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Highly efficient retinal gene delivery with helper-dependent adenoviral vectors

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Abstract There have been significant advancements in the field of retinal gene therapy in the past several years. In particular, therapeutic efficacy has been achieved in three separate human clinical trials conducted to assess the ability of adeno-associated viruses (AAV) to treat of a type of Leber's congenital amaurosis caused by *RPE65* mutations. However, despite the success of retinal gene therapy with AAV, challenges remain for delivering large therapeutic genes or genes requiring long DNA regulatory elements for controlling their expression. For example, Stargardt's disease, a form of juvenile macular degeneration, is caused by defects in *ABCA4*, a gene that is too large to be packaged in AAV. Therefore, we investigated the ability of helper dependent adenovirus (HD-Ad) to deliver genes to the retina as it has a much larger transgene capacity.

Using an EGFP reporter, our results showed that HD-Ad can transduce the entire retinal epithelium of a mouse using a dose of only 1×10^5 infectious units and maintain transgene expression for at least 4 months. The results demonstrate that HD-Ad has the potential to be an effective vector for the gene therapy of the retina.

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Introduction

There have been significant advancements in the field of retinal gene therapy in the past several years. Three

separate human clinical trials were conducted to assess the ability of AAV to treat of a type of Leber's congenital amaurosis caused by *RPE65* mutations. All three trials reported no safety concerns after treatment and noticeable

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increases in visual function.^{1–3} Improvements in visual perception compared to the baseline were still observed 1 year after treatment⁴ and immune response continued to be minimal.⁵ The group with the largest cohort of 12 then selected three patients for administration of the vector into the contra-lateral eye that was not treated in the initial trial. Both subjective visual function assessments and objective measurements demonstrated improved visual abilities in the newly treated eye and minimal immune response.⁶ These data were very encouraging in the development of retinal gene therapy as they demonstrated the possibility of retinal gene therapy mediated by viral vectors. The clinical trials also proved that the immune response is minimal, likely due to the immune privileged status of the eye.

However, the DNA carrying capacity of AAV is limited to 4.7 kb, and thus is not suitable for all applications. For example, Stargardt's disease is an autosomal recessive form of juvenile macular degeneration caused by defects in the gene *ABCA4*. Despite this contradiction, Allocca et al has claimed success in expressing *ABCA4* via AAV.⁷ However, this finding was not commonly accepted and three individual groups have followed up to find that the original study likely observed an aberration of multiple, incomplete parts of *ABCA4* being carried by separate virions and that co-transduction within the host cell led to random recombination.^{8–10} As such, AAV cannot confer expression of the *ABCA4* protein to host cells. Although a lentiviral vector has been shown to be capable of delivering the *ABCA4* gene to mouse photoreceptor cells,¹¹ the insertion of integration vectors is still a concern for human gene therapy.¹²

The *ABCA4* gene, with a CDS of 6.8 kb, encodes an ATP-dependent flipase that is closely tied to phototransduction. If this flipase is defective, its substrate, N-retinylidene-PE, accumulates within the disc lumen. N-retinylidene-PE can then react with a second molecule of all-trans retinal to form di-retinoid-pyridinium-PE (A2PE). When the outer segment of the PR cell is shed and phagocytosed by the RPE, A2PE present in the segment's disc lumen are also taken up. Lysosomal degradation of this results in the hydrolytic product di-retinoid-pyridinium-ethanolamine (A2E), which cannot be further degraded. Consequently, A2E accumulates to form the lipofuscin deposits characteristic of Stargardt's disease, acting as a detergent that compromises the membrane integrity,^{13,14} and converting into free radical epoxides that are capable of killing the retinal pigment epithelial (RPE) cells.^{15,16} With the loss of the RPE, the corresponding PR cells lose the necessary support required to sustain their function and cannot survive. As a result, a defect in *ABCA4*, a gene that functions in the PR cells, results in the build-up of a toxic substrate that does not affect the PR cells, but causes RPE apoptosis, thus indirectly causing the death of the PR cells. This loss of the PR cells is the mechanism by which Stargardt's disease causes a progressive retinal degeneration. Gene therapy would bring clinical benefits to the patients if *ABCA4* can be effectively delivered to photoreceptor cells.

To examine vectors with a large DNA carrying capacity for retinal gene delivery, our lab became interested in the potential of using the helper-dependent adenoviral vector (HD-Ad). With a packaging capacity of ~30 kb, it can carry

single very large, or multiple small transgenes, along with their associated promoters and other regulatory regions. HD-Ad differs from traditional adenoviral vectors in that the vector genome does not contain any viral coding sequences, but retains the inverted terminal repeats (ITR) for DNA replication, and the viral packaging signal for encapsidation into viral particles. This allows for a larger payload for gene delivery. In addition, the efficiency of transduction is also increased, resulting in a higher number of cells successfully transduced and increased transgene expression for a given dose because the lack of viral genes equates with a lack of viral proteins being produced within the transduced cell. The presence of viral proteins would increase the immune response to transduced cells and cause them to be cleared by the immune system, hence reducing the strength and duration of transgene expression.¹⁷ In support of this, a previous study has shown that HD-Ad vectors show reduced toxicity when delivered to mouse lungs when compared with first generation adenoviral vectors.¹⁸

In this study, we developed HD-Ad carrying the EGFP reporter gene in expression cassettes under the control of the ubiquitously expressed CAG promoter, or the combination of the rhodopsin and interphotoreceptor retinoid binding protein enhancer (IRBPE) element to restrict expression to photoreceptor cells. We then introduced these vectors to mouse retinas via subretinal injection to demonstrate the ability of HD-Ad to deliver transgenes to the retina. Our results demonstrate that HD-Ad can transduce the entire retinal pigment epithelium at very low doses, with expression maintained for a minimum of 4 months.

Materials and methods

Cloning of the expression cassettes

EGFP from pEGFP-C1 was cloned into pBluescript II SK (+) by PCR and restriction digest using the forward primer (5'-ATCTGCAGCGCCACCATGGTGA-3), and the reverse primer (5'-ATGGATCCTCACTTGTACAGCTCGTCC-3), the latter of which also inserted a stop codon. It was inserted using the restriction sites PstI and BamHI.

The CAG promoter¹⁹ from a plasmid previously used in our laboratory was cloned by restriction digest into this plasmid to drive *EGFP* expression.

Separately, the rhodopsin promoter was cloned into the same plasmid by PCR of the 1553 bp immediately upstream of the start codon using the forward primer (5'-GCATCTC-GAGTGTGGTCCCTGTG-3), and the reverse primer (5'-GCATGATATCGGCTGTGGCCCTTG-3). The PCR template was the Roswell Park Cancer Institute Human BAC Library RP11-529F4 (The Center for Applied Genomics, Toronto). The promoter was inserted using the restriction sites XhoI and EcoRV. The IRBPE was then cloned into the rhodopsin promoter carrying plasmid by PCR using the forward primer (5'-GCATGGTACCTGGAGGCAGAGGAGAAG-3), and the reverse primer (5'-GCATCTCGAGGCTTTATGAAGGCCAAAGA-3). It was inserted using the restriction sites KpnI and XhoI.

The BGH poly-A sequence from pCDNA3 (Life Technologies Inc., Burlington) was cloned downstream of *EGFP* in

both constructs. This yielded the CAG-EGFP expression cassette (P_{CAG} -EGFP-polyA) and the Rho-EGFP expression cassette (P_{Rho} -IRBPE-EGFP-polyA), both in pBluescript. These cassettes were then transferred into pC4HSU²⁰ for HD-Ad production.

HD-Ad production

HD-Ad production took place using previously published protocols.^{21,22} Briefly, the plasmids carrying the expression cassettes in pC4HSU backbone were linearized to expose the ITR and transfected into 116 cells. NG163 helper virus was added to these cells and incubated to produce the HD-Ad vector. The vector was released by lysing the cells by repeated freeze–thaw cycles. The lysate was then used to co-infect 116 cells with helper virus, generating more HD-Ad which were again released by lysing. This was repeated several times with increasing cell culture sizes to increase HD-Ad titer. The final production cycle was carried out in 3 L suspension cultures and the HD-Ad was purified from the lysate by CsCl gradient ultra-centrifugation. After dialysis in 10 mM Tris–HCl at pH 8.0, the purified HD-Ad was quantified by measuring DNA content at OD₂₆₀.

Flow cytometry

Cells in each well from a 24 well plate were trypsinized using 200 μ L of 0.25% trypsin with 2.21 mM EDTA. 800 μ L of PBS were then added to the well and the entire volume transferred to a test tube and analyzed on a BD FACSCalibur (BD Biosciences, Mississauga). Data analysis was performed using FlowJo software (Tree Star Inc, Ashland).

Subretinal injection

All animal work described herein was performed at the Laboratory Animal Services of the Hospital for Sick Children with supervision by the veterinary staff and with the approval of the Animal Care Committee. All animals were mice of CD1 strain, and purchased through Charles River Laboratories.

24 h before injection, the right eye of each mouse was administered 1% atropine sulfate. Within 30 min of injection, 1% tropicamide and 2.5% phenylephrine HCl were administered to the right eye, and readministered immediately before injection if the pupil dilation was deemed inadequate. The above ophthalmic solutions were purchased from Alcon (Alcon Canada Inc., Mississauga). To protect the eye and improve visualization into the eye, the right eye was covered using a solution of 2.5% hypromellose (Akorn, Lake Forest). For anesthesia, a mixture of 1 mg ketamine and 0.1 mg xylazine per 10 g body weight was administered by intraperitoneal injection. These were sourced from Zoetis (Zoetis Canada, Kirkland) and Bayer (Bayer HealthCare, Toronto) respectively.

All injections took place when the mice were 3–6 weeks of age. Before injection, fluorescein (Sigma–Aldrich Canada Ltd., Oakville) was added to a final concentration of 0.1 mg/mL to give color to the vector suspension such that the injection could be better visualized.

The subretinal injection was done in a trans-corneal manner as described by Timmers et al.²³ The plunger was then depressed at a rate of less than 1 μ L/min. After the plunger had been fully depressed, the needle was held in place for an additional 60 s. After the needle had been withdrawn, a small amount of Cortimyxin antibiotic ointment (Sandoz Canada, Boucherville) was applied.

Sectioning and confocal microscopy

After enucleation, the eyes were fixed in 4% paraformaldehyde in PBS and incubated for 2 h. After fixation, the eye was dissected. The cornea was cut away using straight 2 mm Vannas spring scissors (Fine Science Tools Inc., North Vancouver). The lens was then extracted slowly using forceps and the remaining tissue was washed 3 times with PBS for 5 min each, and an increasing concentration of sucrose up to 20% w/v in 5% steps with 15 min between each step at room temperature. After embedding in freezing medium, the blocks were cut into 12 μ m thick sections.

For confocal microscopy, a Nikon A1R Si point scanning confocal microscope was used. To obtain high resolution while covering the large area of an entire mouse eye, each image consists of many individual confocal images, stitched together by software to form a single image. Before scanning the large image, each section was mapped for location and a focus map formed to compensate for leveling. Up to 120 images (10 \times 12 fields) were then acquired by the acquisition software Nikon NIS Elements, and stitched with 15% overlap. After acquisition and stitching, image manipulation was performed using Volocity 6.1 (PerkinElmer Inc, Woodbridge).

Results

Analysis of promoter preference and transduction efficiency in cultured cells

To determine the infectious unit titer and to confirm cell-specificity of the Rho-EGFP construct, ARPE-19 and WERI-Rb cells transduced with either CAG-EGFP or Rho-EGFP carrying HD-Ad were analyzed by flow cytometry in order to obtain a quantitative comparison as shown in Fig. 1. ARPE-19, a retinal pigment epithelium derived cell line, was used as an analog of the retinal pigment epithelium while WERI-RB, a retinoblastoma derived cell line, was used to for its ability to express genes restricted to photoreceptor cells. Both cell types produced EGFP when transduced with CAG-EGFP. However, when transduced with Rho-EGFP, only WERI-Rb yielded fluorescence, while there was absolutely no EGFP production from Rho-EGFP transduced ARPE-19 cells despite the high doses applied. This indicates that the combination of rhodopsin promoter and IRBPE has sufficient cell-specificity. These results are reflected both in the percentage of positively gated cells (Fig. 1A) as well as mean fluorescence intensity. (Fig. 1B and C).

To determine the infectious titer of vector particles, we extrapolated the number of VP/cell that yields 50% transduction by curve fitting. The curve-fit function applied was $Y = B_{max} \cdot X / (K_d + X)$ where $X = VP/cell$; $Y =$ percentage positive cells; $B_{max} =$ binding maximum (assumed to be

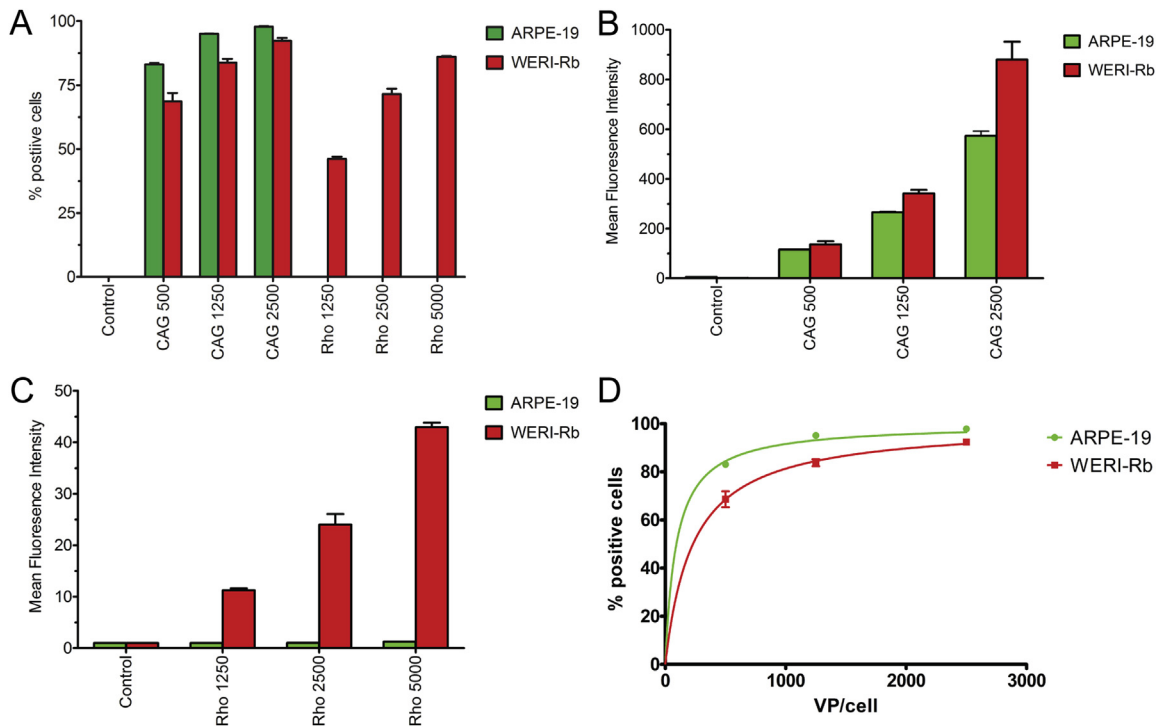


Fig. 1 Flow cytometry results of cultured cells transduced with CAG-EGFP or Rho-EGFP HD-Ad. Flow cytometry indicates that (A) the percentage of positively gated cells increased in dose dependent manner when either cell type was transduced with CAG-EGFP. WERI-Rb but not ARPE-19 responded to Rho-EGFP. (B, C) Similar results were obtained when examining the mean fluorescence intensity with increases observed with dose in all cases except for when ARPE-19 was transduced with Rho-EGFP, again confirming the cell-specificity of Rho-EGFP. Statistical analyses to compare the percentage of positive cells were not performed as they were clearly dose dependent, as were the mean fluorescence intensities. Curve fit for the percentage of positively gated cells for ARPE-19 and WERI-Rb after transduction with CAG-EGFP (D) is used for determining the infectious titer. The results reveal that 1 in 133 vector particle is capable of conferring transgene expression. (The numbers following CAG/Rho indicate VP/cell applied. 3 days post-transduction. $n = 3$. Error bars represent standard error of the mean. 2-way ANOVA of the CAG promoter (B) found statistically significant difference on dose only while 2-way ANOVA of the Rho promoter found statistically significant interaction between dose and cell-type. Pairwise comparisons were not conducted).

100%); K_d = equilibrium binding constant (the value of X required to achieve 50% binding) (Fig. 1D).

The R^2 value for the curve fit for ARPE-19 and WERI-Rb cells were 0.9316 and 0.9082 respectively. The K_d was calculated to be 92.40 (77.84–107.0, 95% CI) for ARPE-19 and 230.1 (191.7–268.4, 95% CI) for WERI-Rb cells, both transduced with CAG-EGFP. These values correspond with the number of vector particles per cell (VP/cell) required to transduce 50% of the cells. The difference between cell lines is indicative of the differences in the ability of each cell-type to be transduced (i.e. transduction efficiency). As ARPE-19 demonstrated a 2.5 fold higher transduction efficiency than WERI-Rb, the calculations below were based on a K_d of 92.40 from ARPE-19. In other words, 92.4 VP/cell is required to infect 50% of the cells.

As defined by the Poisson distribution, the multiplicity of infection required to infect 50% of the cells is $-\ln(0.5)$ or 0.693. Since 92.4 VP/cell transduced 50% of the cells, a conservative estimate of the number of active vector particles per total number of vector particles was calculated to be approximately $92.4/0.69$ or 133. In other words, 1 in 133 VP was infectious. It is likely that the actual ratio of infectious particles per vector particle was even higher as there were multiple assumptions and simplifications

involved and that transduction of ARPE-19 did not occur at 100% efficiency. Therefore, we estimated that approximately 1 in 100 vector particles was infectious in our transduction experiments.

CAG-EGFP confers expression of EGFP throughout the entire retinal epithelium using only 1×10^5 IU and can be maintained for at least 4 months

Having confirmed the *in vitro* function of the vectors, we injected the vector into the subretinal space of mice to determine whether the vectors transduce mouse retinas efficiently. Fig. 2 demonstrates the typical results of CAG-EGFP injection with 1×10^8 IU. 10 separate sections were taken approximately 100 μm apart, progressing from the inferior to the superior side of the eye. It is clear from this set of images that the RPE has been completely transduced across all three dimensions. A high magnification view (Fig. 3) demonstrates that all cells in the RPE exhibited fluorescence.

Given the complete transduction of the retinal epithelium, $10\times$ serial dilutions of the vector were injected to determine the lowest dose required to establish complete

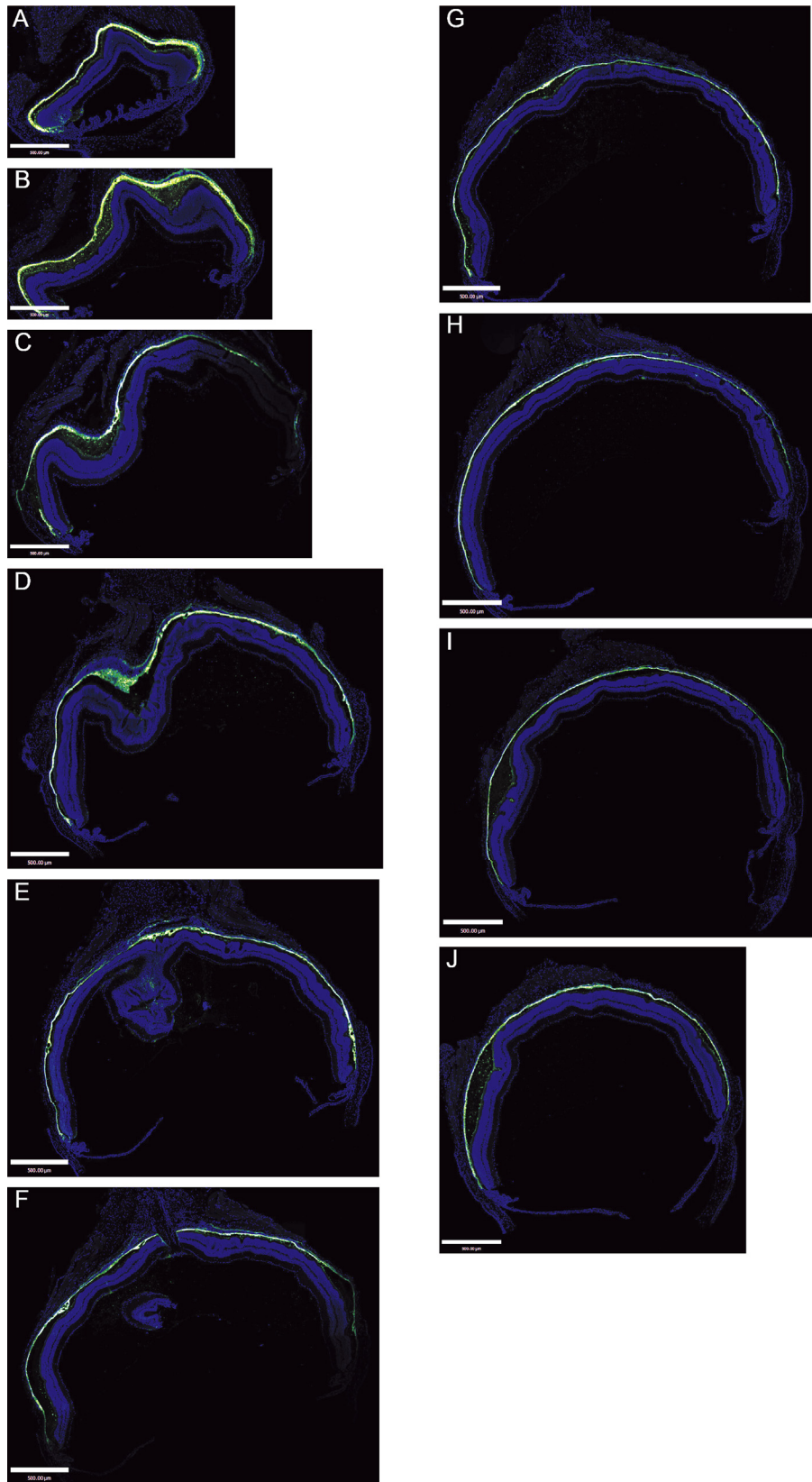


Fig. 2 Fluorescence images of sequential sections throughout a CAG-EGFP HD-Ad injected eye. Sequential sections of CAG-EGFP injected mouse eyes reveal that the RPE was transduced across the entire retina. The sections are 12 μm each, separated by approximately 100 μm . The sections are in the transverse plane, progressing from the inferior to the superior side. The eye was injected with 1×10^8 IU in 1 μL of HDAd carrying CAG-EGFP. The animal was sacrificed 1 week post-injection. (Scale bar = 500 μm).

epithelial transduction (Fig. 4A). The results show that significant amounts of fluorescence can be detected using as little as 1×10^3 IU, and complete epithelial transduction can be assured at 1×10^5 IU.

Having determined the minimal dose for reliable complete epithelial transduction, this dose was used to determine the maximum duration of long term transgene expression (Fig. 4B). The results demonstrate that strong EGFP can be observed for a minimum of 4 months, the longest time-point planned in the experiment.

Expression is largely limited to the RPE, but there are patches of neural-retinal transduction

The results presented thus far show that transduction across the retina was robust, but was only observed in the RPE as was observed in the vast majority of cases. However, in approximately 20% of the CAG-EGFP injected eyes, cells in the neural retina could be observed to give a strong fluorescent signal. This had been observed in large patches of cells covering up to 20% of the retina (Fig. 5). There is no obviously discernible pattern of where these patches occur as they are random in location and not localized to the injection site nor any anatomical feature.

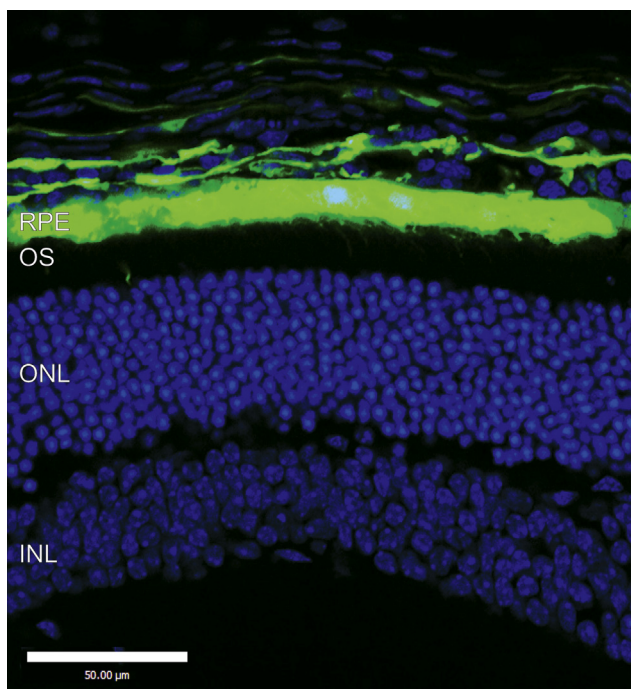


Fig. 3 High magnification confocal photomicrograph of a CAG-EGFP HD-Ad injected retina. A high magnification view of the retina after injection with CAG-EGFP reveals that the fluorescence was exclusive to the RPE layer, indicating that the vector did not transduce the photoreceptor cells. (RPE – retinal pigment epithelium; OS – outer segment (photoreceptors); ONL – outer nuclear layer; INL – inner nuclear layer; 1×10^8 IU in $1 \mu\text{L}$ of HDAd encoding CAG-EGFP; 1 week post-injection; Scale bar = $50 \mu\text{m}$).

Injections of Rho-EGFP results in sporadic, limited transduction restricted to photoreceptor cells

In order to assess the photoreceptor specificity of the Rho-EGFP construct *in vivo*, HD-Ad vector carrying the construct was injected into the subretinal space using methods identical to CAG-EGFP injections. In the majority of cases, no transduction could be detected in the retina (Fig. 6A). However, in approximately 20% of the cases, small areas of transduction can be found (Fig. 6B and D). Under high magnification, it is apparent that in these areas, fluorescence is restricted exclusively to the photoreceptor cells (Fig. 6C and E). These areas of transduction indicate that the rhodopsin promoter construct confers cell-specific transgene expression. This is consistent with the complete lack of transduction of the RPE, including in areas where successful photoreceptor transduction can be found.

Discussion

Adenoviral gene therapy has been in development for many decades, and this study was not the first in attempting to apply adenoviral gene therapy for the treatment of retinal disease. However, it is the first to demonstrate the high efficacy with which HD-Ad can transduce the retinal epithelium at very low doses, and prove that strict cell specificity can be obtained using transcriptional regulation. We have also identified challenges to the *in vivo* application of the vector that have not been previously encountered in the transduction of the neural retina.

Potential application of HD-Ad in RPE diseases

While large area views of the retina show the complete transduction of the RPE across the entire retina, the high magnification views demonstrate that all of the cells of the RPE are successfully transduced with no visible breaks in the transduction. However, these high magnification views also demonstrate that there is no transduction in cell types other than the RPE.

The titer of the vector was determined in this study via flow cytometry of transduced cultured cells. Earlier versions of adenoviral vectors can replicate in a permissible cell line, thus a precise titer based on plaque forming units (PFU) can be determined. However, as HD-Ad lacking all viral genes, the titer must be determined by expression of transgenes after transduction. We chose to determine the infectious unit (IU) titer by flow cytometry because it avoids inaccuracies associated with counting individual cells during a limiting dilution assay, as well as accounting for multiple simultaneously transduced cells by employing the Poisson distribution. This yields a more accurate quantitation of vector activity than using either viral particle (VP) titer or by limiting dilution assays.

Previous dosage studies with first-generation (E1 deletion only) adenoviral vectors in mice have shown that 1×10^7 PFU is the minimum required to obtain complete RPE transduction carrying a CMV-LacZ expression cassette.²⁴ Other studies have subsequently used this dose as an absolute minimum, often with doses as high as 1×10^9 PFU and have repeatedly demonstrated that doses under

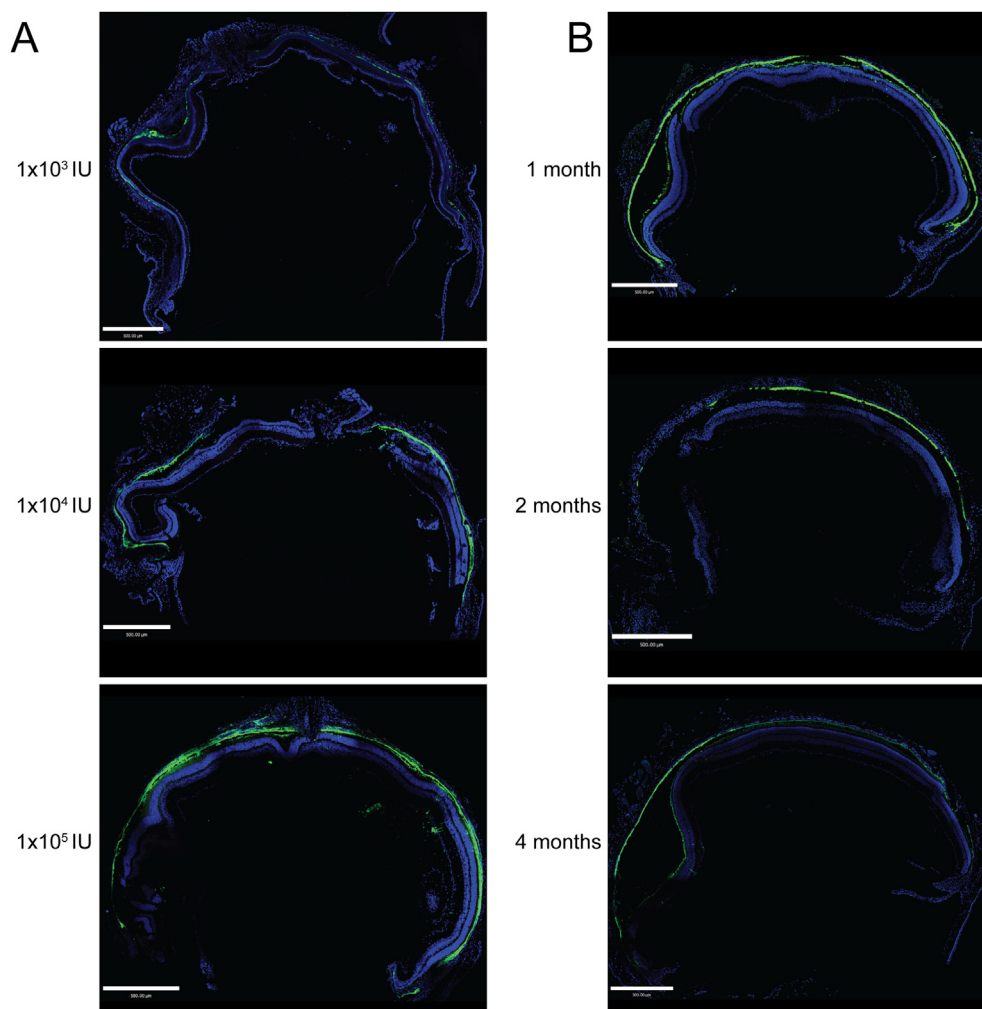


Fig. 4 Fluorescence photomicrographs of retinas injected with very low doses of CAG-EGFP HD-Ad after 1, 2 and 4 months. (A) Injections of low doses of CAG-EGFP reveal that the fluorescence is detectable in the RPE across the entire retina at 1×10^5 IU but became more sparse with lower doses. (B) Long-term monitoring of mice injected with 1×10^5 IU of CAG-EGFP indicates that strong transgene expression can be detected for a minimum of 4 months in the majority of the retinal epithelium. (A: All doses at $1 \mu\text{L}$ of HDAd encoding CAG-EGFP; 1 week post-injection; b: 1×10^5 IU in $1 \mu\text{L}$ of HDAd encoding CAG-EGFP; both: Scale bar = $500 \mu\text{m}$).

1×10^7 PFU are inadequate for complete transduction.^{25,26} Our data clearly demonstrates that complete transduction can be obtained at a dose of 1×10^5 IU or lower. The high magnification examination of these injected retinas reveals that there are no breaks or untransduced areas across the entire retinal epithelium at these low doses.

A lower viral vector titer naturally lends itself to lower toxicity and combined with the lower toxicity inherent in HD-Ad, offers a tantalizing possibility for retinal gene therapy targeting the RPE given our ability to transduce the entire retinal epithelium at very low doses. Of the many causes of inherited retinopathies, the RPE-specific genes that have been recognized so far are *RPE65*, *LRAT*,²⁷ *MERTK*²⁸ and *RDH5*.²⁹ Of these four genes, only *MERTK* is not directly involved in the visual cycle. *RPE65* has been well studied and is well into human clinical trials using AAV.^{1,2,4–6,30} *LRAT* gene therapy has been studied with significant electro-physiological results but poor distribution of gene expression.³¹ Furthermore, it has been argued that the regulatory regions required for *LRAT* expression

cannot be packaged using the AAV vector.²⁷ *MERTK* has been treated in animal studies using lentivirus,³² adenovirus,³³ as well as AAV.^{34,35} A human clinical trial using AAV has been initiated but the results are as yet unpublished.²⁸ A lesser known disease is also caused by mutations in *RDH5*, a retinal-pigment epithelial specific gene that can cause retinal degeneration with no known treatment.²⁹ Due to its low prevalence, treatment for this disease has not been well studied. All such RPE specific diseases could potentially be treated with helper-dependent adenoviral vectors, especially given that the distribution and expression levels observed in this study are unprecedented.

Patches of complete retinal transduction

In the majority of injection of vectors carrying CAG-EGFP, no transduction of the neural retina could be observed while the entirety of the RPE was fluorescent. However, in

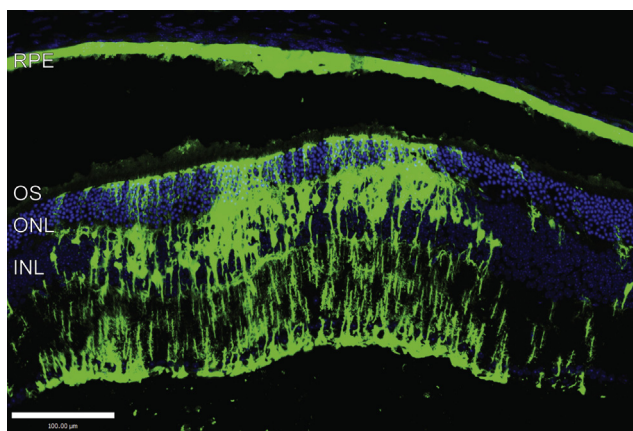


Fig. 5 High magnification confocal photomicrograph of a patch of complete retinal transduction in a CAG-EGFP HD-Ad injected retina. Confocal microscopy of CAG-EGFP injected mice reveal that occasional transduction of cells other than the RPE can be observed. These can occur as patches where many cell-types are transduced across the retinal layers. (RPE – retinal pigment epithelium; OS – outer segment (photoreceptors); ONL – outer nuclear layer; INL – inner nuclear layer; 1×10^5 IU in $1 \mu\text{L}$ of HDAd encoding CAG-EGFP; 1 week post-injection; Scale bar = $100 \mu\text{m}$).

some cases, patches of transduction within the neural retina could be observed. Similarly, injections of vectors carrying Rho-EGFP displayed no fluorescence in the majority of cases, although occasional patches of fluorescence limited to the photoreceptor cells could be observed.

Because the location of these patches varies, it is apparent that the patches do not correspond to any particular anatomical feature in the eye. They are also not localized to the injection site as the patches can occur near the edge of the retina while the injection is targeted towards the center of the retina. However, although these patches do not correspond to the injection site, it is nonetheless possible that they arise from injection injury. Specifically, as the use of a dissection microscope gave only limited depth perception and the physical resistance provided by the eye is minimal, it is possible that the needle tip had scraped and damaged the retina in those areas unintentionally. This indicates that HD-Ad is able to transduce the photoreceptor cells. If we consider the possibility that these patches indicate areas where there was inadvertent injury to the retina, the logical explanation would be that the lack of transduction in other cases represents an inability to access and/or infect the cells, while the physical injury imparted provided a route of infection to the neural retina.

It is possible that the trauma caused a release of cytokines that resulted in the movement of receptors and/or co-receptors to become accessible on the surface of the target cells, making the cells susceptible to transduction. This is supported by evidence from adenoviral infection of the respiratory tract.³⁶ In that study, it was shown that the release of cytokines from infected macrophages causes the migration of integrins to the apical surface of cultured polarized epithelial cells, giving opportunity for adenovirus to infect the epithelium. It is possible that the retinal injury

causes a similar release in cytokines, resulting in changes in the neural retinal cells to become susceptible to infection or transduction.

It is also possible that the barrier to infection is physical. The neural retina is sandwiched by two limiting membranes; the inner limiting membrane that is in contact with the vitreous, and the outer limiting faces the subretinal space and the RPE. The outer limiting membrane forms a barrier between Müller cells and photoreceptor inner segments, leaving the outer segments exposed to the subretinal space. From the complete transduction of the RPE, we can surmise that there is a very large number of vector particles present in the subretinal space between the RPE and the outer limiting membrane. The presence of these patches of transduced cells indicates that the vector is capable of transducing the neural retina, including photoreceptor cells. Therefore, the most logical explanation is that the vector is unable to transduce photoreceptor cells by entering via the photoreceptor cell segments. Rather, the vector must be exposed to the cell-body in the outer nuclear layer in order to establish transduction. Meanwhile, the barrier formed by the outer limiting membrane results in the inability of the HD-Ad vector to travel into the outer nuclear layer. For future study, we propose the injection of α -amino adipic acid (AAA) with the vector. AAA has been documented as a method by which the outer limiting membrane can be disrupted to allow for increased integration of transplanted stem cell precursors from the subretinal space.³⁷ Therefore, the injection of AAA before or during the injection of the vector would allow for the transient, reversible disruption of the outer limiting membrane, thus providing access for the vector to transduce the photoreceptors.³⁸

In summary, it is clear from the results that helper-dependent adenoviral vectors are highly effective for delivering genes to the RPE at a very low dose, resulting in prolonged expression lasting for a minimum of 4 months. However, it is disappointing that effective, widespread transduction of the neural retina could not be achieved using our methods. Based on the presence of the patches of transduction observed, there appears to be an issue with access to the relevant cells by the vector rather than the ability of the vector to transduce such cells. With modifications to the method of delivery of the vector, such as pre-treating the retina with AAA, it may be possible to transduce the neural retina. The results with Rho-EGFP vector have shown that if a method of delivery could be found to introduce the vector to the neural retina, the IRBP enhancer – rhodopsin promoter construct is capable of limiting gene expression to only the photoreceptors.

Moving forward in the application of helper-dependent adenoviral vectors in retinal gene therapy, the focus should shift towards the use of this unique vector to deliver genes to the RPE as the results herein has proven it to be highly effective with long duration of transgene expression at very low doses. If a method of delivery can be developed for introducing the vector to the neural retina, it would also unlock HD-Ad vectors for transduction of photoreceptor cells. It would allow for the delivery of large genes to the cells of the neural retina to treat diseases that involve genes too large to be packaged in AAV. Such progress in the study of HD-Ad would give hope to the patients of a variety

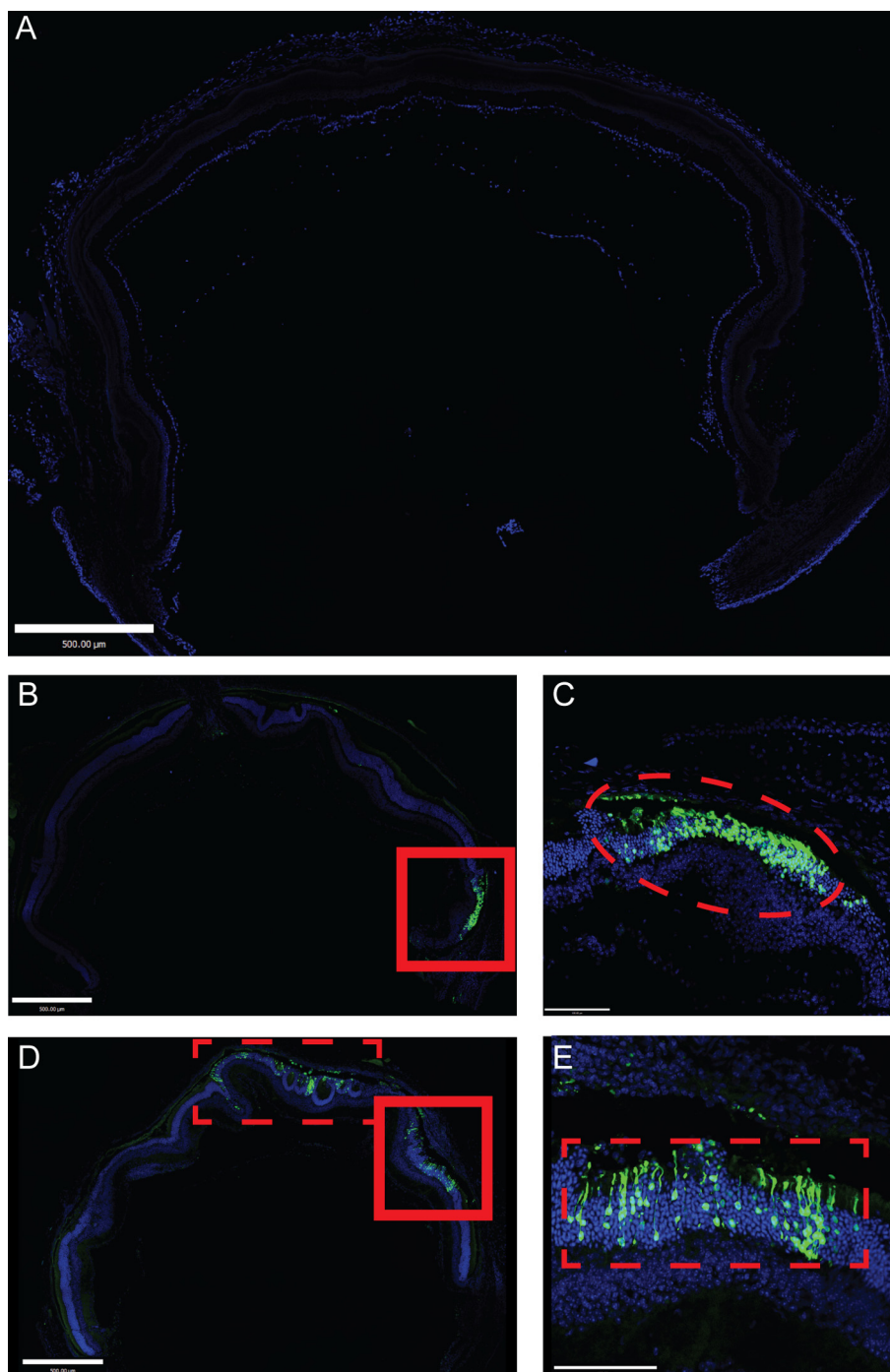


Fig. 6 Low and high magnification confocal photomicrographs of Rho-EGFP HD-Ad injected retinas. Injections of Rho-EGFP usually resulted in no visible transduction (A). However, in approximately 20% of the cases, areas of transduction can be found (B and D; each from different mice; areas of transduction are highlighted with dashed red lines). High magnification views of these areas reveal that transduction is exclusive to photoreceptor cells (C and E, corresponding to the red boxed area of B and D respectively). (RPE – retinal pigment epithelium; OS – outer segment (photoreceptors); ONL – outer nuclear layer; INL – inner nuclear layer; Scale bars = 500 μm (A, B, D), Scale bars = 100 μm (C and E); 1×10^8 IU in 1 μL of HDAd vector carrying Rho-EGFP; 1 week post-injection).

of retinal degenerative diseases, such as Stargardt's disease, for whom there is no present cure.

Conflicts of interest

The authors declare no conflict of interest.

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