Retinal Penetrating Adeno-Associated Virus

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Purpose. The most common method of delivery of genes to the outer retina uses recombinant adeno-associated virus (AAV) injected into the subretinal space using a surgical procedure. In contrast, most drugs are delivered to the retina using an intravitreal approach in an office setting. The objective of the current study was to develop AAV vectors that can reach the outer retina via intravitreal injection.

Methods. Recently, we described a molecular chaperone (Nuc1) that enhanced the penetration of small and large molecules, including AAV, into the retina. The Nuc1 amino acid sequence or a truncated version of Nuc1 (IKV) was genetically incorporated into an exposed loop of AAV2/9 VP1 protein. These novel recombinant AAV vectors expressing green fluorescent protein (GFP) or nuclear factor erythroid 2 p45-related factor 2 (Nrf2) were injected into the vitreous of C57Bl/6J or Nrf2 knockout mice, respectively. The amount of GFP expression or oxidative stress as measured by 8-Hydroxy-2′-deoxyguanosine staining in C57Bl/6J or Nrf2 knockout mice, respectively, was quantified.

Results. Incorporation of Nuc1 into AAV2/9 did *not* lead to significant expression of GFP in the murine retina. However, incorporation of IKV into AAV2/9 led to robust expression of GFP in photoreceptors and retinal pigment epithelium (RPE) via the intravitreal and subretinal routes of delivery. Furthermore, expression of Nrf2 using an IKV vector led to a reduction in oxidative stress in the retina of C57Bl/6J and Nrf2 knockout mice.

Conclusions. We have developed a novel AAV vector that enables delivery of transgenes to the outer retina of mice, including photoreceptors and RPE following intravitreal injection.

Keywords: adeno-associated virus (AAV), photoreceptors, intravitreal

Significant progress in the field of gene therapy has led to the development of a treatment for a form of blindness known as Leber's congenital amaurosis (RPE65-LCA).[1] This treatment is comprised of a recombinant adeno-associated virus serotype 2 (AAV2) vector delivered between the retina and the retinal pigment epithelium (RPE) via a surgical procedure known as "subretinal injection."[2] This procedure results in a puncture of the retina as well as a retinal detachment, risking damage to the retina[3] and, consequently, exacerbating potential loss of vision. In contrast, the most common method of delivery of drugs to ocular tissues involves injection of the drug directly into the vitreous, performed in an office setting. Intravitreal injections are routinely administered to millions of individuals suffering from diseases such as age-related macular degeneration (AMD).[4] However, intravitreal delivery of AAV2 does not generally result in virus or gene delivery to the outer retina, tha is, the photoreceptors or RPE, that are the target cells for the majority of retinal diseases.[5] In order to overcome this limitation, there is significant interest in the development of AAV vectors that can penetrate the retina via the intravitreal route of delivery.

In order to develop retinal penetrating AAV vectors, several approaches have been previously used. The most common approach is library screening,[6] whereby a highly diverse group of peptides are encoded into an exposed loop of an AAV capsid and such AAV libraries undergo multiple rounds of enrichment for infectivity of the outer retina following intravitreal injection. As the selection is typically performed in mice, such approaches have led to some AAVs that are potent in mice,[7] but less effective in nonhuman primates (NHPs), and, thus, likely not ideal for use in humans. For example, one of the first AAV capsids developed using the above approach, referred to as 7m8, was found to be highly efficient in penetrating the retina of mice,[7] but required very high titers to be effective in NHPs, leading to immune responses and damage to the retina.[8] Furthermore, this approach can suffer from strain selectivity. For example, the AAV variant AAV-PHP.B is highly neurotropic in the C57BL/6J strain of mice (in which it was selected) but fails to exhibit neurotropism in the BALB/cJ strain of mice or in NHPs.[9] In a recent study, multiple rounds of AAV selection were performed in the retina of NHPs, leading to generation of AAVs that are more promising,[10,11] but the dramatic results achieved in mice have not yet been replicated in NHPs, leaving significant room for further development of retinal penetrating AAV vectors.

A different approach, as used in this study, is to consider design of recombinant AAV capsids based on knowledge of human retinal cell surface receptors. Previously, we have

found that targeting of heparan sulphate proteoglycans (HSPGs) on the retinal cell surface allows for the design of peptides that can be effectively endocytosed by retinal cells.[12] Previously, we have described the targeting of nucleolin on the photoreceptor cell surface by peptides or aptamers.[13–16] Although nucleolin is generally expressed on the surface of rapidly dividing cells, such as cancer cells,[17,18] we have found that nucleolin is unexpectedly highly expressed on the photoreceptors of the mouse, NHP, and human retinas.[15]

Most recently, we described a novel peptide referred to as Nuc1 designed to target both HSPGs and nucleolin on the retinal cell surface.[19] Nuc1 peptide was endocytosed by a large class of retinal cells, including photoreceptors within 4 hours after intravitreal injection. Nuc1 significantly enhanced delivery of small molecules, proteins, antibodies, and very large molecules, such as AAV, into murine retinal tissues following intravitreal delivery without the need for chemical conjugation between peptide and cargo, that is, Nuc1 behaved akin to a chaperone for uptake of macromolecules by photoreceptors and other cells in the retina. Of specific interest was our observation that co-delivery of Nuc1 with AAV2/9 enhanced the infectivity of AAV2/9 for photoreceptors and RPE via both the subretinal and intravitreal routes of injection.[19] The major objective of the current study was to investigate whether Nuc1 may be genetically incorporated into the AAV2/9 capsid to generate a retinal penetrating AAV. Results of our studies, reported herein, indicate that this is indeed possible – leading to a highly potent retinal penetrating AAV.

Materials and Methods

VP1 Structure Modeling and Recombinant AAV Plasmids

A protein fragment comprised of AAV9 VP1 (PDB: 3UX1; amino acids 263:383)[20] with an Nuc1 or truncated Nuc1 (IKV) sequence inserted between amino acids 370 and 371 of AAV9 VP1, was modeled in AlphaFold 3[21] (Google Deepmind) and the resulting predicted structure was aligned and superimposed on the previously published crystal structure of AAV9 VP1[20] using UCSF ChimeraX version 1.2.5.[22]

For production of an AAV9 backbone containing the Nuc1 or IKV peptide sequences, G blocks containing the Nuc1 or IKV peptide sequences were cloned into the backbone of AAV9 VP1 between codons 370 and 371 of AAV9 VP1 using standard recombinant DNA cloning techniques, generating AAV9 backbone plasmids (AAV9 RepCap) referred to as pAAV9Nuc1 and pAAV9IKV, respectively. The CBA-green fluorescent protein (GFP) expression cassette has been described previously[23] and this was cloned into an AAV2 ITR plasmid (pAAVCAGGFP) described previously.[23] The Nrf2-expressing construct was generated by replacing the GFP cDNA in this cassette with a synthetic cDNA expressing human Nrf2,[24] generating pAAVCAGNRF2.

Production and Purification of Recombinant AAV

Recombinant AAV was produced essentially as described previously.[25] In brief, a transgene containing plasmid (pAAVCAGGFP or pAAVCAGNRF2) were co transfected with a backbone plasmid (pAAV9 or pAAV9Nuc1 or pAAV9IKV) and an adenovirus helper plasmid in HEK293T cells at approximately 70% to 80% confluence in 150 mm culture plates. Two hours prior to transfection, DMEM medium was replaced with fresh DMEM. Plasmids were precipitated using calcium phosphate and added dropwise to the culture plates. The medium was replaced with DMEM containing 10% FBS at 24 hours post-transfection. Cells and media were harvested 96 hours later. The collected cell pellet was lysed, and the clarified lysate was layered onto an iodixanol gradient (OptiPrep; Sigma-Aldrich, St. Louis, MO, USA) in centrifuge tubes. The tubes were centrifuged at 69,000 rpm in a 70Ti rotor (Beckman Instruments) at 18°C and 4 mL fractionated solution from 40% iodixanol layer was removed. The fraction was further diafiltered and concentrated using final lactated ringer's solution and glycerol added to 5% and stored the aliquoted at −80°C until further use.

AAV Titration

Viral genomes were titered by real-time quantitative PCR (RT-qPCR) using primers targeting the GFP or NRF2 transgenes, respectively. A total of 5 µL of AAV suspensions were digested with DNaseI to remove aggregated genomic DNA and then further incubated with Proteinase K to digest the AAV capsid. The remaining DNA was extracted and purified using Phenol-Chloroform and dissolved in Tris-EDTA buffer. Subsequently, the pAAVCAGGFP and pAAVCAGNRF2 plasmids were digested with SmaI and gel extracted to generate a standard curve for the GFP or NRF2 transgenes. Quantitative PCR was performed to quantify copies/µL against the standard curve using the following PCR: denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 55°C for 30 seconds. Genome copies of each vector were quantified against the standard curve.

Animals

This study was carried out in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research, set out by the Association of Research in Vision and Ophthalmology (ARVO) and was approved by Tufts University Institutional Animal Care and Use Committee (IACUC). Six-week-old C57Bl/6J mice, which are homozygous for Nrf2, and Nrf2-KO (B6.129 × 1-Nfe2l2^{tm1Ywk}/J; Stock No: 017009) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed under a 12-hour light/dark cycle. The Nrf2-KO mice were bred in an institutional animal facility and 6 to 8 weeks old mice from third and fourth filial generations were used for the study.

Subretinal and Intravitreal Injections

Mice were anesthetized by intraperitoneal injection using Ketamine (100 mg/kg; Phoenix, St. Joseph, MO, USA) and Xylazine (10 mg/kg; Lloyed Inc., Shenandoah, IA, USA) and followed by topical application of 0.5% proparacaine hydrochloride (Akorn Inc., Lake Forest, IL, USA) to the cornea. Intravitreal or subretinal injections were performed using a 32-gauge needle and a 5 µL glass syringe, as previously described.[12] Then, 1 µL of AAV vector (1.1 × 10E9 gc/µL for AAVIKVGFP and AAV9GFP or 7.6 × 10E8 gc/µL for AAVNuc1) was injected per mouse eye with 1 µg Nuc1.

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Quantitation of Transgene Expression

Expression of GFP in the retina of AAV-injected animals with or without Nuc1 was quantified by RT-qPCR. Two weeks following injection, total RNA from the retina was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was synthesized from 1 µg of RNA using the one-step high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). GFP expression was quantified using a SYBR Green (Applied Biosystems) assay on a BioRad thermocycler system (iQ5 Multicolor real-time PCR detection system) using primers 5′-GACCCCCGCCCATTGAC-3′ and 5′-GCACCCCAGGCTTTACACTT-3′ for GFP or 5′-CGGTTCCGATGCCCTGAGGCTCTT-3′ and 5′-CGTCACACTTCATGATGGAATTGA-3′ for β-Actin. Data obtained in the form of Ct-values were normalized against β-Actin internal control and fold-change was calculated using the ddCt-method.

Fundus Imaging

Mice were anesthetized as described above, pupils were dilated with 1 drop of 1% tropicamide (Sandoz Inc., Princeton, NJ, USA) and 2.5% phenylephrine (Akorn, Lake Forest, IL, USA), and the cornea was kept moist by topical application of eye lubricant (GenTeal; Alcon, Fort Worth, TX, USA). Fundus imaging was performed once weekly following intravitreal and subretinal injection using a Micron IV Retinal Imaging Microscope and StreamPix software (Phoenix Research Labs, Pleasanton, CA, USA), as described previously.[26]

N-Methyl-Nitrosourea–Induced Oxidative Stress

Mice were injected intravitreally with AAVIKV expressing Nrf2 in combination with Nuc1. Three weeks following injection, mice were administered intraperitoneally with 50 mg/kg N-methyl-nitrosourea (MNU). PBS injected mice were used as controls. After 24 hours, the eyes were harvested and cryosectioned. To detect MNU induced oxidative stress, 8-OHdG immunostaining was performed on cryosections. The sections were imaged and used for quantification of 8-OHdG positive nuclei using ImageJ (FIJI version and ImageJ-macros plug in), as described previously.[27]

Immunohistochemistry

Enucleated eyes were fixed in 4% paraformaldehyde and sectioned to 14 µm thickness using a Microm HM550 cryostat (Kalamazoo, MI, USA). Following rehydration in 1X Phosphate-Buffered Saline (PBS) for 15 minutes, cryosections were blocked with either 6% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS or using the Mouse on Mouse Detection Kit (Vector Laboratories, Burlingame, CA, USA), as necessary, for 1 hour. Following blocking, sections were incubated overnight in a moist chamber with the relevant primary antibody, that is, anti-glutamine synthetase (Rabbit polyclonal ab73593; Abcam), anti-β-III-Tubulin (chicken polyclonal ab9354; Millipore Sigma), anti-rhodopsin (a gift from Dr. Roberty Molday), anti-opsin (rabbit polyclonal AB5407; Millipore Sigma) and anti-PKCα (mouse monoclonal MA1-157; Invitrogen), anti-Nrf2 (Rabbit monoclonal ab62352; Abcam), and anti-8-OHdG (mouse monoclonal ab48508; Abcam). Sections were washed with 1X PBS and incubated with respective secondary antibodies labeled with either Alex-fluor 544 or 488 (Molecular Probes, Eugene, OR, USA). Sections were mounted in anti-fade medium containing DAPI (Vectashield-DAPI; Vector Laboratories, Burlingame, CA, USA) to counterstain the nuclei, and confocal images were captured using a Leica TCS SPE microscope (Leica Microsystems, Wetzlar, Germany).

Quantification of N-Methyl-Nitrosourea–Induced Oxidative Stress

Image analyses and quantification of 8-OHdG specifically in the outer nuclear layer (ONL) of the retina was determined using ImageJ macros plugin. After splitting the RGB-images into separate channels, the blue channel (DAPI-staining of the nuclear DNA) was used as a mask to isolate the nuclear 8-OHdG signal (red channel). The signal above the threshold and outside of that mask was defined to be cytosolic, whereas the signal outside of the mask and below the threshold was defined as the background.

Analysis of Data/Statistics

All data are presented as mean ± SD. All statistical analysis was performed using Prism 5 (GraphPad Software, Inc.), except where indicated. Studies measuring 8-OHdG positive nuclear spots were quantified using image J software and the relative recovery from the MNU induced oxidative stress was quantified by 1-way ANOVA. Any P values < 0.05 was considered as statistically significant.

Results

Incorporation of Nuc1 Peptide Sequence Into the AAV2/9 Capsid

Recently, we found that the infection of retinal cells by AAV2/9 may be enhanced if co-injected with a 16 amino acid peptide termed Nuc1 (Fig. 1A). We hypothesized whether retinal infectivity of AAV2/9 may be yet further enhanced by the genetic incorporation of the Nuc1 peptide sequence into the AAV2/9 capsid. The Nuc1 amino acid sequence (flanked by a glycine residue, total 18 aa) inserted into a fragment of AAV9 VP1 (PDB: 3UX1 amino acids 263:383) was modeled in AlphaFold 3[21] and the predicted three-dimensional structure superimposed on the previously described crystal structure of AAV9 VP1 capsid.[20] The results indicated a large but potentially viable insertion (in blue in Fig. 1B) into an exposed loop of AAV9 VP1 (in green in Fig. 1B, Supplementary Movie S1). An AAV2/9 backbone representing this recombinant predicted VP1 structure was generated using standard cloning techniques. This modified backbone was utilized to construct a recombinant AAV expressing GFP regulated by the chicken beta actin (CAG) promoter and is hereafter referred to as AAVNuc1CAGGFP.

In Vivo Delivery of AAVNuc1CAGGFP

Upon subretinal injection of AAVNuc1CAGGFP in adult (6 weeks old) C57BL/6J mice, we found that addition of the Nuc1 peptide sequence into the AAV9 VP1 capsid protein did *not* lead to significantly improved infectivity of the retina relative to AAV9CAGGFP (Fig. 2). However, cell morphology and co-staining of GFP-positive retinal cells suggested that in *some* retinas there was infection of cone photoreceptor cells

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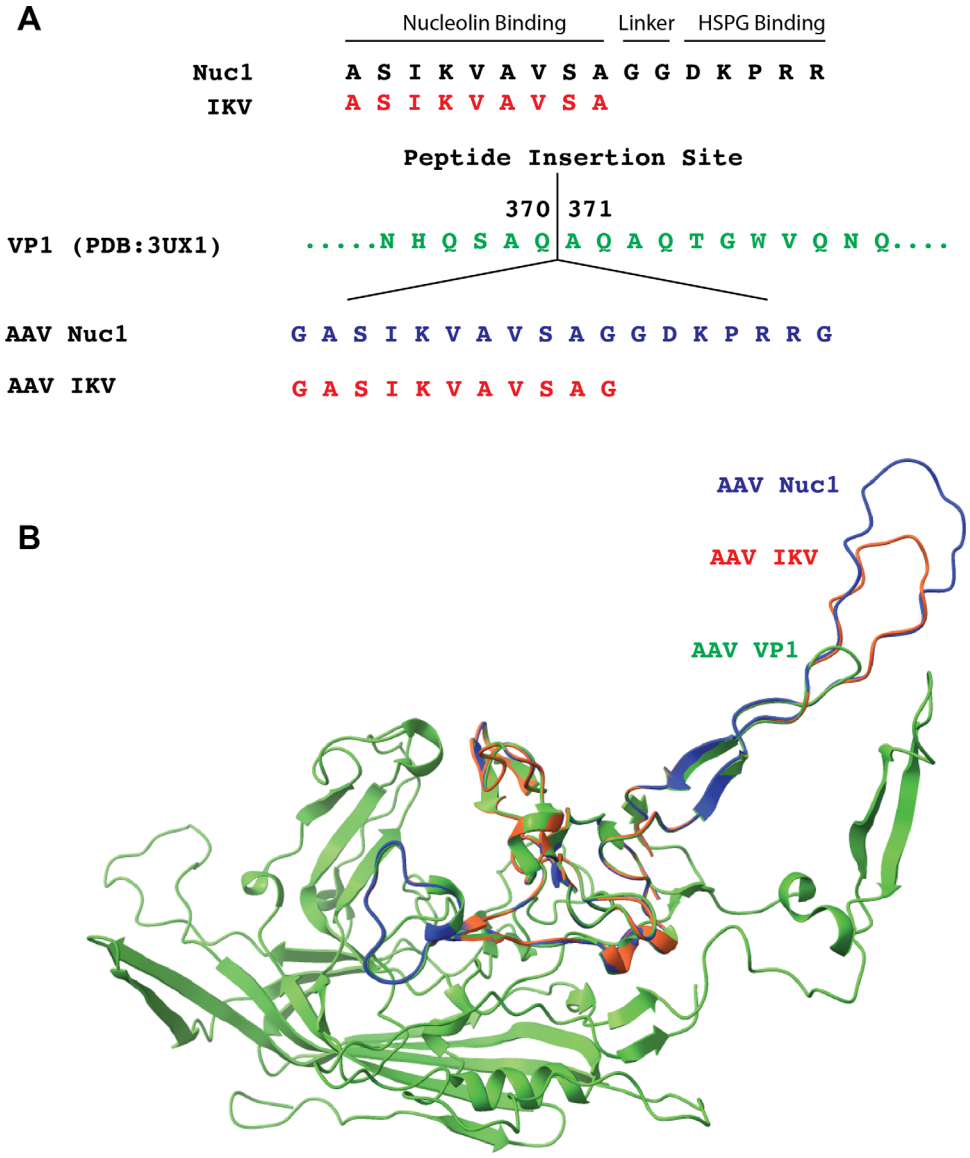

Figure 1. Modeling of Nuc1 and IKV sequences into AAV9 VP1. (**A**) The Nuc1 or truncated Nuc1 (IKV) sequences flanked by glycine residues were inserted between amino acids 370 and 371 of AAV9 VP1 (PDB: 3UX1). (**B**) A fragment of VP1 (amino acids 263:383) containing either Nuc1 or IKV was modeled in AlphaFold 3 (Google Deepmind) and the results were superimposed on the previously published crystal structure of AAV9 VP1 using UCSF ChimeraX version 1.2.5.

(see Fig. 2, see GFP section co-stained with cone opsin). Co-staining of frozen sections with PKC, glutamine synthase, cone, or rod opsin indicated that AAVNuc1CAGGFP infected primarily the rod and cone photoreceptors as well as the RPE, but all rather poorly relative to AAV9CAGGFP (see Fig. 2).

Following intravitreal injection, AAVNuc1CAGGFP failed to infect either the inner or outer retina, a result similar to that of AAV9CAGGFP (Fig. 3). However, infection of the outer retina using AAVNuc1CAGGFP or AAV9CAGFP could be enhanced when they were co-injected with Nuc1 peptide via the intravitreal route of injection (see Fig. 3B), but this result was also significantly variable for both virus constructs, where some retinas examined had limited expression of GFP in the outer retina (see Fig. 3, GFP inset). One possible explanation for this variability is competition between the Nuc1 peptide and the Nuc1 sequence in the viral capsid for binding cell surface receptors. We conclude that incorporation of an Nuc1 sequence into the AAV2/9 capsid does *not* lead to significant infection of the retina via either the subretinal or intravitreal routes of delivery.

Incorporation of a Truncated Nuc1 Sequence Into the AAV Capsid

Heparan sulphate proteoglycans are a receptor for AAV2 binding and AAV2 does not effectively penetrate the retina, possibly due to HSPGs mediated sequestration.[28,29] We considered whether the HSPG binding region within Nuc1 was possibly hindering or interfering with infectivity of AAVNuc1CAGGFP. To address this, we generated a version of AAVNuc1CAGGFP that had the heparan sulphate binding sequence (DKPRR; see Fig. 1A) of Nuc1 deleted, that is, the

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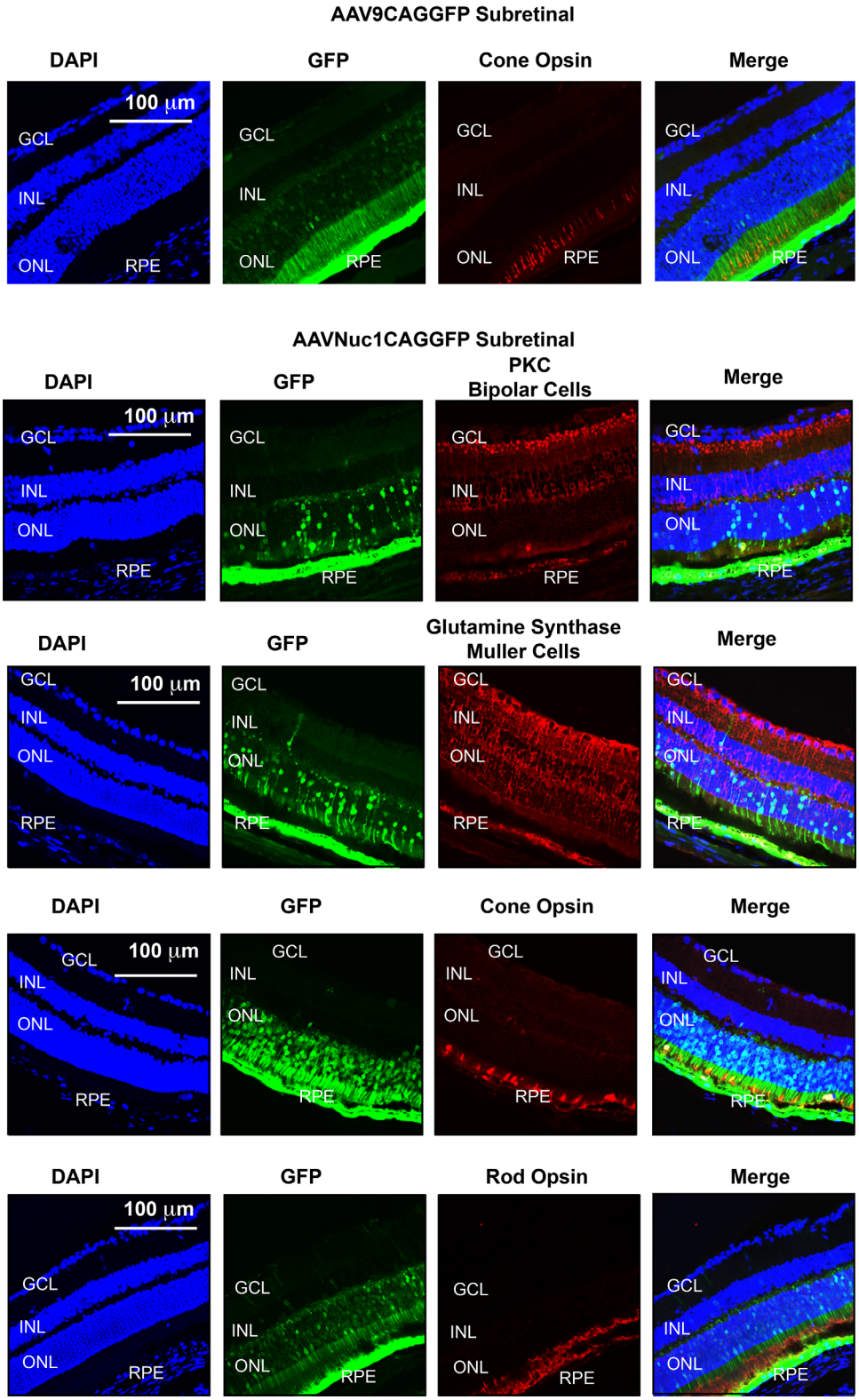

Figure 2. Incorporation of Nuc1 into the AAV capsid does not enhance infection of AAV2/9 via the subretinal route of injection. Subretinal injection of AAVNuc1CAGGFP did *not* lead to a significant infection of retinal cells relative to the parental virus AAV9CAGGFP. Co-staining of frozen sections with PKC (bipolar cells), Glutamine Synthase (Muller cells), cone, or rod opsin (photoreceptors) indicated that AAVNuc1CAGGFP infected primarily the rod and cone photoreceptors but in all cases, rather poorly relative to AAV9CAGGFP. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

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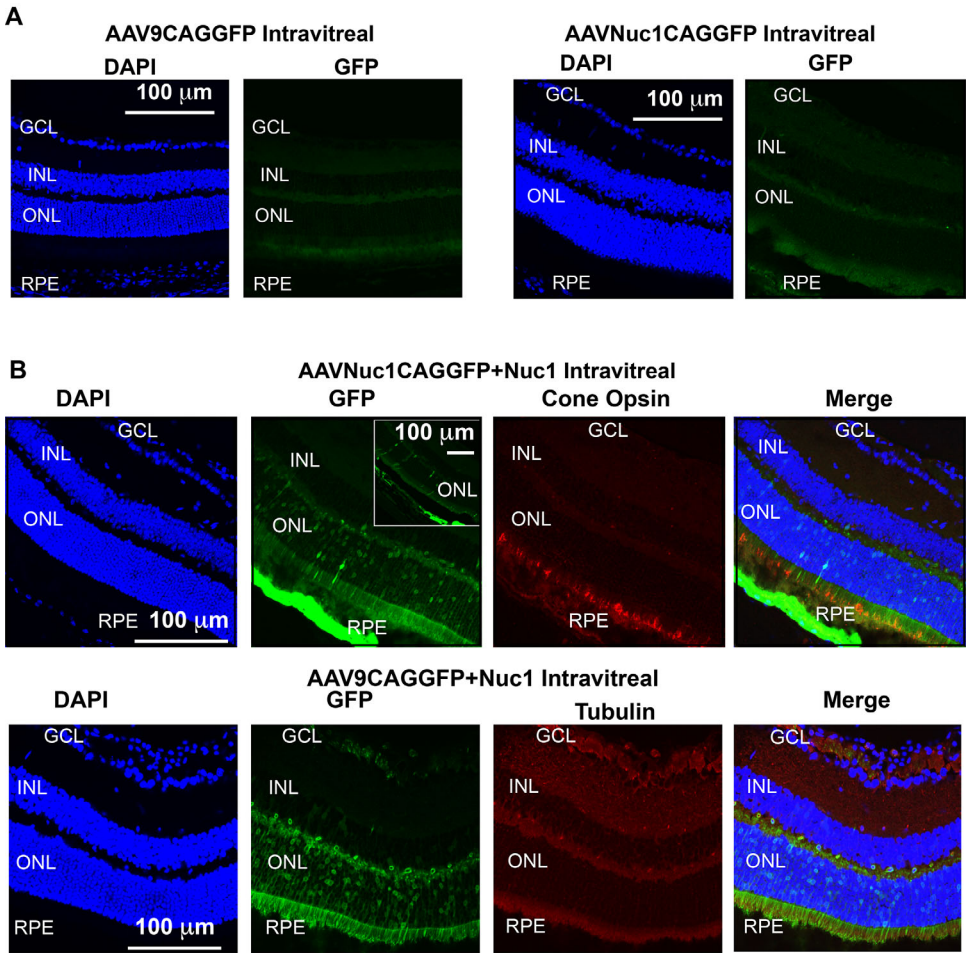

Figure 3. Incorporation of Nuc1 into the AAV capsid does not enhance infection of AAV2/9 via the intravitreal route of injection. (A) Intravitreal injection of AAVNuc1CAGGFP or AAV9CAGGFP did not lead to any significant expression of GFP in the retina. (**B**) In contrast, AAVNu1CAGGFP or AAV9CAGGFP could penetrate the retina when co injected with Nuc1. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

novel recombinant virus now contained only the nucleolin-binding sequence ASIKVAVSA. DNA encoding this shorter peptide flanked at each end by a glycine residue (total 11aa) was inserted between codons 370 and 371 of VP1 (PDB: 3UX1; see Figs. 1A, 1B) and modeled as described above; and a recombinant GFP-expressing virus generated and referred to as AAVIKVGFP.

Upon subretinal injection, we now found that AAVIKVGFP injected into 6 weeks old C57Bl/6J mice had significant infection of retinal cells (Figs. 4A–D). In all retinas examined, the photoreceptors were consistently strongly GFP positive. However, in some retinas, we could observe additional cell types that were also GFP positive. Counter staining of AAVIKVGFP infected retinal sections with antibody against rod opsin (see Fig. 4A), cone opsin (see Fig. 4B), glutamine synthase (see Fig. 4C), and PKCα (Fig. 4D) exhibited infection of rod and cone cells of the outer retina by AAVIKVGFP, with some notable transduction also of Muller and bipolar cells.

AAVIKVGFP was injected intravitreally into 6 weeks old C57BL/6J mice and frozen sections examined for expression of GFP. Again, in all retinas examined, the photoreceptors were consistently strongly GFP positive (Fig. 5A). However, in some retinas, we could observe additional cell types that were also GFP positive. Counterstaining with cone opsin (Fig. 5B), rod opsin (Fig. 5C), PKCα (Fig. 5D) or tubulin (Fig. 5E) and glutamine synthase (Fig. 5F) revealed that AAVIKVGFP infected rods and cones in addition to bipolar cells, ganglion cells, and Muller cells, respectively. These data suggest that the HSPG binding motif of Nuc1 in AAVNuc1CAGGFP is antagonistic to the binding of cell surface nucleolin by the IKV motif. Quantitation of GFP expression in the retina of the different recombinant viruses described in this study is presented below.

Nuc1 Mediated Enhancement of Retinal Penetration by AAVIKV

Previously, we have observed increasing retinal uptake of molecules by Nuc1 following intravitreal injection when co-administered with increasing amounts of exogenous Nuc1. We considered whether retinal transduction by AAVIKVGFP following intravitreal injection could be enhanced further by co-administration with Nuc1. We found that AAVIKVGFP co-injected intravitreally with Nuc1 had the most potent infection of outer retinal cells observed thus far in our studies, with robust expression throughout the retina (Fig. 6A).

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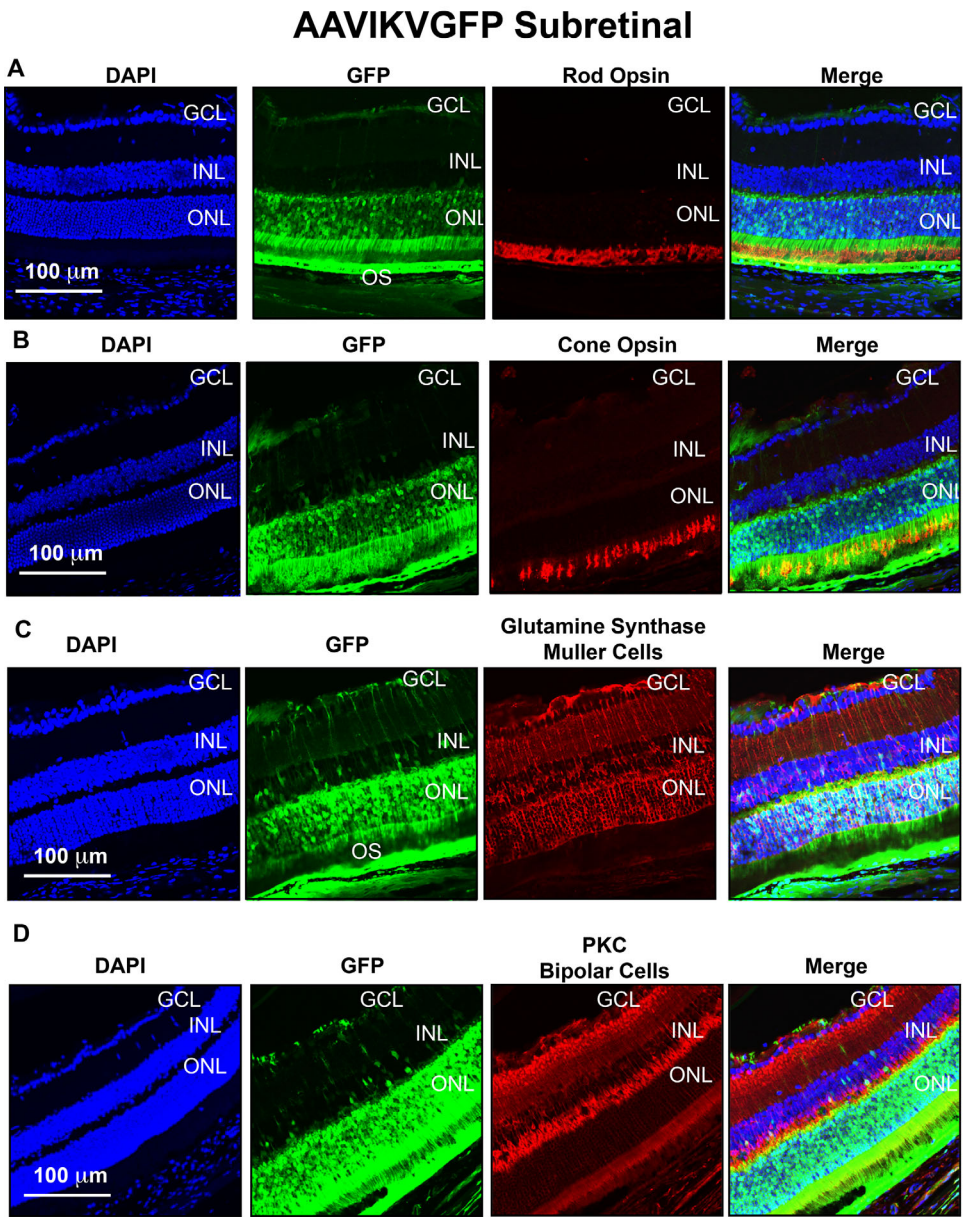

Figure 4. AAVIKVGFP infection following subretinal injection. A single subretinal injection of AAVIKVGFP leads primarily to transduction of photoreceptors in addition to a variety of other retinal cells. Retinal cryostat sections were co-stained with rod opsin (**A**), cone opsin (**B**), glutamine synthase (**C**), and PKC (**D**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

Closer examination (see the boxed region in Fig. 6A) revealed that a high density of photoreceptors and RPE were GFP positive (Fig. 6B). This pattern of GFP expression was not limited to a specific region of the retina, as fundus photography of live animals revealed GFP across a significant field of the retina (Fig. 6C). Furthermore, longer exposure (of the boxed region in Fig. 6B) revealed that the inner plexiform layer (IPL) and ganglion cell layer (GCL) were also GFP positive, albeit significantly less than the ONL or the RPE (Fig. 6D). Counter staining with PKCα for bipolar cells revealed an abundant number of bipolar cells were GFP positive in addition to ONL and RPE (Fig. 6E). A significant number of cells in the choroid were also observed to be GFP-positive (see Fig. 6B); we consider that this is possibly due to transscleral penetration of the virus due to backflow of the virus suspension during or following injection, rather than penetration of the tight junctions of the RPE.

Quantitation of GFP Expression

In order to quantify GFP expression in the retina from the different AAV constructs described in this study, we performed RT-PCR of infected retinal tissues following intravitreal injection. We found that co-injection of Nuc1 peptide significantly enhanced the levels of mRNA expression from each virus tested. Relative to AAV9CAGGFP, co-injection of Nuc1 enhanced mRNA levels by approximately 4.3-fold. Co-injection of Nuc1 peptide also enhanced expression of AAVIKVGFP by approximately 8.5-fold (Fig. 7). Relative to AAVCAGGFP, IKVGFP + Nuc1 had approximately 300-fold greater GFP mRNA expression. In conclusion, insertion

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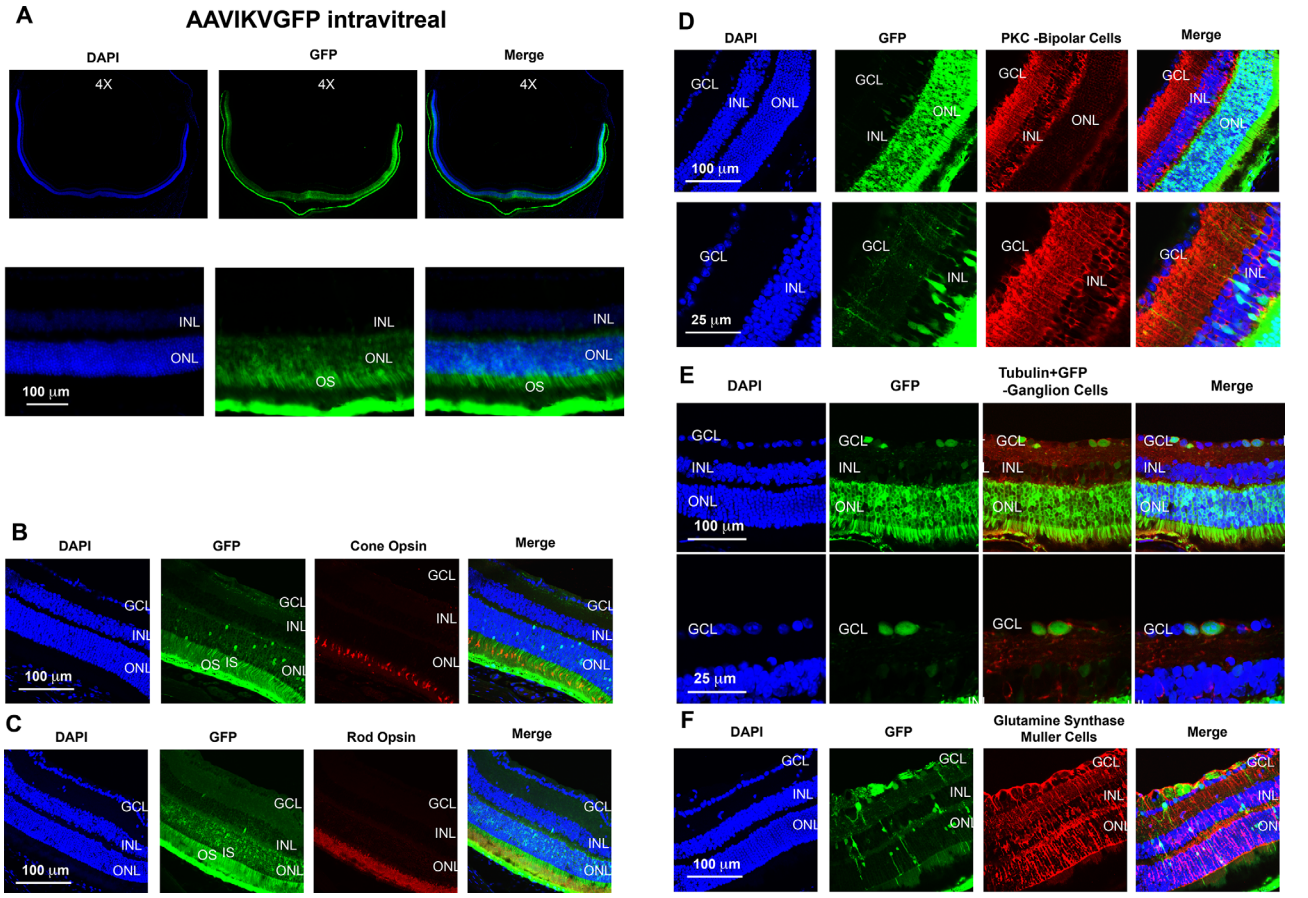

Figure 5. AAVIKVGFP infection following intravitreal injection. A single intravitreal injection of AAVIKVGFP leads primarily to infection of photoreceptors (**A**) in addition to a variety of other retinal cells. Retinal cryostat sections were co-stained with cone opsin (**B**), rod opsin (**C**), PKC/ bipolar cells (**D**), tubulin (**E**), or glutamine synthase (**F**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar 100 µm. Where relevant, higher magnification images are also presented for some panels.

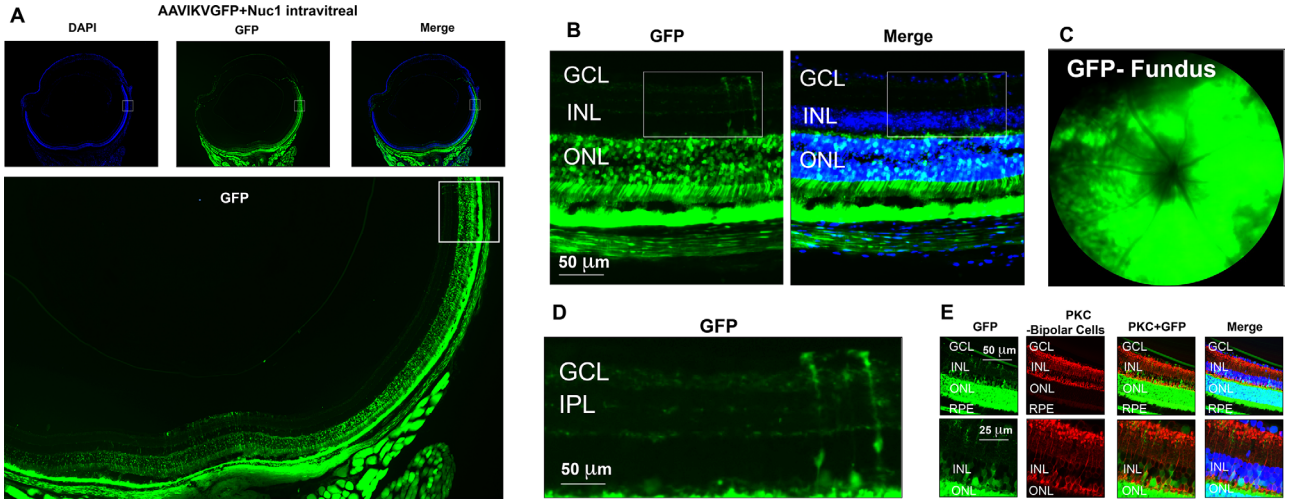

Figure 6. Nuc1 mediated enhancement of retinal penetration by AAVIKV. Co-injection of AAVIKVGFP with Nuc1 peptide led to maximum transgene expression following intravitreal injection, occurring over the entire retinal surface (**A**). Higher magnification images indicated that photoreceptors were highly GFP-positive, including the retinal pigment epithelium (RPE) and choroid (**B**). Fundus imaging of live animals also revealed that transgene expression was across the entire retinal surface (**C**). Higher exposure of inner retina revealed GFP-positive inner plexiform layer (IPL) and ganglion cell layer (GCL), including some Muller cells (**D**). Longer exposure images of retinal sections revealed that cells in the inner nuclear layer (INL) were also positive, co-stained with PCK for bipolar cells (**E**). Scale bar 50 µm and 25 µm, respectively.

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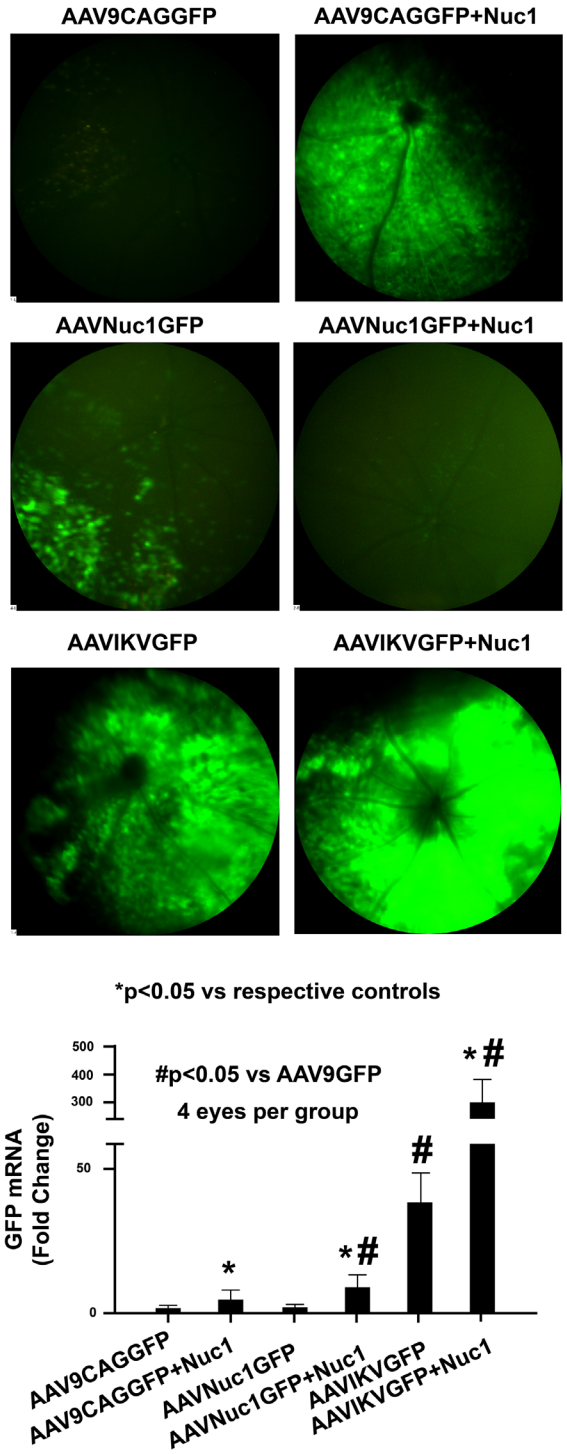

Figure 7. Quantitation of GFP mRNA following intravitreal injection of AAV. Representative fundus images of GFP expression and quantitation of GFP mRNA following intravitreal injection of the various viruses described in this study. Relative to AAV9CAGGFP, co-injection of Nuc1 enhanced mRNA levels by over 4-fold. Nuc1 also enhanced expression of AAVIKVGFP by over eight-fold. Relative to AAV9GFP, IKVGFP + Nuc1 had approximately 300-fold greater levels of mRNA. Values are represented as mean ± SD ($N = 4$/eyes per group). *$P < 0.05$ versus controls (AAV9GFP and AAVIKVGFP, respectively) and #$P < 0.05$ versus AAV9GFP. Fundus image for AAVIKVGFP + Nuc1 is taken from Figure 6, presented here for easier comparison.

of the nucleolin-binding region of Nuc1 (IKV) into the VP1 capsid protein of AAV9 significantly enhances retinal penetration and infection; and the magnitude of infection can be yet further enhanced through co-injection of Nuc1 peptide.

Inhibition of Oxidative Stress in the Outer Retina via Intravitreal AAV Delivery

For proof of concept, we wished to address whether the AAVIKV vector may be applied in a disease-relevant animal model. Specifically, we wished to utilize the AAVIKV backbone described above to express a transgene that may have utility in a number of retinal diseases. NRF2 (nuclear factor erythroid 2 -related factor 2) is a master transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation.[30,31] Over 250 genes are targeted by NRF2. In a state of homeostasis, NRF2 is sequestered in the cytoplasm by Kelch like ECH-associated protein 1 (KEAP1) and Cullin 3, that target NRF2 for degradation by ubiquitination. Oxidative stress disrupts ubiquitination, allowing NRF2 translocation to the nucleus where it binds to the antioxidant response element (ARE) upstream of anti-oxidative genes, initiating their transcription.[32] NRF2 has been previously found to play a significant role in the pathophysiology of animal models of retinal degeneration, including AMD,[33] retinitis pigmentosa,[34] glaucoma,[35] uveitis[36,37] and diabetic retinopathy[38–40]; as well as in many diseases of aging, including Alzheimer's disease.[41–43]

Intraperitoneal injection of N-methyl-N-nitrosourea (MNU) in mice leads to significant oxidative stress in the retina.[44] In nuclear and mitochondrial DNA, this leads to oxidation of the nucleotide deoxyguanosine to 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of oxidative stress. We wished to determine whether co-intravitreal injection of Nuc1 peptide and an AAVIKV encoding human Nrf2 (AAVIKV-Nrf2) in MNU-injected mice could allow for sufficient Nrf2 expression in the outer retina to inhibit oxidative stress. In order to test this hypothesis, adult C57Bl/6J mice were co-injected intravitreally with either AAVIKV-Nrf2 + Nuc1 or the control virus, AAVIKV-GFP + Nuc1. After 3 weeks of transgene expression, the mice were injected with MNU. Eyes were harvested 24 hours post-injection of MNU, sectioned, and antibody-stained for the presence of 8-OHdG and Nrf2. As previously observed,[45] 8-OHdG staining is detected mostly in the ONL following intraperitoneal injection of MNU. In MNU-treated mice that were injected intravitreally with AAVIKV-Nrf2 + Nuc1, we observed approximately 71% ($P < 0.05$) less 8-OHdG staining relative to AAVIKV-GFP + Nuc1 in the ONL (Fig. 8A).

We considered whether the ameliorative effect of AAVIKV-Nrf2 + Nuc1 may be greater in a mouse deficient in Nrf2. To test this hypothesis, we co-injected Nrf2 knockout mice with either AAVIKV-Nrf2 + Nuc1 or with AAVIKV-GFP + Nuc1, as above, allowed 3 weeks for expression of Nrf2, and then injected mice intraperitoneally with MNU. Twenty-four hours later, the eyes were harvested for sectioning and staining. As for the C57BL/6J mice, 8-OHdG staining was observed mostly in the ONL of Nrf2-deficient mice (see Fig. 8B), following intraperitoneal injection of MNU in AAVIKV-GFP + Nuc1 injected Nrf2 knockout mice. In MNU-injected Nrf2 knockout mice injected intravitreally with AAVIKV-Nrf2, however, we observed a significant reduction in 8-OHdG staining in the ONL (see Fig. 8B). This was

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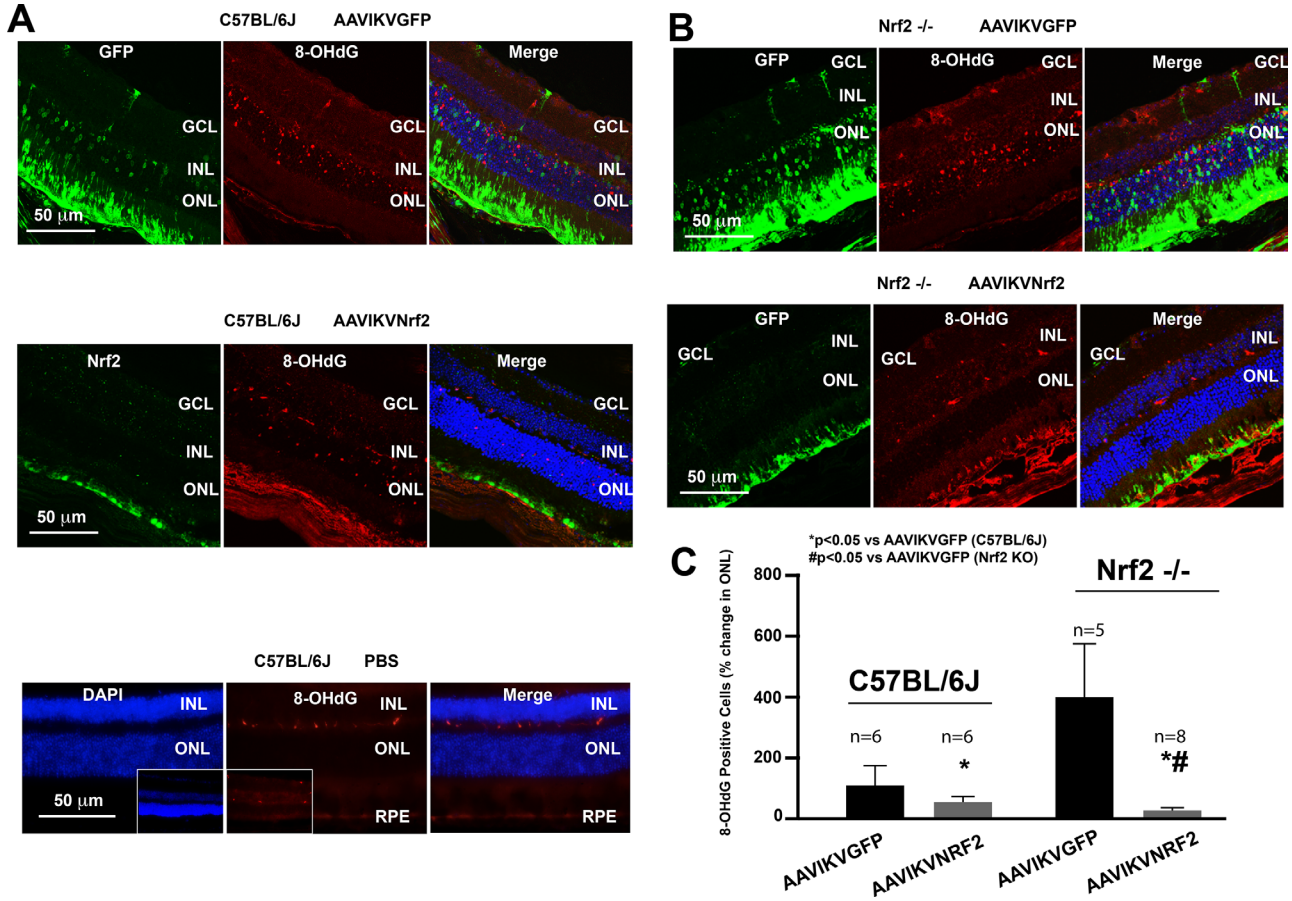

Figure 8. Inhibition of oxidative stress in the outer retina via intravitreal AAV delivery. AAVIKV-Nrf2 injected C57/Bl6J eyes exhibited significantly less 8-OHdG staining relative to AAVIKV-GFP injected eyes (**A**). There was no detectable 8-OHdG staining in the ONL of PBS injected eyes. Similarly, AAVIKV-Nrf2 injected NRF2 knockout mice exhibited significantly less 8-OHdG staining relative to AAVIKV-GFP eyes (**B**). Quantitation of these retinas demonstrated a significant reduction in 8-OHdG staining in the ONL (**C**). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Values are represented as mean ± SD (N = 5-6/retinas per group). $^{*}P < 0.05$ versus AAVIKVGFP for C57BL/6J mice and $^{\#}P < 0.05$ versus Nrf2 knockout mice. Lower power images are included as insets for PBS, showing minimal 8-OHdG staining throughout the retina.

confirmed by quantitation of 8-OHdG staining in the ONL of injected mice wherein a significant ($P < 0.05$) reduction of 98% in the levels of 8-OHdG compared to AAVIKV-GFP in MNU treated Nrf2-deficient mice, following co-injection with AAVIKV-GFP + Nuc1 relative to AAVIKV-GFP + Nuc1 (Fig. 8C). In conclusion, intravitreal delivery of an AAVIKV-Nrf2 vector significantly reduces outer retinal MNU-induced oxidative stress in both C57Bl/6J as well as Nrf2 knockout mice.

Discussion

AAV vectors have shown some potential for treatment of retinal diseases. However, the overwhelming majority of retinal diseases affect cells of the outer retina that can only be accessed by AAV vectors following subretinal injection. Such injections necessitate detachment of the photoreceptors from the underlying RPE in order to create a space in which the vector suspension can be delivered. Subretinal injections are an invasive surgical procedure that can result in complications, such as retinal detachment, subretinal hemorrhage, and retinal atrophy.[46] In contrast, millions of intravitreal injections have been performed in the United States alone since 2016. In that time frame, the frequency of complications, namely infection and discomfort, following intravitreal injection have significantly declined due to persistent adherence to procedural guidelines.[46] Furthermore, unlike subretinal injection, intravitreal injections can be performed as an outpatient procedure. The development, therefore, of AAV vectors that can capitalize on the success of the intravitreal injection for the treatment of retinal diseases is pressing.

Previously, we have described a peptide, Nuc1, that can act as a chaperone for the co-internalization of macromolecules, including proteins and AAV into the retina. The purpose of the current study was to determine whether Nuc1 sequence incorporated genetically into the AAV9 VP1 capsid protein may enable the penetration of AAV from the vitreous to the outer retina. Although our initial attempt at AAV capsid modification using the entire Nuc1 peptide sequence (AAVNuc1CAGGFP) was not successful, our refinement of the Nuc1 amino acid sequence to eliminate the heparan sulphate binding region and, subsequently, incorporation of the shorter Nuc1 peptide into the VP1 capsid protein of AAV9 (to generate AAVIKVGFP) was highly successful in achieving efficient transduction of the outer retina via

intravitreal injection. Why we observed little or no transduction of any cells in the retina following intravitreal injection of AAVNuc1CAGGFP could be due to strong binding of the heparan sulphate binding region of Nuc1 to HSPGs of the inner limiting membrane, as has been observed for AAV serotype 2.[47] Nuc1 was originally designed to bind HSPGs, and whereas this property was conducive to delivery of macromolecules, it appeared to hinder infectivity of the Nuc1-containing AAV (AAVNuc1CAGGFP). Deletion of the HSPG binding region of Nuc1 in the context of the AAV capsid (AAVIKVGFP) resulted in significant penetration and transduction of the retina following intravitreal injection strongly supports this hypothesis.

It is possible, however, that in addition to any negative effect of the heparan sulphate binding region of Nuc1 on AAV penetration of the inner limiting membrane, the poor performance of AAVNuc1CAGGFP following both subretinal and intravitreal injection is due to the incorporation of a large insert (18 amino acids) relative to prior studies in which peptides have been inserted into this same region of the AAV9 VP1 capsid protein,[48] resulting in reduced binding efficiency of the capsid protein to its receptor, or an unstable capsid structure. What was somewhat unexpected was that co-injection of full-length Nuc1 further enhanced retinal penetration by AAVIKVGFP, although it further reduced retinal delivery of AAVNuc1CAGGFP. This effect of co-injection of exogenous Nuc1 was also observed in our studies using full-length Nuc1 peptide to deliver a variety of proteins to the outer retina following intravitreal injection.

Using the novel AAVIKV + Nuc1 gene delivery platform developed herein, we have demonstrated significant reduction in the levels of a marker of oxidative stress in the outer retina. The reduction in the levels of the oxidized nucleotide, 8-OHdG was considerably greater in an Nrf2-deficient mouse exposed to MNU than in an Nrf2 wild-type mice exposed to MNU following intravitreal injection with AAVIKV-Nrf2 + Nuc1 peptide relative to AAVIKV-GFP +Nuc1. This is not surprising, given the capacity of the endogenous Nrf2 of the wild-type C57BL/6J mice to translocate to the nucleus under conditions of oxidative stress. Oxidative stress is an acutely relevant phenomenon in photoreceptors of the retina, cells with a high metabolic rate that are continuously assaulted by light and oxidative stress,[49] and, thus, oxidative stress plays an important role in the progression of retinal diseases. NRF2 is an essential protein for regulation of cellular redox balance and has been implicated in RPE degeneration following chronic cigarette smoking, a risk factor for AMD.[49]

In summary, we have developed a novel AAV vector (AAVIKV) that efficiently transduces the outer retina of mice following intravitreal injection. The transduction by AAVIKV can be further enhanced by co-injection in the vitreous with Nuc1. Testing the AAVIKV with and without Nuc1 peptide in larger animals such as pigs or NHPs is beyond the scope of the current study and such studies in larger animals will be conducted as a next step. Although we have not performed toxicity studies for AAVIKV, we did not observe any gross toxicity in the retina, as measured by morphology of the retina, and by the absence of infiltrating cells in the vitreous of eyes of mice injected with any of the viruses described herein. Whereas additional work needs to be conducted to further characterize AAVIKV, the novel AAV developed in this study warrants further investigation as a gene delivery vector platform for treatment of retinal diseases.

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Author Contributions: B.K. was responsible for generating the data presented in Figures 2 to 7; M.M. was responsible for generating the data presented in Figure 8. S.C. was responsible for guiding the studies, acquiring funding, and writing the manuscript. R.K.-S. was responsible for overall project design, designing and modeling Nuc1 and its derivatives (Fig. 1, Supplementary Movie S1), acquiring funding, and writing the manuscript.

Disclosure: **B. Kumar**, Visiogene LLC (C); **M. Mishra**, Visiogene LLC (C); **S. Cashman**, None; **R. Kumar-Singh**, Is a listed inventor of US patent application US20220204562A1 and the data submitted in the current manuscript has been included in part in the patent application. Patent is assigned to Tufts University (P), Visiogene LLC (C)

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Supplementary Material

Supplementary Movie. Rotation of the predicted recombinant AAV structures depicted in Figure 1.

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