Improvement of Photoreceptor Targeting via Intravitreal Delivery in Mouse and Human Retina Using Combinatory rAAV2 Capsid Mutant Vectors

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PURPOSE. Effective intravitreal gene delivery to cells of the central retina (i.e., photoreceptors) would be of substantial benefit for treating patients with retinal diseases, such as achromatopsia, where retinal detachment from a subretinal may be harmful. Previous studies demonstrated that mutation of the recombinant adeno-associated virus (rAAV) capsid through introduction of peptide insertions or amino acid substitutions dramatically alters vector tropism. Herein, we evaluate the photoreceptor transduction efficiency of three rAAV2/2-based capsid mutant vectors: rAAV2/2[7m8], rAAV2/2[QuadYF+TV], and a chimeric vector incorporating both mutations (termed rAAV2/2[MAX]) following intravitreal delivery in mice. Furthermore, we evaluate the transduction efficiency of rAAV2/2[MAX] using explanted human central retinal samples to address clinical translatable.

METHODS. Vectors containing a GFP or mCherry reporter gene were intravitreally injected into C57BL/6J or Nrl-EGFP mice, respectively. Transduction was assessed in vivo utilizing a custom multilume confocal scanning laser ophthalmoscope. Injected Nrl-EGFP mouse retinas were used to quantify transduced photoreceptors using flow cytometry. Postmortem human retinal tissue was cultured following administration of rAAV2/2[MAX]. C57BL/6J retinas and human explants were cryosectioned to determine vector tropism.

RESULTS. The chimeric vector rAAV2/2[MAX] transduced significantly higher proportions of the retina than did either single mutant serotypes following intravitreal delivery in murine retina, including inner retinal cells and photoreceptors. Vector rAAV2[MAX] demonstrated transduction of human photoreceptors and ganglion cells.

CONCLUSIONS. Transduction observed via rAAV2/2[MAX] indicates that combining mutations with complementary mechanisms of action in a single vector results in enhanced transduction. rAAV2/2[MAX] also presented the ability to transduce human photoreceptors and ganglion cells, indicating potential for efficient intravitreal vector delivery.

Keywords: gene therapy, adeno-associated virus, capsid mutation, intravitreal injection

Vectors based on recombinant adeno-associated virus (rAAV) have emerged as useful clinical tools for delivering genetic material to the RPE and the neurosensory retina, with Phase I/II clinical trials for the treatment of Leber’s congenital amaurosis,1,2 choroideremia,3 Leber’s hereditary optic neuropathy,4 and achromatopsia, demonstrating both safety and efficacy. As the majority of inherited retinal diseases occur due to dysfunction in photoreceptors or RPE,5,6 clinical trials have relied on subretinal delivery approaches to administer vector to the targeted cells.1,5,7,8 Unfortunately, performing subretinal injections in patients with a degenerative retinal disease has led to surgical complications in the past, such as central retinal thinning and macular hole formation.1,9 Consequently, the development of rAAV vectors that can efficiently deliver genetic material to photoreceptors and RPE cells following a minimally invasive intravitreal injection is of great importance.

Early work demonstrated that transduction of mouse retina is possible via intravitreal injection using an unmodified rAAV serotype 2 vector (termed herein rAAV2/2); however, expression is limited to ganglion and inner retinal cells.10 The inability of rAAV2/2 to penetrate farther into the retina is largely due to its strong affinity for heparin sulfate proteoglycan (HSPG), which results in the majority of injected rAAV2/2 virions becoming irreversibly bound to the inner limiting membrane (ILM) before reaching the photoreceptors.11 Recently, efforts have been made to alter the properties of rAAV vectors to enhance photoreceptor transduction following intravitreal injection through modification of the capsid, the external protein shell of the virus that mediates cell binding and entry. Capsid modifications fall broadly into two classes: point mutations and peptide insertions. The former class involves substitution of one or more surface-exposed amino acids that are prone to phosphorylation with residues that have similar physical properties (e.g., size and shape), but lack a terminal hydroxyl group.12–14 These substitutions—for example, tyrosine to phenylalanine (Y-F) and threonine to valine (T-V)—help to prevent ubiquitin-mediated proteasome degradation of the virion upon cellular entry and therefore increase the efficiency of transgene delivery to the nucleus. The later class involves the insertion of short (7–12 amino acid) peptide sequences into an
external loop domain (typically loop IV) of the virus structural protein(s) (VP1-3), which causes altered cell surface/membrane binding interactions, such as the reduction in HSPG binding, leading to an improvement in tissue penetration and cell targeting.15–18

In recent years, a rAAV2/2 capsid mutant variant containing five point mutations (Y272E, Y444F, T491V, Y500E and V730E, termed herein, rAAV2/2[QuadYF+TV])14 and a capsid mutant containing a retina-specific 7m8 peptide insertion (N587_R588insLALGETTRPA; termed herein, rAAV2/2[7m8])19 have both been shown to exhibit improved photoreceptor transduction following intravitreal injection compared to unmodified rAAV2/2. As the mechanistic action of each mutation class is largely independent, we hypothesized that inclusion of both a peptide insertion and multiple amino acid substitutions into a single rAAV2/2 capsid would result in an additive increase in photoreceptor transduction from the vitreous.

To evaluate our hypothesis, we generated a rAAV2/2-based hybrid vector containing five point mutations (Y272E, Y444F, Y500E, V730E, T491V) in addition to the 7m8 peptide (termed herein, rAAV2/2[MAX]). We subsequently compared the transduction profile of rAAV2/2[MAX] with that of rAAV2/2 (unmodified vector), rAAV2/2[QuadYF+TV] (point mutation class vector), and rAAV2/2[7m8] (peptide insertion class vector) following intravitreal injection in mice using a combination of confocal scanning laser ophthalmoscope (cSLO) imaging, histology, and flow cytometry. Lastly, due to the observed variance in transduction pattern following intravitreally delivered rAAV in mouse and non-human primate models,19,20 we sought to assess the potential tropism of rAAV2/2[MAX] in the human retina ex vivo using postmortem human central retinal explants.

METHODS

Animals
Animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research Guidelines and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Four- to 6-week-old C57BL/6j and B6.Cg-Tg(Nrl-EGFP)1Aaw/j (Nrl-EGFP) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and housed in a 12-hour-light and 12-hour-dark photoperiod with food and water ad libitum.

Cell Culture
HEK293T cells were obtained from ATCC (no. CRL-11268; Manassas, VA, USA) and cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA). Media was supplemented with 2% FBS and 1% antibiotic-antimycotic. Seventy-two hours post transfection, purification of AAV vector was determined by using a PicoGreen assay (Thermo Fisher Scientific, Waltham, MA, USA)22; all vectors were titer matched to 5.2 × 10^{12} vector genomes (vg)/mL using HBSS as a diluent prior to injection.

Intraocular Injections
C57BL/6j mice or Nrl-EGFP mice (Jackson Laboratory) were anesthetized by a single intraperitoneal injection of ketamine (60 mg/kg; Biomiche, Galway, Ireland) and xylazine (10 mg/kg; Lloyd, Inc., Shenandoah, IA, USA). Pupil dilation was carried out by application of topical mydriatics, namely 2.5% phenylephrine HCl (Paragon BioTech, Portland, OR, USA) and 1% tropicamide (Akorn, Lake Forest, IL, USA). All study animals received bilateral intravitreal injections of purified rAAV vector, packaging either a ubiquitously expressing GFP or mCherry reporter construct, using a Hamilton syringe and attached 33-G needle via a transscleral injection route. All animals received a total of 1.0 × 10^10 vg per eye in 2 μL total volume.

cSLO Imaging
Animals were imaged using a custom multiline cSLO (modified Spectralis HRA; Heidelberg Engineering, Heidelberg, Germany) as described previously.23 Camera alignment was performed using the near-infrared (820 nm) reflectance-imaging mode; EGFP expression was visualized using a 486-nm excitation laser and 502- to 537-nm band pass emission filter. rAAV-derived mCherry fluorescence was visualized using a 561-nm excitation laser and 582-nm high-pass emission filter. Images were pseudocolored in ImageJ (developed by Wayne Rasband and provided in the public domain by the National Institutes of Health, Bethesda, MD, USA, at http://rsb.info.nih.gov/ij/index.html).

Histology
Whole eyes were enucleated from mice post mortem, with the anterior chamber, iris, and lens dissected off and the posterior pole fixed in 4% paraformaldehyde overnight at 4°C. Eyecups were subsequently cryoprotected in 30% sucrose in 10 mM PBS at 4°C for 24 hours before being embedded in optical cutting temperature (OCT) media. Retinal explants were similarly fixed and cryoprotected in their tissue culture inserts before being transferred onto a glass slide using a hippocampal tool to enable imaging. Explants were embedded in situ on the glass side following imaging; briefly, sufficient OCT media was added to the explant to cover it before the slide was placed on dry ice until the OCT media hardened. The embedded retinal explant was then transferred to an appropriate mold for cryosectioning. All tissues were subsequently cryosectioned (14-μm slices) using a Microm HM 505 E cryostat (Thermo Fisher Scientific), and each section was collected and stored at −20°C until use. For samples that underwent immunohistochemical staining, slides were thawed for 2 hours at room temperature. Sections were permeabilized in PBS + 0.1% Triton X-100 (Sigma Aldrich) for 20 minutes. Slides were subsequently blocked with a solution of 10% normal goat serum (ab74481; Abcam, Cambridge, MA, USA), 0.1% Tween-20 (Sigma Aldrich), and PBS for 1 hour. Following blocking, sections were
incubated for 24 hours at 4°C with the primary antibody (1:1000 anti-recoverin: Millipore AB5585 or 1:500 glial fibrillary acidic protein [anti-GFAP] (G3893; Sigma Aldrich) diluted in a solution containing 1% normal goat serum, 0.1% Tween-20, and PBS. The next day, the samples were washed four times for 20 minutes with 0.1% Tween-20 and PBS (PBST). Samples were incubated for 1 hour at room temperature in 1:500 fluorescent-conjugated secondary antibody (donkey anti-rabbit Alexa Fluor-555 or donkey anti-mouse Alexa Fluor-555) in PBST and washed for 20 minutes four times. Samples were counterstained with Hoechst 33342 (Thermo Fisher Scientific) and subsequently washed once. Histologic sections were imaged on a confocal microscope (Nikon Eclipse 80i; Nikon, Minato, Tokyo, Japan) with a 20× objective. Explant flat mounts were imaged initially on an inverted fluorescent microscope (Leica DMIL; Leica, Wetzlar, Germany) using a 10× objective. All images were processed in ImageJ software, creating stacked maximum-intensity projections.

Flow Cytometry

Dissociation of mouse retinas was carried out using the Worthington Biochemical Corporation Papain Dissociation System (Lakewood, NJ, USA) with the following modifications. Mice were euthanized with CO2 and eyes enucleated for dissection. Each retina was individually removed from the eyecup, dissected into small fragments using Vannas scissors, and dissociated at 37°C on a rocking platform for 1.5 hours in 715 μL papain with 35 μL DNase. Each sample was manually agitated until retinal pieces were no longer visible and the solution appeared homogeneous. Samples immediately underwent fixation through the addition of 250 μL 10% paraformaldehyde (to a final concentration of 4%), omitting centrifugation, resuspension, and discontinuous density gradient separation steps. GFP and mCherry expression were quantified using a BD LSRII flow cytometer. C57BL/6J retinas (no fluorescence) and uninjected Nrl-EGFP (green fluorescence only) served as gating controls. Ten thousand events were recorded for each sample using the LSRII flow cytometer. C57BL/6J mice at postnatal week (PW) 4. Four weeks post injection (PWS), GFP intensity and distribution was assessed in vivo using cSLO imaging (Supplementary Fig. S1A–H). Retinal GFP fluorescence was present in all injected eyes, with distribution and overall transduction levels varying between each capsid. Notably, rAAV2/2[MAX] demonstrated substantially greater levels of transduction than both the unmodified (rAAV2/2) and single mutation class vectors (rAAV2/2[QuadYF+TV] and rAAV2/2[7m8]), with high levels of GFP expression in both the central and peripheral retina. This finding strongly indicated an additive effect on transduction from an intravitreal delivery when combining capsid mutations with different mechanisms of action. In order to determine the retinal cell types transduced by each of the vectors, postmortem histologic retinal sections were examined for all mice. In line with previous studies, intravitreal injection of unmodified rAAV2/2 vector resulted in widespread transduction of ganglion cells and Müller glia (Fig. 1B). Photoreceptor transduction was only apparent in eyes (n = 3, all groups) injected with capsid mutant vectors, with rAAV2/2[MAX] demonstrating substantially higher levels of photoreceptor transduction than either the rAAV2/2[QuadYF+TV] or rAAV2/2[7m8] single-class mutants (Fig. 1B–E). We subsequently confirmed photoreceptor gene expression by performing immunohistochemical staining for recoverin, a photoreceptor-specific calcium-binding protein. A GFP reporter driven either by a strong ubiquitously expressing promoter (CBA) or a photoreceptor-specific promoter (GRK1) was packaged into rAAV2[MAX] and intravitreally injected into PW6 C56BL/6j wild-type mice (1.0 × 1010 vg/eye; n = 3 eyes per group) in C57BL/6 j mice at postnatal week (PW) 4. Postmortem histology revealed consistently high numbers of GFP-positive cells located in the outer nuclear layer (ONL) and that colocalized with recoverin staining for both vectors (Fig. 2A, B), indicative of high-level photoreceptor transduction. In rAAV2/2[MAX]–GRK1.GFP–injected eyes, GFP expression was largely restricted to the ONL, with substantially fewer inner

RESULTS

Intravitreal Transduction Profile of rAAV2/2-based Capsid Mutants

We first sought to investigate whether combining capsid mutation classes within a single rAAV capsid has an additive effect on transduction efficiency following intravitreal injection using a rodent model. To this end, we modified the canonical rAAV2/2 capsid via insertion mutagenesis to generate a hybrid vector (termed herein, rAAV2/2[MAX]) that incorporates five single amino acid substitutions (Y272F, Y444F, T491V, Y500F, and Y730F) derived from rAAV2/2[QuadYF+TV]14 and a peptide insertion (NS878_RS588sILAGETTRPA)19 derived from rAAV2/2[7m8] (Fig. 1A). A ubiquitously expressing fluorescent reporter construct (CBA-GFP) was packaged into unmodified rAAV2/2, rAAV2/2[QuadYF+TV], rAAV2/2[7m8], or rAAV2/2[MAX] and injected intravitreally (1.0 × 1010 vg/eye; n = 3 eyes per group) in C57BL/6 j mice at postnatal week (PW) 4. Four weeks post injection (PWS), GFP intensity and distribution was assessed in vivo using cSLO imaging (Supplementary Fig. S1A–H). Retinal GFP fluorescence was present in all injected eyes, with distribution and overall transduction levels varying between each capsid. Notably, rAAV2/2[MAX] demonstrated substantially greater levels of transduction than both the unmodified (rAAV2/2) and single mutation class vectors (rAAV2/2[QuadYF+TV] and rAAV2/2[7m8]), with high levels of GFP expression in both the central and peripheral retina. This finding strongly indicated an additive effect on transduction from an intravitreal delivery when combining capsid mutations with different mechanisms of action. In order to determine the retinal cell types transduced by each of the vectors, postmortem histologic retinal sections were examined for all mice. In line with previous studies, intravitreal injection of unmodified rAAV2/2 vector resulted in widespread transduction of ganglion cells and Müller glia (Fig. 1B). Photoreceptor transduction was only apparent in eyes (n = 3, all groups) injected with capsid mutant vectors, with rAAV2/2[MAX] demonstrating substantially higher levels of photoreceptor transduction than either the rAAV2/2[QuadYF+TV] or rAAV2/2[7m8] single-class mutants (Fig. 1B–E). We subsequently confirmed photoreceptor gene expression by performing immunohistochemical staining for recoverin, a photoreceptor-specific calcium-binding protein. A GFP reporter driven either by a strong ubiquitously expressing promoter (CBA) or a photoreceptor-specific promoter (GRK1) was packaged into rAAV2[MAX] and intravitreally injected into PW6 C56BL/6j wild-type mice (1.0 × 1010 vg/eye; n = 3 eyes per group). Postmortem histology revealed consistently high numbers of GFP-positive cells located in the outer nuclear layer (ONL) and that colocalized with recoverin staining for both vectors (Fig. 2A, B), indicative of high-level photoreceptor transduction. In rAAV2/2[MAX]–GRK1.GFP–injected eyes, GFP expression was largely restricted to the ONL, with substantially fewer inner
retinal or ganglion cell neurons transduced compared to rAAV2/2[MAX]-CBA.GFP-injected eyes. These data strongly support the ability of the rAAV2/2[MAX] capsid mutant vector to mediate robust transduction of photoreceptor cells following intravitreal administration. It is interesting that retinal sections of rAAV2/2-injected animals displayed minimal GFAP staining (Supplementary Fig. S2), indicating a relative absence of glial cell activation 4 weeks post intravitreal delivery.

Quantification of Rod Photoreceptor Transduction

To quantify the level of photoreceptor transduction for each capsid mutant vector following intravitreal injection, we employed a transgenic mouse model (B6/CgTg(Nrl-EGFP)1ASW/J, known herein as Nrl-EGFP) that selectively expresses EGFP in rod photoreceptors, which comprise approximately 97% of the photoreceptors in a mouse retina. It is interesting that retinal sections of rAAV2/2[MAX]-injected animals displayed minimal GFAP staining (Supplementary Fig. S2), indicating a relative absence of glial cell activation 4 weeks post intravitreal delivery.

**FIGURE 1.** Evaluation of retinal transduction in wild-type C57BL/6J mice following intravitreal delivery. (A) Diagrammatic representation of point mutations and insertions in AAV2-based rep and cap plasmid for each capsid mutant. Representative confocal fluorescent microscopy images of retinal cross-sections of C57BL/6J animals injected with 1.0 × 10^10 vg (B) rAAV2/2, (C) rAAV2/2[QuadYF+TV], (D) rAAV2/2[7m8], and (E) rAAV2/2[MAX], encapsulating a ubiquitous expressing GFP reporter. Counterstain: Hoechst33342. Scale bar: 40 μM, 20X objective.

red fluorescent reporter construct (CBA-mCherry). Four weeks post injection (PW8), infrared reflectance, intrinsic (i.e., transgenic) EGFP and rAAV-derived mCherry fluorescence was assessed using a custom multiline cSLO instrument that allows fluorophores with green (e.g., EGFP) and red (e.g., mCherry) emission spectra to be imaged independently within the same retina (Fig. 3; see Methods for specifications). Infrared reflectance imaging was initially employed to align the cSLO with the fundus and locate the ONL, which is identified in vivo as the confocal plane having the highest level of reflectance (Fig. 3 A–D). cSLO imaging of intrinsic EGFP expression revealed a uniform expression pattern throughout the retina in all groups; the absence of any reduced EGFP signal within the retina in Nrl.EGFP mice following intravitreal injection of rAAV indicates a lack of photoreceptor toxicity arising from vector delivery (Fig. 3E–H). Consistent with our previous findings (Supplementary Fig. S1), imaging of mCherry fluorescence revealed poor transduction throughout the retinas of rAAV2/2 vector-injected animals (Fig. 3I), while increasing levels of pan-retinal expression was detected in animals injected with rAAV2/2[QuadYF+TV], rAAV2/2[7m8],
and rAAV2/2[MAX] vectors (Fig. 3J–L). Following cSLO imaging, retinas were papain-dissociated individually and the number of rod photoreceptor (GFP+ mCherry+) and nonrod photoreceptor (GFP-mCherry+) cells transduced for each capsid mutant serotype quantified by flow cytometry, as previously described.25,26 Papain dissociation of each retina generated a suspension of single cells of differing size and granularity, as expected, indicating recovery of a heterogeneous population comprising cells from all retinal cell layers (Fig. 4A). Dissociated retinas from uninjected C57BL/6J wild-type (nonfluorescent) and Nrl-EGFP (GFP+) mice were used as gating controls (Fig. 4B, 4C). Intravitreally delivered unmodified rAAV2/2 (n = 7) displayed only modest transduction (3.9% ± 1.5%) of rod photoreceptors (Fig. 4D, Supplementary Fig. S3A). By contrast, rAAV2/2[QuadYF+TV] (n = 5) and rAAV2/2[7m8] (n = 5) showed dramatically increased levels of rod photoreceptor transduction (11.2% ± 2.5% and 14.0% ± 3.8%, respectively; Fig. 4E, 4F, Supplementary S3B, S3C). In line with our in vivo imaging and prior histologic observations, rAAV2/2[MAX] (n = 5) exhibited significantly higher rod photoreceptor transduction (32.4% ± 10.6%) compared to all other capsid variants tested (Fig. 4G, 4H, Supplementary Fig. S3D; P < 0.001, 1-way ANOVA with Tukey’s post hoc test), with an 8.2-fold increase in rod transduction compared to unmodified rAAV2/2. Furthermore, a significantly greater number of nonrod cells in the retina were also transduced by rAAV2/2[MAX] (Fig. 4I; P < 0.01, 1-way ANOVA with Tukey’s post hoc test, n = 5–7) with a 6.8-fold increase in transduction compared to rAAV2/2. Taken together, these findings clearly demonstrate that combining capsid mutations with differing modes of action into a single vector results in an additive increase in retinal transduction. The resulting rAAV2/2[MAX] vector is able to mediate effective gene delivery to a significant proportion of photoreceptors following intravitreal delivery.

Evaluating the Tropism of rAAV2/2[MAX] Capsid Mutant Vector in ex vivo Human Retinal Culture

To determine whether the rAAV2/2[MAX] capsid mutant vector is able to transduce human central retina, postmortem...
posterior poles with no ocular phenotype were obtained from Lion’s Eye Bank of Wisconsin. An approximately 5 × 5-mm section of the central retina containing the fovea was dissected out using the optic disk, superior/inferior arcades, and macular luteal pigment as anatomic references (Fig. 5A). The central retinal explant was immediately transferred to organotypic culture and placed photoreceptor-side down prior to the addition of 2.4 × 10^{11} vector genomes of rAAV2/2[MAX] packaging a ubiquitously expressing GFP construct (sc-CBA-GFP) to the ganglion cell aspect. Successful transduction of the central retina was observed by flat-mount fluorescent microscopy following 7 days of culture (Fig. 5B). Histologic sections of the human tissue revealed expression in the ganglion and photoreceptors cells; however, no transduction was evident in the inner nuclear layer (INL) (Fig. 5C). While this finding indicates that rAAV2/2[MAX] is able to effectively transduce human ganglion cells and photoreceptors, it remains unclear whether preferential targeting of these cell types is an artifact of the organotypic culture environment (see Discussion).

**DISCUSSION**

Herein, we describe a hybrid rAAV2/2 vector (rAAV2/2[MAX]) that incorporates capsid mutations of two independent classes—single amino acid substitutions (QuadY+TV) that mediate virion escape from proteolytic degradation and a peptide insertion (7m8) that improves cellular entry—leading to a significant increase in photoreceptor transduction following intravitreal delivery.

We initially evaluated the retinal transduction profile of rAAV2/2[MAX] against that of unmodified rAAV2/2 and rAAV2/2-based capsid mutant vectors rAAV2/2[QuadYF+TV] and rAAV2/2[7m8] in wild-type C57BL/6j and transgenic Nrl-EGFP reporter mice using cSLO imaging. Imaging of wild-type C57BL/6j mice following intravitreal injection of a ubiquitously expressing GFP reporter construct demonstrated that rAAV2/2[MAX] mediated an altered transduction pattern of the retina compared to unmodified rAAV2/2 or either AAV2/2-based single mutant class vectors. Using a custom multiline cSLO instrument, it was possible for the first time to image both green (i.e., EGFP) and red (i.e., mCherry) fluorescent proteins independently within a single mouse retina in vivo. Dual color imaging of Nrl-EGFP mice following intravitreal delivery of an mCherry reporter construct crucially revealed no evidence of toxicity for any vector evaluated, including rAAV2/2[MAX], in spite of its improved retinal penetrance. Although cSLO images of rAAV2/2[MAX]-injected eyes appear to have higher signal strength than either singly modified capsid mutant vector, it is
not possible to determine in vivo which cell types are transduced with any certainty. To more accurately determine the transduction profile of each capsid we utilized postmortem histology of C57BL/6J-injected mice. rAAV2/2 sections demonstrated minimal photoreceptor transduction, while rAAV2/2[QuadYF+TV] and rAAV2/2[7m8] both had moderate levels of transduction, primarily in the inner retina and ganglion cell layers (GCLs). By contrast, rAAV2/2[MAX]-injected eyes appeared to have widespread transgene expression evident in the all layers of the retina, including substantially higher levels of transduction in the photoreceptor layer. Using recoverin as a marker of the photoreceptor cell layer, we were able to confirm that rAAV2/2[MAX] delivery to the vitreous resulted in high levels of transduction in photoreceptor cells. To validate this finding, we then injected the rAAV2/2[MAX] vector packaging a photoreceptor-specific reporter construct (GRK1-GFP) that is known to substantially reduce off-target (i.e., nonphotorecep-

**Figure 4.** Transduction of rod photoreceptors following intravitreal injection of rAAV capsid mutants quantified by flow cytometry. (A–C) Dot plots showing gating controls and complete data sets of samples individually run for (D) rAAV2/2 (n = 7), (E) rAAV2/2[QuadYF+TV] (n = 5), (F) rAAV2/2[7m8] (n = 5), and (G) rAAV2/2[MAX] (n = 5) injected eyes; all vectors packaged a ubiquitously expressing mCherry reporter and were injected intravitreally in adult (PW4) Nrl-EGFP mice. Transduction efficiency of each vector is expressed as the percentage of rod photoreceptor (GFP+) cells that demonstrated reporter gene expression (mCherry+, Q2; D–F). A significant difference in rod photoreceptor transduction (H) and non-rod neural retinal transduction (I) was seen between rAAV2/2[MAX] and rAAV2/2, rAAV2/2[QuadYF+TV], or rAAV2/2[7m8] (1-way ANOVA with Tukey’s post hoc test, **P < 0.01, ***P < 0.001, ****P < 0.0001).
Eyes injected intravitreally with rAAV2/2[MAX] packaging GRK1-GFP demonstrated substantial levels of photoreceptor transduction and decreased levels of off-target expression. Our in vivo imaging studies and subsequent histologic examinations strongly support the hypothesis that inclusion of capsid mutations of different class within a single capsid results in an additive increase transduction that allows more effective photoreceptor targeting from the vitreous.

In order to accurately quantify the number of photoreceptors transduced for by each capsid mutant vector we utilized flow cytometry. Flow sorting of transgenic Nrl-EGFP–expressing rods following rAAV-mediated intravitreal delivery of an mCherry reporter construct was found to be a highly reproducible method of quantifying transgene delivery efficiency. Photoreceptor transduction was observed to be significantly improved following intravitreal delivery of rAAV2/2[MAX] vector (1.0 × 10¹⁰ vg/eye), with approximately 34% of rod photoreceptors transduced. In addition to improved rod photoreceptor transduction, flow cytometry revealed that transduction of nonrod cells (e.g., inner retinal neurons and ganglion cells) was also significantly increased following rAAV2/2[MAX] injection compared to rAAV2/2 or rAAV2/2-based vectors containing only a single class of mutation (rAAV2/2[QuadYF+TV] or rAAV2/2[QuadYF+TV]). The improvement of transduction throughout the retina strongly indicates that the two mechanisms contributing to improved rAAV transduction efficiency, namely decreased proteasome degradation and increased cellular entry, are independent and additive.

Both rAAV2/2[QuadYF+TV] and rAAV2/2[QuadYF+TV] single-class vectors were found to be broadly similar with regard to their transduction of rod (8.6% vs. 11.3%) and nonrod cells (11.2% vs. 14%) following intravitreal administration. This finding appears to be at variance with data generated during our earlier imaging studies (Fig. 3 and Supplementary Fig. S1), where intravitreal injection of rAAV2/2[QuadYF+TV] appeared to result in more extensive retinal transduction. It is worth noting, however, that interpretation of in vivo cSLO fluorescence images is complicated by two main factors. First, cSLO imaging using a standard 55-degree lens allows visualization of only approximately the central third of the fundus and as such would not record any differences in the extent of peripheral retinal transduction. Given the divergent mechanisms by which the QuadYF+TV and 7m8 capsid mutations confer improved retinal transduction and known anatomic variations in retinal structure between central and peripheral eccentricities (e.g., thickness of the inner plexiform layer, GCL, and nerve fiber layer), it is conceivable that the rAAV2/2[QuadYF+TV] vector more efficiently transduced peripheral retina, which would not be observed on cSLO imaging. Second, signal strength in fluorescence cSLO imaging is directly proportional to the amount of fluorophore within a cell. As a consequence, large, highly arborized neurons (e.g., ganglion cells) or glia (e.g., Müller cells) with proportionally larger cytoplasmic volumes generate greater signal than smaller, non- or minimally arborized cells (e.g., photoreceptors). It is important that while the size and intensity of cellular fluorescence can dramatically impact the appearance of a cSLO image, they are largely irrelevant in flow cytometry once correct gating has been established relative to controls. Indeed, in a dissociated suspension, a small dimly fluorescent cell and a large brightly

**FIGURE 5.** Transduction of human central retina by rAAV2/2[MAX] packaging a ubiquitously expressing GFP reporter. (A) Representative infrared reflectance cSLO image showing the tissue dissection area: an approximately 5-mm² square of retina bordered by the arcades and containing the macula at the center. (B) Distinct single cells (punctate white dots) were visible when viewed from the photoreceptor aspect of the macular explant (10X objective). (C) Photoreceptor and ganglion cell transduction was observed upon histologic examination of the fixed and cryopreserved explant (20X objective). (A) Scale bar: 1.77 mm, (B, C) Scale bar: 50 μm.
fluorescent cell are both counted by a cytometer as a single event. The apparent discrepancies highlighted herein strongly reinforce the critical necessity of confirming findings obtained through qualitative assessments, such as in vivo cSLO imaging, with a reproducible quantitative approach, such as flow cytometry, that allows unambiguous and objective measurement of transduction efficiency.

While we were able to largely restrict transgene expression to photoreceptors using a cell-specific promoter (Fig. 2), the ability of the rAAV2/2[MAX] vector to transduce multiple different cell types within the retina may in many cases be beneficial. Indeed, one expects that it would be fundamental for the development of successful gene-based therapies for diseases where gene expression is required in multiple tissue layers, such as X-linked retinoschisis, a retinal dystrophy characterized by foveal schisis and delamination of the retina due to the absence of RS1 protein secretion from photoreceptors and bipolar cells.27 Similarly, the improved retinal penetrance of the rAAV2/2[MAX] vector may facilitate treatment of nondegenerative diseases originating from dysfunction of the inner retina, such as congenital stationary night blindness.

Although pan-retinal expression was attained from an intravitreal injection of rAAV2/2[MAX], transduction was not uniform throughout the retina. Indeed, cSLO imaging revealed transgene expression to be unevenly distributed in all capsid mutant vectors tested, with the highest level of expression regularly seen near the optic disk and retinal vasculature. It is likely that these differences are due largely to anatomic differences across the retina with respect to the ILM, a physical barrier comprised of HSPG that separates the retina and vitreous and is known to represent a substantial barrier to rAAV penetration.11 The transduction pattern observed in our murine-based experiments supports previous studies in non-human primates, where transduction was observed to be limited to areas where the ILM is thinner (i.e., macula, optic nerve, and major blood vessels).20 Moreover, earlier studies have demonstrated that proteolytic digestion of the ILM prior to intravitreal injection results in significantly improved photoreceptor transduction in both mouse and non-human primate animal models, confirming the role of the ILM as the major barrier to intravitreal targeting of photoreceptors.11,20,21 While effective in animal models, ILM degradation or removal (e.g., by peeling) is unlikely to be clinically feasible in the context of gene delivery to human patients with already compromised retinas, such as individuals with progressive degenerative diseases.20 The development of a vector capable of mediating improved photoreceptor targeting from the vitreous without toxicity is therefore of substantial clinical utility.

As preservation/correction of central vision is of paramount importance in patients suffering retinal dysfunction (e.g., achromatopsia) or degeneration (e.g., retinitis pigmentosa), targeting of foveal cone photoreceptors is essential for the development of an effective gene therapy treatment. We propose that this may be best achieved using a minimally invasive intravitreal delivery technique, wherein a small volume of undiluted capsid mutant virus vector with high penetrance, such as rAAV2/2[MAX], is delivered directly onto the fovea following vitrectomy and the eye filled with gas (to maintain pressure). By having patients remain recumbent for a prolonged period (minutes to hours) following administration of the virus to the foveal region where the ILM is thinnest (20–4000 nm),31 we hypothesize that substantial levels of photoreceptor transduction could be achieved in vivo. As virus would no longer be diluted by vitreous and is delivered directly to the target tissue (i.e., the fovea), we believe that the effective vector dose could be substantially reduced, helping to prevent off-target gene expression and limit host immunogenicity.2,32 Unfortunately, due to anatomic limitations of the mouse eye, including small size, the inability to remove vitreous effectively, the small number of cone photoreceptors, and the absence of a fovea, it was not possible to examine this hypothesis in the present study. In view of these constraints, we instead turned to an ex vivo organotypic culture system that allows for the tropism of the hybrid vector rAAV2/2[QuadYF+TV] to be determined on human retina directly. In contrast with previous studies where fresh peripheral retina was obtained during a retinectomy,33,34 we cultured central retina acquired post mortem from donor eyes. While obtaining viable postmortem retinal samples proved to be challenging, the opportunity to evaluate the transduction profile of rAAV2/2[QuadYF]+ in samples containing the macula was deemed to be of substantial clinical relevance. In order to crudely recapitulate an intravitreal delivery approach, large (5 × 5 mm) explants containing the fovea were carefully dissected out of the eyecup in a manner that ensured substantial amounts of vitreous remained associated with the retina. Explants were placed in organotypic culture, ganglion cell-side up, and the vector was administered directly into the associated vitreous rather than added freely to the culture medium. We observed that expression was limited ganglion and photoreceptor cells 7 days post infection, indicating that, unexpectedly, transduction of photoreceptors represents an artifact of the culture system, with virus accessing photoreceptors around the edges of the explant rather than traversing through the retinal layers. We propose to overcome this issue using a mock intravitreal injection, wherein the vitreous is digested from the posterior pole (e.g., using hyaluronidase) and virus incubated in the eyecup prior to dissection. Furthermore, we propose that culture time of the retinal explants be extended (>14 days), as even using self-complementary vectors with a strong, ubiquitous CMV-enhanced CBA promoter, maximal transgene expression is not observed for 2 to 3 weeks.

Due to global use of the ubiquitin-proteasome pathway throughout the body, substitution of amino acids on the capsid surface that are prone to phosphorylation (i.e., serine, threonine, and tyrosine) should improve transduction in the majority of tissues, regardless of serotype. Indeed, tyrosine-to-phenylalanine point mutations have also been incorporated in rAAV2/8 and rAAV2/9 vectors, improving the transduction in a wide range of tissues, including the retina, kidney, and pancreas.35–39 Although the 7m8 peptide was chosen due to its previously demonstrated impact on rAAV-mediated photoreceptor transduction, other cell-penetrating or cell-specific targeting peptides, such as the epidermal growth factor receptor tyrosine kinase inhibitor or human immunodeficiency virus Tat peptide,40 could be incorporated in place of the 7m8 peptide in order to improve transduction of nonretinal cell types. The insertion of some peptides can have deleterious effects on the capsid stability41 and therefore cannot be incorporated into the capsid.

Although the novel rAAV2/2[QuadYF+TV] capsid has multiple modifications, it is unlikely that it would be able to evade AAV2-derived neutralizing antibodies (i.e., monoclonal fragment antibodies) as the modifications do not affect the structure of the protrusions near the 3-fold axis (AAV2 epitopes).42 Supporting this theory, in a previous study we demonstrated that rAAV2/2[QuadYF+TV] was neutralized by serum containing antibodies raised against AAV2.43 In summary, the work presented here demonstrates that a significant improvement in retinal transduction following intravitreal delivery can be achieved by combining two classes of capsid mutations within a single rAAV2/2 capsid and that the
resultant rAAV2/2[MAX] is capable of transducing human photoreceptors in ex vivo culture.

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