improved models will become available, allowing more rapid screening of promoters and rAAV capsids in human systems with high biological relevance (Figure 6. Section 5.1-5.6).

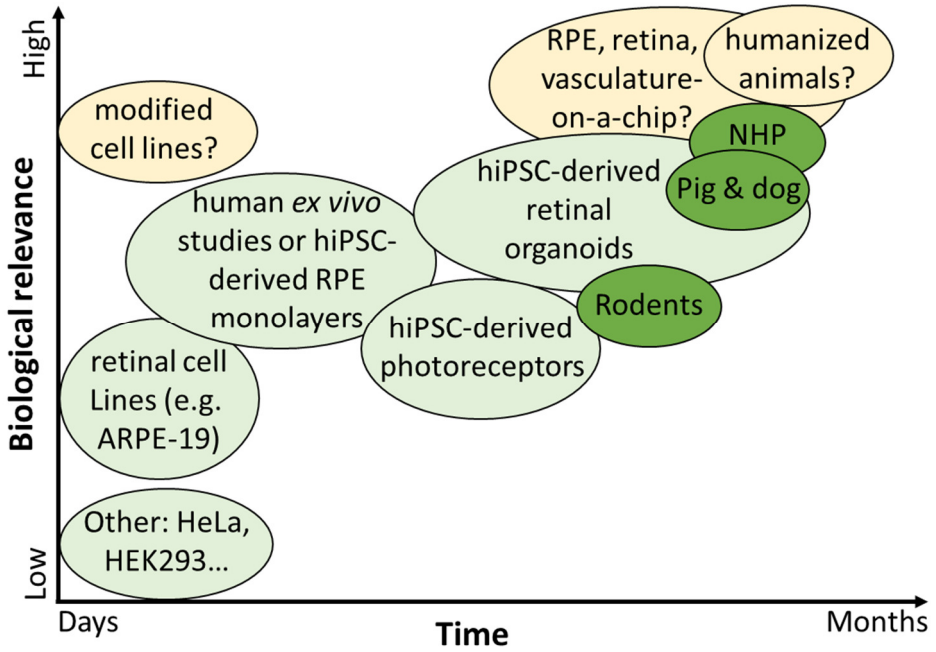

Figure 6. Qualitative assessment of biological relevance and time of assay for retina-specific rAAV potency assay models. hiPSC, human induced pluripotent stem cell; NHP, non-human primates; RPE, retinal pigment epithelium.

7. Methods

A meta-analysis on pro-viral plasmids and production platforms for ocular rAAV therapies in clinical trials (Table 1 & S1)

We analyzed current reviews on gene therapy on clinical trial identifiers. Then, we searched for the keywords (AAV, gene therapy, retinitis pigmentosa, RP, Leber congenital amaurosis, LCA, AMD, CHM) on https://clinicaltrials.gov/ (last date 01-April-2020). We limited the search to gene therapies expressing the gene-of-interest in the eye. We further cross-checked the results with reported clinical trials in the news and current reviews. If sequences or rAAV production platforms were insufficiently reported in research papers the search was widened: (1.) Patents based on researchers and companies involved; (2.) Ph.D. thesis; (3.) Company registration documents; (4.) Company websites; (5.) Company-provided presentations; (6.) Posters on international conferences; (7.) Abstracts on international conferences. All related information was gathered in the supplementary word document. All clinical trial identifiers were added to each unique rAAV product to identify the unique products. Lentiviral products, cell therapy products, antibodies, and antisense oligonucleotide (AONs) products were removed. The unique ocular gene therapy products were then analyzed on prokaryotic plasmid backbone, inverted terminal repeat AAV serotype, enhancers, promoters, introns, genes, codon-optimization, human or other species-related sequences, post-transcriptional stabilizing sequences, polyadenylation sequences, and rAAV production platform. Not present information was indicated as “UntoldR”.

Conflict of interest statement

The LUMC is owner of a pending patent on the use of *CRB2* gene therapy vectors (WO2015020522A1) and received license income and research funds from HORAMA. JW is full-term employee of LUMC and acts for LUMC as temporary consultant for HORAMA. JW is mentioned as an inventor on the patent application. TMB declares no conflict of interest.

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1.3 Aim and outline of this thesis

Chapter 1 reviews (a) the origin and models of *CRB1*-associated retinal dystrophy, (b) the current recombinant adeno-associated viral (rAAV) plasmid vectors in ocular clinical trials, (c) ubiquitous and retinal cell-specific promoters, (d) bioactivity assays, and (e) transgene assays.

Chapter 2 describes (a) the morphological phenotype and visual deficits of the *Crb1*^{KO}*Crb2*^{LowMGC} mouse (a *CRB1*-like retinitis pigmentosa mouse model) in which one *Crb2* allele is made nonfunctional in Müller glial cells on a *Crb1*-null genetic background and compared to *Crb1*^{KO} littermates, (b) a novel DL-AAA Müller glial-cell stress-induced *CRB1*-like RP mouse model, and (c) how rAAV-h*CRB2* and rAAV-h*CRB1* therapy to Müller cells protects against retinal degeneration but only rAAV-h*CRB2* protecting against vision deficits in the DL-AAA exposed *CRB1*-like retinitis pigmentosa mouse model.

In **Chapter 3** we developed and describe an assay where one can screen more than eight rAAV vectors in parallel from one human cadaver neuroretina which can be kept viable for 21 days *ex vivo*. Several rAAV-vectors require 3-4 weeks of incubation for maximal transgene expression but *ex vivo* human neuroretinal material tends to degenerate quickly. The described transgene expression assay can evaluate the tropism and potency on human tissue and provides tips&tricks in assay setup (such as controlling for photoreceptor outer segement presence during rAAV infection).

In **Chapter 4** we describe how CRB proteins are expressed in the developing human retina (foetal explants and human retinal organoids). We differentiated three human control induced pluripotent (hiPSC) lines and three *CRB1*-RP patient hiPSC lines to mature retinal organoids. The three patient lines developed vulcanic cell eruptions similar as seen in *CRB1*-like RP mouse models. We also screened three different rAAV capsids on tropism and potency on human retinal organoids *in vitro* and human cadaver neuroretinas *ex vivo*. The rAAV-vector potency assay indicated that rAAV serotype 5 capsids can efficiently infect the target cells of a rAAV-based gene supplementation therapy (human photoreceptors and Müller glial cells) outperforming rAAV9. Further, we show that the loss of outer segments may decrease the photoreceptor rAAV-infectivity.

Chapter 5 describes the development of isogenic hiPSC lines of two patient *CRB1* RP lines and how the *CRB1* RP phenotype develops in *CRB1*-RP retinal organoids over time after the onset of *CRB1* expression at the outer limiting membrane. We hypothesized that CRB1 variant protein may aggregate or be less efficiently shuttled to the outer limiting membrane. Interestingly, we found little variant CRB1 protein throughout the neuroretina in patient-derived retinal organoids. We show that (a) the decrease of CRB1 protein upregulates degradative protein markers (p62, LC3, LAMP1, ARL8A/B), (b) that the extracellular domain of CRB1 can interact with NOTCH1 which by large is lost in *CRB1* patient organoids, (c) a strong decrease of recycling endosomal marker RAB11A and an increase of early endosomes (EEA1+ particles), and (d) an increase of WDFY1 protein at the OLM/ONL. We propose that the decrease of CRB1 at the outer limiting membrane reduces the turnover of receptor recycling shifting early/late endosomes maturation towards degradation (lysosomes, autophagosomes).

Chapter 6 is a discussion of the thesis. **Chapter 7** includes a summary and the Nederlandse samenvatting, acknowledgements, curriculum vitae of the author of this thesis and a list of publications.

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Supplementary information

Table S1 Description of clinical trial rAAV gene therapy products in ophthalmology

Sponsor – Product – Clinical trial phase – Gene

Achromatopsia (ACHM)
MeiraGTx – AAV-CNGA3 – I/II – *CNGA3*
Clinical trial start date (clinical trial identifier): 29-Nov-2018 (NCT03758404) rAAV: AAV2/8.hG1.7p.h*CNGA3*co.SV40polyA Proviral plasmid: pAAV.untoldR.ITR2.hG1.7p.h*CNGA3*co.SV40polyA.ITR2 hG1.7p=Novel synthetic cone specific promoter. Core green opsin promoter including a mutation (0.5 kb) + Locus Control Region (LCR; 1.2 kb) upstream of the red opsin gene. Production platform: HEK293. Citation: [1–3]
STZ Eyetrial – rAAV.h*CNGA3* – I/II – *CNGA3*
Clinical trial start date (clinical trial identifier): 20-Nov-2015 (NCT02610582) rAAV: AAV2/8.hCAR.h*CNGA3* Proviral plasmid: pSub.KanR.ITR2.hCARp.h*CNGA3*co.WPREm.bGHpolyA hCAR= human cone arrestin (aka hArr3) 405 bp CNGA3= 2085 bp full-length human CNGA3 cDNA WPREm= WPRE is a mutated WPRE (WPREm) comprising non-expressible woodchuck hepatitis virus X protein (WHX) open reading frame (WHX OR). 543 bp bGHpolyA= bovine growth horome 207 bp Production platform: AAV cis (pSub-hArr3-hCNGA3-WPREm-KanR) and trans (pDP8-KanR) in HEK293 cells. Citation: [4,5]
Applied Genetic Technologies Corp. (AGTC) – AGTC-402 – I/II – *CNGA3*
Clinical trial start date (clinical trial identifier): 17-Oct-2019 (NCT02935517) rAAV: AAV2tYF.PR1.7p.SV40 SD/SA.h*CNGA3*co.SV40polyA Proviral plasmid: pAAV.untoldR.ITR2.PR1.7.SV40 SD/SA.h*CNGA3*co.SV40polyA.ITR2 AAV2tYF=AAV serotype 2 with surface-exposed tyrosine mutations: Y275F, Y733F, Y447F. Production: (rHSV) complementation system in suspension-cultured baby hamster kidney (sBHK) cells Citation: [6,7]
Applied Genetic Technologies Corp. (AGTC) – rAAV2tYF-PR1.7-h*CNGB3* – I/II – *CNGB3*
Clinical trial start date (clinical trial identifier): 9-Nov-2015 (NCT02599922) rAAV: AAV2tYF.PR1.7.SV40 SD/SA.h*CNGB3*co.SV40polyA Proviral plasmid: pAAV.untoldR.ITR2.PR1.7p.SV40 SD/SA.h*CNGB3*co.SV40polyA.ITR2 AAV2tYF= AAV serotype 2 with surface-exposed tyrosine mutations: Y275F, Y733F, Y447F. Production: (rHSV) complementation system in suspension-cultured baby hamster kidney (sBHK) cells Citation: [6–8]
MeiraGTx – either AAV-CNGB3 or AAV-*CNGA3*– I/II – *CNGB3/CNGA3*
Clinical trial start date (clinical trial identifier): 23-Dec-2016 (NCT03001310); 12-Sept-2017 (NCT03278873) rAAV: AAV2/8.hCARp.h*CNGB3*.SV40polyA or AAV2/8.hG1.7p.h*CNGA3*co.SV40polyA Proviral plasmid: pAAV.untoldR.ITR2.hCARp.h*CNGB3*.SV40polyA.ITR2 + pAAV.untoldR.ITR2.hG1.7p.h*CNGA3*co.SV40polyA.ITR2 Production platform: HEK293. Citation: [1–3]

Wet Age-Related Macular Degeneration (wet AMD)
Sanofi Genzyme – AAV2-s*FLT01* – I/II Clinical trial start date (clinical trial identifier): 3-Dec-2009 (NCT01024998) rAAV: rAAV2/2.CAGp.s*FLT01*.bGHpolyA Proviral plasmid: pAAVSP70.untoldR.ITR2.CBA.s*FLT01*.bGHpolyA.ITR2 CBA: hybrid CMV/CBA promoter, derived from the pDRIVE CAG plasmid (Invivogen, San Diego, Calif.; having 100% sequence homology with the pCAGGS) with an upstream extension of about 49 nucleotides of CMV enhancer (1661 bp). CBA= proximal chicken β actin promoter and human beta-globin exon 1 and intron 1. The University of Pennsylvania considers CBA and CAGGS the same. pAAVSP70 derived from pAV1 derived from pBR322. Citation: [9–14]
Adverum Biotechnologies – ADVM-022 - I Clinical trial start date (clinical trial identifier): 21-Nov-2018 (NCT03748784) rAAV: AAV2/7m8.eCMV.CMVp.s*FLT01*.hSAR.hGHpolyA Proviral plasmid: pBAC-AAV.untoldR.ITR2.eCMV.pCMV.TLP.eMLP.SyntheticIntron.Kozak.s*FLT01*co. hSAR.hGHpolyA.ITR2 Production: Baculovirus, Sf9. (plasmids: rBAC-AAV. rBAC-RepCap) eCMV = human early CMV enhancer. pCMV = human CMV promoter. TLP = adenovirus tripartite leader sequence. eMLP = major late promoter. SI = synthetic intron. pSI chimeric intron. 5 -donor site from the first intron of the human β-globin gene and the branch and 3 -acceptor site from the intron that lies between the leader and the body of an immunoglobulin gene heavy chain variable region [15] s*FLT01*co = codon-optimized aflibercept (recombinant chimeric protein consisting of the vascular endothelial growth factor (*VEGFA*) binding portion of human *VEGFR-1* (domain 2) and *VEGFR-2* (domain 3 or KDR) fused to the Fc portion of human *IgG1* immunoglobulin.). hSAR = human scaffold attachment region. Citation: [15–17]
Oxford BioMedica – OXB-201 / RetinoStat – I Clinical trial start date (clinical trial identifier): 23-Feb-2011 (NCT01301443); 5-Sept-2012 (NCT01678872) Lenti: EIAV.CMVp.hEndo.IRES.hAngio.WPRE.LTR. Citation: [18]
Lions Eye Institute (Perth, Western Australia, Australia) / Adverum Biotechnologies – rAAV.s*Flt-1* – I/II Clinical trial start date (clinical trial identifier): 19-Dec-2011 (NCT01494805) rAAV: AAV2/2.CMVp.chimericIntron.s*Flt-1*.SV40polyA Proviral plasmid: pSSV9.untoldRAmpR.ITR2.CMV.ChimericIntron.sFlt-1.SV40polyA.ITR2 sFlt-1 = full-length soluble fms-like tyrosine kinase 1 (non-membrane associated splice variant of VEGF receptor 1 [s*VEGFR-1*]). Citation: [19–22]
Hemera Biosciences – AAVCAGs*CD59* (HMR59) – I Clinical trial start date (clinical trial identifier): 13-Jul-2018 (NCT03585556) rAAV: AAV2/2.CAGp.s*CD59*.hGHpolyA Proviral plasmid: pUC.untoldR.MCS(Stratagene).ITR2.CAG.s*CD59*.hGHpolyA.ITR2 sCD59 = soluble CD59 antigen binds C5b678 terminal complement protein complex and prevents incorporation of multiple C9 molecules (blockage of the Membrane attack complex [MAC] in the alternative complement cascade/pathway). Citation: [23,24]
Regenxbio – RGX-314 – I Clinical trial start date (clinical trial identifier): 28-Feb-2017 (NCT03066258) rAAV: AAV2/8.ITR.CB7p.*aVEGFAfabH*.(F)/F2A.*aVEGFfabL*.rβ-globin-polyA.ITR Proviral plasmid: pAAV.untoldR.ITR2.CAG/CB7.*aVEGFAfabH*.(F)/F2A.*aVEGFfabL*.rβ-globin-polyA.ITR2 aVEGFAfabH = anti-VEGFA Heavy chain. Self-cleaving furin (F)/F2A linker. aVEGFAfabL = anti-VEGFA Light chain. rβ-globin-polyA = rabbit β-globin polyA. Citation: [25]

Dry Age-Related Macular Degeneration (dry AMD) incl. Geographic Atrophy

Hemera Biosciences – AAVCAGsCD59 (HMR59) – I

Clinical trial start date (clinical trial identifier): 9-May-2017 (NCT03144999)
rAAV: AAV2/2.CAGp.s*CD59*.hGHpolyA
Proviral plasmid: pUC.untoldR.MCS(Stratagene).ITR2.CAG.s*CD59*.hGHpolyA.ITR2
sCD59 = soluble CD59 antigen binds C5b678 terminal complement protein complex and prevents incorporation of multiple C9 molecules (blockage of the Membrane attack complex [MAC] in the alternative complement cascade/pathway). Citation: [23,24]

Gyroscope Therapeutics – GT005 – I/II

Clinical trial start date (clinical trial identifier): 19-Feb-2019 (NCT03846193)
rAAV: rAAV2/2.CBAp.h*CFI*co.WPRE.bGHpolyA
Proviral plasmid: pBR322.UntoldR.ITR2.CBA.*CFI*.WRPE.bGHpolyA.ITR2
hCFIco= human complement factor I codon-optimized (C3b/C4b inactivator). Increases the level of C3b-inactivating and iC3b-degradation activity in the RPE. Citation: [26,27]

Choroideremia

Biogen / Nightstar Therapeutics / University of Oxford (Oxford, OFE, UK) / University of Miami (Miami, FL, USA) / University of Alberta (Edmonton, AB, Canada) / STZ eyetrial (Tübingen, BW, Germany) – rAAV2-REP1 – I/II; II – *CHM*

Clinical trial start date (clinical trial identifier): 28-Oct-2011 (NCT01461213); 4-Mar-2014 (NCT02077361); 3-Apr-2015 (NCT02407678); 17-Sept-2015 (NCT02553135); 2-Feb-2016 (NCT02671539); 25-Apr-2018 (NCT03507686); 12-Apr-2018 (NCT03496012); 12-Jul-2018 (NCT03584165);
rAAV: rAAV2/2.CBA.rabbit β-globin SD/SA.h*CHM*.WPRE.bGHpolyA
Proviral plasmid: pAAV.untoldR.ITR2.CBA.rabbit β-globin SD/SA.h*CHM*.WPRE.bGHpolyA.ITR2
Production: Triple co-transfection of HEK293 cells (AAV2 rep-cap helper plasmid; adenovirus helper plasmid containing E2A, E4ORF6, and VA RNA ORFs; pAAV). Citation: [28–30]

Spark Therapeutics – AAV2-REP1 – I/II – *CHM*

Clinical trial start date (clinical trial identifier): 19-Jan-2015 (NCT02341807)
rAAV: AAV2/2.CBAp.h*CHM*.bGHpolA.
Proviral plasmid: pAAV.Stuffer.KanR.ITR2.CBA.h*CHM*.bGHpolA.ITR2. Citation: [31]

Leber Congenital Amaurosis

Allergan / Editas Medicine Inc– AGN-151587 (EDIT-101) – I/II – LCA10 – *CEP290*

Clinical trial start date (clinical trial identifier): 13-Mar-2019 (NCT03872479)
rAAV: AAV2/5.U6.CEP290gRNAs323/.U6.CEP290gRNAs64.hGrk1.Kozak.SV40 SA/SD.*SaCas9*.NLS.spA
Proviral plasmid: pAAV.untoldR.ITR2.U6.CEP290gRNAs323/.U6.CEP290gRNAs64.hGrk1p.Kozak.SV40 SA/SD.*SaCas9*.NLS.SyntheticPolyA.ITR2
CEP290 rescue= in intron 26 of CEP290 (IVS26 c.2991+1655 A>G) gene editing (removal) of the mutation (p.Cys998X). Modified synthetic polyA sequence: (bold+underlined=synthetic polyA sequence. 60 bp).
TAGC**AATAAAGGATCGTTTATTTTCATTGGAAGCGTGTGTTGGTTTTTTG**ATCAGGCGCG
Production: HEK293. Citation: [32,33]

ProQR – QR-110 – I/II & II/III – LCA10 – *CEP290*

AON technology. p.Cys998X mutation correction on mRNA level
Clinical trial start date (clinical trial identifier): 20-Sept-2018 (NCT03140969); 12-Apr-2019 (NCT03913143); 2-Aug-2019 (NCT03913130). Citation: [34]

Genzyme / Sanofi - SAR439483 – I/II – *GUCY2D*

Clinical trial start date (clinical trial identifier): 18-Apr-2019 (NCT03920007)
rAAV: AAV2/5.hGRK1p.SV40 SA/SD.*GUCY2D*.bGHpolyA
Proviral plasmid: pAAV.puromycinR.ITR2.hGRK1p.SV40 SA/SD.*GUCY2D*.bGHpolyA.ITR2
Production platform: HeLaS3. Citation: [35,36]

Spark Therapeutics – AAV2-*RPE65* (h*RPE65*v2) – III – *RPE65* *FDA & EMA approved
Clinical trial start date (clinical trial identifier): 15-Aug-2007 (NCT00516477); 24-Sep-2010 (NCT01208389); 26-Mar-2018 (NCT00999609); 24-Jul-2018 (NCT03597399); 27-Jul-2018 (NCT03602820);
rAAV: AAV2/2.CBAp.h*RPE65*.bGHpolA
CBA: hybrid CMV/CAG promoter, derived from the pDRIVE CAG plasmid (Invivogen, San Diego, Calif.; having 100% sequence homology with the pCAGGS) with an upstream extension of about 49 nucleotides of CMV enhancer (1661 bp). CBA= proximal chicken β-actin promoter and human β-globin exon 1 and intron 1. The University of Pennsylvania considers CBA and CAGGS the same.
Proviral plasmid: pAAV.Stuffer.KanR.ITR2.CBA.h*RPE65*.bGHpolA.ITR2. Citation: [37]

Applied Genetic Technologies Corp (AGTC) / Hadassah Medical Organization (Jerusalem, Israel) / University of Pennsylvania (Pennsylvania, USA) – AAV2-CBSB-*RPE65* – I + I/II – *RPE65*
Clinical trial start date (clinical trial identifier): 1-Jun-2007 (NCT00481546); 13-Jan-2009 (NCT00821340); 10-Sept-2008 (NCT00749957);
rAAV: AAV2/2.CB-SBp.h*RPE65*.SV40polyA
Proviral plasmid: pAAV.KanR.ITR2.CB-SBp.h*RPE65*.SV40polyA.ITR2. CB-SB: 152 bp-shortened CBA promoter on CMV enhancer 5' end. Production: HEK293 cells, two-plasmid system. Citation: [38,39]

MeiraGTx – AAV2/5-OPTI*RPE65* – I/II - *RPE65*
Clinical trial start date (clinical trial identifier): 27-Okt-2016 (NCT02946879) 24-May-2016 (NCT02781480)
rAAV: AAV2/5.NA65p.SV40Intron.h*RPE65*co.SV40polyA
Proviral plasmid: pAAV.untoldR.ITR2.NA65p.SV40Intron.h*RPE65*co.SV40polyA.ITR2
NA65p= optimized human RPE65 promoter stronger than CBA
Production: HEK293T cells, three-plasmid system. Citation: [40]

Nantes University Hospital (Nantes, France) – rAAV-2/4.*hRPE65*– I/II - *RPE65*
Clinical trial start date (clinical trial identifier): 21-Dec-2011 (NCT01496040)
rAAV: AAV2/4.hRPEp.*hRPE65*.bGHpolyA
Proviral plasmid: pAAV.untoldR.ITR2.hRPE65p.h*RPE65*.bGHpolyA.ITR2
Production: HEK293T cells, two-plasmid system (pDP4-Kana helper plasmid)
Citation: [41]

University College (London, UK) – tgAAG76 (rAAV 2/2.hRPE65p.h*RPE65*) – I/II - *RPE65*
Clinical trial start date (clinical trial identifier): 26-Mar-2008 (NCT00643747)
rAAV: AAV2/2.hRPE65p.hRPE65.bGHpolyA
Proviral plasmid: pAAV.untoldR.ITR2.hRPE65p.h*RPE65*.bGHpolyA.ITR2
hRPE65p= 1.6 kb human RPE65 promoter
Production: B50 cell line utilizing helper adenovirus. Citation: [42]

Leber Hereditary Optic Neuropathy (LHON)

GenSight Biologics – GSO10 – III – G11778A mtDNA
Clinical trial start date (clinical trial identifier): 17-Feb-2014 (NCT02064569); 12-Jan-2016 (NCT02652780); 12-Jan-2016 (NCT02652767); 26-Sept-2017 (NCT03293524); 23-Jan-2018 (NCT03406104); 17-Sep-2018 (NCT03672968)
rAAV: AAV2/2.*ND4*
Proviral plasmid: pAAV2.untoldR.ITR.CMVp.HBB2.MTS-COX10.h*ND4*.3'COX10.ITR
HBB2=human β-globulin intron
Production: HEK293 cells. Citation: [43–46]

University of Miami (Miami, FL, USA) – scAAV2-P1*ND4*v2 – I – G11778A mtDNA
Clinical trial start date (clinical trial identifier): 11-Jun-2011 (NCT02161380);
rAAV: scAAV2/2-tyF.smCMVp. ATP1mt(MTS).h*ND4*.bGHpolA
Proviral plasmid: pBS.UntoldR.ITR2Δ.Sc-trs.smCMVp.ATP1mt(MTS).h*ND4*.WPRE.bGHpolA .ITR2
(P1ND4v2). smCBA= a truncated chimeric CMV/CBA promoter (953 bp)
MTS=ATP1-based mitochondrial targeting sequence
AAV2-tyF= AAV2 mutated capsids Y444F+Y500F+Y730F
Production: HEK293 cells. Citation: [47,48]

Huazhong University of Science and Technology (Huazhong, Hubei Sheng, China) – rAAV2-*ND4*– not applicable – G11778A mtDNA
Clinical trial start date (clinical trial identifier): 28-Dec-2010 (NCT01267422); 15-May-2017 (NCT03153293); 9-Feb-2018 (NCT03428178)
rAAV: AAV2/2.CMVp.COX10(5'UTR).*ND4*.COX10(3′UTR).bGHpolyA
Proviral plasmid: pSNAV.neoR.ITR2.CMV.COX10(5'UTR).*ND4*.COX10(3′UTR).bGHpolyA.ITR2
COX10(5'UTR)= mitochondrial targeting sequence
Production: HEK293 + HSV1-rc/ΔUL2. Beijing FivePlus Molecular Medicine Institute. Citation: [49–51]

Retinitis Pigmentosa

Allergan / RetroSense Therapeutics – RST-001 – I/II – advanced RP
Clinical trial start date (clinical trial identifier): 22-Sep-2015 (NCT02556736)
rAAV: AAV2/2.CBAp.*Chop2/ChR2*.WPRE.bGHpolyA
Proviral plasmid: pAAV.untoldR.ITR2.CBAp.*Chop2/ChR2*.WPRE.bGHpolyA.ITR2
Chop2/ChR2= Microbial type rhodopsins. Light-gated cation-selective membrane channel rhodopsin-2
CBA= hybrid CMV/CBA promoter, derived from the pDRIVE CAG plasmid (Invivogen, San Diego, Calif.; having 100% sequence homology with the pCAGGS) with an upstream extension of about 49 nucleotides of CMV enhancer (1661 bp). THE CBA= proximal chicken β actin promoter and human beta-globin exon 1 and intron 1. The University of Pennsylvania considers CBA and CAGGS the same.
Production: HEK293. Citation: [12,52–54]

jCyte, Inc – jCell – I, II –RP
Clinical trial start date (clinical trial identifier): 19-Dec-2014 (NCT0232081)2; 5-Mrt-2017 (NCT03073733)
Single intravitreal injection of 0.5 - 3.0 million human retinal progenitor cells (hRPC). Citation: [55,56]

ReNeuron Limited – hRPCRP– I/II –RP
Clinical trial start date (clinical trial identifier): 8-Jun-2015 (NCT02464436)
Participants will undergo vitrectomy surgery and subretinal implantation of human retinal progenitor cells (hRPC) in the study eye.

GenSight Biologics– rAAV2.7m8-CAG-*ChrimsonR-tdTomato* (GS030) – I/II – non-syndromic
Clinical trial start date (clinical trial identifier): 31-Oct-2017 (NCT03326336)
rAAV: rAAV2/7m8.CAGp.ChrimsonR-tdTomato.bGHpolyA
Proviral plasmid: pAAV.KanR.ITR2.CAG.*ChrimsonR-tdTomato*.bGHpolyA.ITR2
AAV2 7m8= AAV serotype 2 mutated capsid variant AAV2~^{588}LALGETTRP. No sialic acid dependence. Lower heparin affinity. Expression of Channelrhodopsin in retinal ganglion cells. Citation: [57–59]

Bionic Sight LLC / Applied Genetic Technologies Corp (AGTC) – BSO1 – I/II
Clinical trial start date (clinical trial identifier): 06-Feb-2020 (NCT04278131)
rAAV: AAV(untold).untold.*ChronosFP*(*Chr90*-fluorescent protein).untold. Proviral plasmid: untold
Expression of channelrhodopsin variant chronos (fast acting) in retinal ganglion cells

Horama SA – AAV2/5-h*PDE6B* – I/II – *PDE6B*
Clinical trial start date (clinical trial identifier): 1-Nov-2017 (NCT03328130)
rAAV: rAAV2/5.hGRK1p.h*PED6B*.bGHpolyA
Proviral plasmid: pAAV.untoldR.ITR2.hGRK1.h*PED6B*.bGHpolyA.ITR2. Citation: [60,61]

King Khaled Eye Specialist Hospital (Riyadh, Saudi Arabia) – rAAV2-VMD2-h*MERTK*– I/II – *MERTK*
Clinical trial start date (clinical trial identifier): 30-Nov-2011 (NCT01482195)
rAAV: rAAV2/2.hVMD2p.SV40 SD/SD.h*MERTK*.SV40polyA.bGHpolyA
Proviral plasmid: pTR.ColE1ori.untoldR.F1(+)ori.ITR2.hVMD2p.SV40 SD/SD.h*MERTK*.SV40polyA.bGHpolyA.ITR2
hVMD2p= human VMD2 promoter (- 585 to + 38 bp region. 623 bp)
Production: HEK293 co-transfection (pTR-VMD2-hMerTK and pDG-KanR). Citation: [62]

Novartis – CPK850 AAV8 – I/II – *RLBP1*
Clinical trial start date (clinical trial identifier): 15-Dec-2017 (NCT03374657)
rAAV: scAAV2/8.sRLBP1p.modSV40.h*RLBP1*.SV40polyA
Proviral plasmid: pAAV.untoldR.ITR2Δ.sRLBP1p.modSV40 SA/SD.h*RLBP1*.SV40polyA.ITR2
modSV40 SA/SD= modified SV40 splice acceptor/donor intron, 157 bp in length, nucleotides 502–561 and 1,410–1,497 of SV40 genomic sequence (NC_001669.1) + connecting sequence CGGATCCGG between two fragments.
Production: AAV293 in CellSTACK (HEK293 subclone; Stratagene). Triple-plasmid transfection (pHelper, pRep2Cap8, pAAV). Citation: [63,64]

Applied Genetic Technologies Corp. (AGTC)/Biogen-rAAV2tYF-GRK1-*RPGR* (AGTC-501/BIIB088)-I/II-*RPGR*
Clinical trial start date (clinical trial identifier): 20-Oct-2017 (NCT03316560)
rAAV: AAV2/2tYF.hGRK1p.SV40 SA/SD.h*RPGR1*co-ORF15.SV40polyA
Proviral plasmid: pAAV.untoldR.ITR2.hGRK1p.SV40 SA/SD.h*RPGR1*co-ORF15.SV40polyA.ITR2
hGRK1p= 292 bp human GRK1 promoter (positions 1793-2087)
SV40 SA/SD= 100 bp mini SV40 splice donor/acceptor intron
h*RPGR1*co = Based on GenBank reference mRNA sequence NM_001034853 *hRPGR* isoform C. Codon optimized based on human codon usage, reduced tandem repeats, adjusted G/C content
2tYF= AAV2 triple YF mutations
Production: recombinant herpes simplex virus (HSV) complementation system in suspension-cultured baby hamster kidney (sBHK) cells
Citation: [65,66]

MeiraGTx UK II Ltd / Janssen – AAV-*RPGR* – I/II – *RPGR*
Clinical trial start date (clinical trial identifier): 17-Aug-2017 (NCT03252847)
rAAV: AAV2/5.hGRK1p.SV40 SD/SA.h*RPGR*-ORF15-L.SV40polyA
Proviral plasmid: pAAV.untoldR.ITR2.hGRK1.SV40 SD/SA.h*RPGR*-ORF15-L.SV40polyA.ITR2
hRPGR-ORF15-L= small-deletion human hRPGR-ORF15 'long form' (codons 862-988del) expresses a human RPGR-ORF15 protein of ~170 kD
Citation: [67,68]

Nightstar Therapeutics / Biogen - AAV-*RPGR* – II/III – *RPGR*
Clinical trial start date (clinical trial identifier): 14-Apr-2017 (NCT03116113)
rAAV: AAV2/8.hGRK1p.h*RPGR*co-ORF15co.bGHpolyA
Proviral plasmid: pAAV.untoldR.ITR2.hGRK1.h*RPGR*co-ORF15.bGHpolyA.ITR2 (AAV2.CBA.eGFP cat. 7072 Vector Biolabs)
Production: HEK293T Hyperflask. Two-plasmid co-transfection: pDP8.ape (PlasmidFactory) + pAAV
Citation: [69,70]

Usher syndrome
ProQR - QR-421a (STELLAR) – I/II – USH2A Exon 13 Clinical trial start date (clinical trial identifier): 19-Dec-2018 (NCT03780257) RNA therapies antisense oligonucleotide exon-13 skipping Citation: [34]
Sanofi / Oxford Biomedical - EIAV-CMV-*MYO7A* (UshStat) – I/II – Usher syndrome type 1B / MYO7A Clinical trial start date + #: 6-Jan-2012 (NCT01505062); 17-Feb-2014 (NCT02065011) Lenti: EIAV.SIN-LTR.NeoR.CMVp.h*MYO7A*.WPRE.SIN-LTR Production: HEK293T co-transfection pHCMVG, pESynGP, pONY8-EIAV.CMV. h*MYO7A*.LTR (based on pLG338-SPORT. Citation: [71–73]
Stargardt disease
Sanofi / Oxford Biomedical - EIAV-ABCA4 (SAR422459) – I/II – *ABCA4* Clinical trial start date (clinical trial identifier): 7-Jun-2011 (NCT01367444); 29-Nov-2012 (NCT01736592) Lenti: EIAV.CMVp.ABCA4.LTR Plasmid: pONY8-EIAV.SIN-LTR.NeoR.CMVp.h*ABCA4*.SIN-LTR Production: HEK293T co-transfection pHCMVG, pESynGP, pONY8-EIAV.CMV.ABCA4.LTR (based on pONY4.0Z and pLG338-SPORT). Citation: [72–74]
X-linked retinoschisis
Applied Genetic Technologies Corp. (AGTC) / Biogen - rAAV2tYF-CB-hRS1 – I/II – *RS1* Clinical trial start date (clinical trial identifier): 15-Apr-2015 (NCT02416622) rAAV: AAV2/tYF.CBAp.hRS1.WPRE.SV40polyA Proviral plasmid: pTR-AAV.untoldR.ITR2.smCB.h*RS1*.WPRE.SV40polyA.ITR2 smCB= 953 bp chimeric CMV-chicken β-actin promoter (shortened hybrid chicken β-actin/rabbit β-globin intron) tYF= AAV serotype 2 with surface-exposed tyrosine mutations: Y275F, Y733F, Y447F. Production: rHSV + sBHK cells. Two-plasmid co-transfection system (pAAV, pHelper(AAV2rep.AAV2tYFCap) Citation: [75–77]
National Eye Institute (Washington DC, USA) - AAV8-RS1 – I/II – *RS1* Clinical trial start date (clinical trial identifier): 17-Dec-2017 (NCT02317887) rAAV: scAAV2/8.IRBPe.hRS1p.hRS1.hBGpolyA Proviral plasmid: pAAV.untoldR.ITR2Δ.IRBPe.hRS1p.h*RS1*.hBGpolyA.ITR2 IRBPe= interphotoreceptor retinoid-binding protein (IRBP) enhancer element hRS1p= modified tissue-selective human retinoschisin promoter hRS1=an intact human retinoschisin cDNA with a truncated first intron located in its authentic position between the exon 1 and 2 sequences hBGpolyA= human β-globin 3' UTR and polyadenylation site Production: triple co-transfection of HEK293 cells Citation: [78–80]

UntoldR, not reported in the primary literature

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