Adenovirus Vectors Targeting Distinct Cell Types in the Retina

J. Harry Sweigard, Siobhan M. Cashman, and Rajendra Kumar-Singh

PURPOSE. Gene therapy for a number of retinal diseases necessitates efficient transduction of photoreceptor cells. Whereas adenovirus (Ad) serotype 5 (Ad5) does not transduce photoreceptors efficiently, previous studies have demonstrated improved photoreceptor transduction by Ad5 pseudotyped with Ad35 (Ad5/F35) or Ad37 (Ad5/F37) fiber or by the deletion of the RGD domain in the Ad5 penton base (Ad5ΔRGD). However, each of these constructs contained a different transgene cassette, preventing the evaluation of the relative performance of these vectors, an important consideration before the use of these vectors in the clinic. The aim of this study was to evaluate these vectors in the retina and to attempt photoreceptor-specific transgene expression.

METHODS. Three Ad5-based vectors containing the same expression cassette were generated and injected into the subretinal space of adult mice. Eyes were analyzed for green fluorescence protein expression in flat-mounts, cross-sections, quantitative RT-PCR, and a modified stereological technique. A 257-bp fragment derived from the mouse opsin promoter was analyzed in the context of photoreceptor-specific transgene expression.

RESULTS. Each virus tested efficiently transduced the retinal pigment epithelium. The authors found no evidence that Ad5/F35 or Ad5/F37 transduced photoreceptors. Instead, they found that Ad5/F37 transduced Müller cells. Robust photoreceptor transduction by Ad5ΔRGD was detected. Photoreceptor-specific transgene expression from the 257-bp mouse opsin promoter in the context of Ad5ΔRGD vectors was found.

CONCLUSIONS. Adenovirus vectors may be designed with tropism to distinct cell populations. Robust photoreceptor-specific transgene expression can be achieved in the context of Ad5ΔRGD vectors. (*Invest Ophthalmol Vis Sci.* 2010;51:2219–2228) DOI:10.1167/iovs.09-4367

Loss of any one of the multiple cell types found in the retina can lead to blindness, the second most feared condition after cancer among Americans. The human retina is a laminated tissue composed of light-sensitive photoreceptors in the outer retina that modulate current with a complex array of inner retinal neurons. Photoreceptors rely on the juxtaposing retinal

pigment epithelium (RPE) for generation and recycling of *retinal* (vitamin A), the chromophore that absorbs light to initiate the phototransduction cascade that is eventually perceived as vision. Retinal neurons are supported by Müller glia, which provide critical support for the maintenance and functioning of the retina.

Loss of RPE cells is characteristic of age-related macular degeneration (AMD), the most common cause of blindness in elderly individuals in the United States.¹ Loss of photoreceptors may lead to retinitis pigmentosa (RP), the most common cause of inherited blindness in the working population, affecting approximately 1 in 3000 individuals.² The rare hereditary disease referred to as Müller cell sheen dystrophy is caused by primary Müller cell dysfunction.³ Hence, there is a substantial need for gene transfer vectors that effectively transduce each of the different cell types in the retina. To date, all gene transfer vectors injected in the outer retina, in a region referred to as the subretinal space, primarily infect the RPE. However, through vector modifications, such as pseudotyping or deletion of integrin binding motifs in the viral capsids, other cell types in the retina, such as photoreceptors, may also be transduced, though still not as effectively as the RPE.

Human adenovirus (Ad) has been previously used in two clinical trials for eye diseases without any serious adverse events.^{4,5} Short-term transgene expression typically observed from adenovirus vectors may be overcome by the use of the helper-dependent adenovirus vectors.^{6–8} Therefore, a clear understanding of the tropism of different adenovirus serotypes in the retina would greatly enhance the progression of adenovirus-mediated ocular gene therapy studies. Adenovirus serotype 5 (Ad5) infects cells by initial binding of the adenovirus fiber protein to the membrane-associated coxsackie adenovirus receptor (CAR), followed by internalization of the virus through an interaction between the RGD motif in adenovirus penton base with $\alpha_v\beta_{3/5}$ integrins.^{9–11} In contrast, Ad35 and Ad37 fibers have been shown to interact with CD46 and sialic acid, respectively.^{12,13} Previously, it had been shown that the transduction of photoreceptors can be enhanced either by deletion of the RGD motif in the penton base or by replacement of the Ad5 fiber with that of Ad35 or Ad37.^{14–16}

Although each of these studies described improved photoreceptor transduction, the data from these studies cannot be directly compared because of the different genes, promoters, or transgene detection methods used. Hence, the relative performance between these vectors is still an unanswered and relevant question for the advancement of Ad-mediated retinal gene therapy. One aim of the present study was to compare the performance of these different adenovirus vectors under conditions that permit their direct comparison in the retina, i.e., the use of the same transgene and promoter combination and a similar detection method for the presence of the gene product. A second aim of this study was to use the most potent vector identified herein to attempt photoreceptor-specific transgene expression in the retina through the use of cell-specific promoters. Significant photoreceptor-specific trans-

From the Department of Ophthalmology, Tufts University School of Medicine, Boston, Massachusetts.

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Corresponding author: Rajendra Kumar-Singh, Department of Ophthalmology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111; rajendra.kumar-singh@tufts.edu.

gene expression has not thus far been described using adenovirus vectors. Our results and conclusions differ significantly from those of previous studies and, hence, highlight the need for continued evaluation of adenovirus tropism in the retina before their advancement into the clinic.

MATERIALS AND METHODS

Cell Lines

HEPA 1c1c7, Y79, and 293 cell lines were obtained from ATCC (Manassas, VA). Cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). Cells (293 and 911)¹⁷ were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (FBS). HEPA 1c1c7 cells in α MEM/10% FBS and Y79 cells in RPMI 1640 supplemented with L-glutamine, D-glucose, Na pyruvate (1 mM), and 15% FBS.

Recombinant Adenovirus Constructs

The hybrid 5/35 fiber was amplified by PCR from an Ad5/F35 plasmid (obtained from Andre Lieber, University of Washington). Using the primers AdE28403F (5' TATTCAGCAGCACCTCCTTGCC 3') and AdE30702R (5' ATGTAGGCGTGGACTTCTCCTTCG 3') to yield a product of 1.4 kb. An 842-bp *AgeI/MfeI* fragment was then cloned into *AgeI/MfeI*-digested p6.2FIB¹⁸ to generate p6.2FIBF35. A 4.9-kb *PacI/SrfI* fragment of p6.2FIBF35 was then cloned into similarly digested Adeasy1¹⁹ to create pAdEasyF35. This was then recombined with pShCAGGFP¹⁴ to generate AdF35CAGGFP. AdF37CAGGFP was generated by recombination of pShCAGGFP and pAd5F37.¹⁸

Ad5CAGGFP Δ RGD has been previously described.¹⁴ The Ad5sRhoGFP Δ RGD vector was made using a similar strategy. The 257-bp mouse opsin promoter was PCR amplified from SB6.25 (a gift from Wolfgang Baehr, The University of Utah) using the primers sRHO-F (5'-CCCA-GATCTCCCGAATTCCAGAGGACTCTGG-3') and sRHO-R (5'-CCCGTC-GACCCGCGCAGCTCAGCCACTGAC-3'). The PCR product was digested with *BglII/SalI* and was cloned into the corresponding sites of pShGFP.¹⁴ The resultant plasmid was named pShsRhoGFP. This was recombined with pAdEasy Δ RGD¹⁴ to generate Ad5sRhoGFP Δ RGD. Ad5F37 and Ad5 Δ RGD were constructed using the AdEasy system¹⁹ and were previously described.^{14,18}

In all cases adenovirus was recovered in 293 cells, as previously described.¹⁸ Viruses were purified using a purification kit (Adenopure; Puresyn, Inc., Malvern, PA) and were concentrated using a 100-kDa filter (Millipore, Billerica, MA). Viral titers were determined using a mass spectrophotometer at 260 nm (DU530 UV/Vis; Beckman Coulter, Hialeah, FL).

Western Blot Analysis

Viral particles (5×10^9) were suspended in 50 μ L of 100 mM Tris-HCl, 0.5 M NaCl, 0.1% SDS, and 10% Triton X-100 containing leupeptin (10 μ g/mL), aprotinin (10 μ g/mL), and phenylmethylsulfonyl fluoride (1 mM). Samples were loaded onto a 10% Tris-glycine polyacrylamide gel under denaturing conditions. The fiber protein was detected using the monoclonal antibody Ab-4 (clone 4D2; Neomarkers, Fremont, CA) at a concentration of 1:1000, followed by horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch, Bar Harbor, ME) secondary antibody.

In Vitro CD46 Transfection and Blocking

pBluescript containing human CD46 cDNA was obtained from ATCC (MGC-26544). The cDNA was released by digestion with *EcoRI* and *SspI* and was cloned into *EcoRI/EcoRV*-digested pCAGEN (a gift of Connie Cepko) to generate pCAGCD46. pCAGntLacZ was generated by insertion of a *NotI* fragment of pBntLacZ (pBluescript containing LacZ cDNA) into *NotI*-digested pCAGEN. Six micrograms of either pCAGCD46 or pCAGntLacZ was transfected into 1×10^5 HEPA 1c1c7 cells using transfection reagent (Lipofectamine 2000; Invitrogen) and was maintained in α MEM supplemented with 2% FBS at

A

B

FIGURE 1. Structure of Ad5 Δ RGD, Ad5/F37, and Ad5/F35. (A) The RGD domain is deleted in the penton base of Ad5 Δ RGD. The Ad5/F37 hybrid fiber is composed of the first 17 amino acids of the Ad5 fiber, followed by amino acids 18 to 365 of the wild-type Ad37 fiber and a BGH pA signal. The Ad5/F35 hybrid fiber is composed of the first 44 amino acids of the Ad5 fiber, followed by amino acids 132 to 991 of the wild-type Ad35 fiber and the Ad5 pA signal (aa 32775–33651). An EGFP expression cassette was cloned in the antisense orientation into the E1 region. Expression is driven by a chicken β -actin promoter/CMV enhancer/rabbit globin intron (CAG). (B) Western blot analysis of the native and denatured fiber proteins of Ad5, Ad5/F37, and Ad5/F35 reveal the expected trimer/monomer band sizes of 192/64, 114/38, and 105/35 kDa, respectively, using a monoclonal antibody to the retained N terminus of the Ad5 fiber. Δ RGD, deleted amino acids arginine, glycine, aspartic acid; pA, polyA tail; LTR, left inverted terminal repeat; RITR, right inverted terminal repeat; Ψ , packaging signal; MLT, major late transcript; E1–E4, early regions 1–4.

37°C/5% CO₂ for 48 hours. Cells were then infected with either Ad5CAGGFP (multiplicity of infection [MOI] 20) or Ad5F35CAGGFP (MOI 750) for an additional 48 hours.

Blocking Experiments

Cells were transfected as described. After 48 hours the cells were treated with either 5 μ g/mL mouse anti-human CD46 (MEM258; Serotec, Raleigh, NC) to block the SCR1 domain of CD46 or rabbit anti-sheep IgG (Jackson ImmunoResearch) as a nonspecific control for 30 minutes at 37°C/5% CO₂. The cells were then infected with either Ad5CAGGFP (MOI 20) or Ad5F35CAGGFP (MOI 750), as described. Green fluorescence protein (GFP) expression was analyzed with a microscope (IX51; Olympus, Tokyo, Japan) and by flow cytometry (FACSCalibur; Becton-Dickinson, Franklin Lakes, NJ).

Y79 Cell Transduction

Cells (4.5×10^5 Y79) were infected with Ad5CAGGFP, Ad5F35CAGGFP, Ad5F37CAGGFP, or Ad5 Δ RGDCAGGFP at an MOI

FIGURE 2. CD46 expression enhances Ad5/F35 transduction of cells. **(A)** Transfecting HEPA 1c1c7 cells with a plasmid expressing CD46 improved transduction of Ad5/F35 but not Ad5. **(B)** Pretransfection of HEPA 1c1c7 cells with a plasmid that expresses CD46 followed by blocking with an antibody that binds to the SCR1 extracellular domain of CD46 reduces the efficiency of Ad5/F35 viral cell entry. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

500. Infected cells were maintained in RPMI media at 37° 5%/CO₂ for 24 hours, after which GFP expression was analyzed (FACSCalibur; Becton-Dickinson).

In Vivo Transduction

All animal studies were conducted in accordance with the policies set forth by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and federal and state guidelines. Unless otherwise specified, for each viral construct, at least six eyes of 6-week-old C57BL/6J mice were injected with 1 μ L containing 1×10^9 viral particles. After 6 days the eyes were harvested and fixed in 4% PFA.

To analyze surface area and efficiency of viral transduction, six eyes were harvested for each construct, the cornea and lens were removed, and the eyes were cut into quadrants and flat-mounted onto a coverglass. An image of each eyecup was captured using a microscope (Nikon, Tokyo, Japan) and a camera (Olympus) under bright field and GFP (2.5-second exposure) filter. To prepare for quantitative analysis, all images were sized to 600 $\times$ 450 pixels using graphics editing software (Photoshop; Adobe, San Jose, CA). The area of transduction was calculated using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). The percentage of area of transduction was calculated by dividing the transduced region by the total surface area. Mean fluorescence of the transduced area was calculated by converting the image to grayscale and calculating the mean pixel intensity using ImageJ. Surface plots were obtained using the ImageJ plug-in feature known as interactive 3D surface plots.

To compare viral transduction in the retina with the RPE/choroid, six eyes were harvested for each construct, the lens and cornea were

removed, and the retina was peeled away from the RPE/choroid. The retina and RPE/choroid were placed in separate 1.5-mL tubes and submerged in liquid nitrogen for 15 seconds. Eyes were not visualized for GFP before dissection to prevent UV damage and to expedite removal of the retina/RPE without protein degradation. RNA was extracted and purified (RNeasy kit; Qiagen, Valencia, CA), and then 3 μ g each sample was DNase treated (Turbo; Ambion, Austin, TX). Samples were loaded in triplicate for RT-PCR (Icycler IQ5; Bio-Rad, Hercules, CA) to detect GFP expression using a custom assay to EGFP obtained from Applied Biosystems (Foster City, CA) with the forward primer EGFP-ANYF (5'-GAGCGCACCATCTTCTTCAAG-3'), the reverse primer EGFP-ANYR (5'-TGTCGCCCTCGAACTTCAC-3'), and the probe EGFP-ANYM1 (5'-ACGACGGCAACTACA-3'). All samples were normalized to mouse β -actin (Applied Biosystems).

To analyze the transduction pattern in the retina, the quadrant containing the GFP region was dissected from each flat-mount used for the transduction study (see Fig. 4) and embedded in OCT. Serial 14- μ m transverse sections were made through the entire quadrant, and images were taken (BX51 microscope [Olympus]; Retiga 2000R FAST camera [QImaging, Surrey, BC, Canada]; QCapture Pro 5.0 software [QImaging]). Exposure times were adjusted independently for the RPE and retina for each virus to optimize visualization of the cell types transduced.

To determine the proportion of transduced cells in the inner nuclear layer (INL) and the outer nuclear layer (ONL) compared with the total number of cells for each layer in Ad5 Δ RGD, we used a modified stereological technique. Eighty-four serial 14- μ m sections spanning the transduced area were used for analysis. The fluorescent images were converted to 8-bit and analyzed with software (StereoIn-

FIGURE 3. Y79 retinoblastoma cells express CD46 and are more efficiently transduced by Ad5/F35 than Ad5, Ad5/F37, or Ad5ΔRGD. (A) Immunostaining indicates that Y79 cells express CD46 and some sialic acid, whereas CAR and α v integrin are expressed at very low levels. (B) FACS analysis of GFP expression after infection at an MOI of 500 reveals that Ad5/F35 more readily transduces Y79 cells than does Ad5, Ad5/F37, or Ad5ΔRGD. * $P \leq 0.05$; ** $P \leq 0.01$.

investigator version 6.5.5.1; Micro BrightField, Williston, VT). The optical dissector probe was used along with a sampling grid of $86.3 \times 20 \mu\text{m}$ and a sampling periodicity of 10. Before sampling, the INL/ONL/RPE was manually traced using the bright-field image to create an ROI for each section. The bright-field image was then used to count the total number of cells, and the fluorescent image was used to count the total number of GFP-positive cells. The raw data (see Fig. 6) was automatically calculated by software (StereoInvestigator version 6.5.5.1; Micro BrightField) using CE Gundersen variance. This provides high, median, and low estimates of cells for each section. The coefficient of error was estimated automatically by the software according to the Scheffer method, which is designed to calculate the CE for data points collected from a traced ROI taken from randomly sampled areas through a nonuniformly shaped tissue. Therefore, the curvature of the tissue is extrapolated from the traced images.

Immunohistochemistry

Y79 cells were plated at a density of 4.5×10^5 cells in poly-D-lysine-coated chamber slides (Becton-Dickinson) for 24 hours and were fixed in 10% formalin for 15 minutes before staining. Tissue sections were fixed in 4% PFA and then taken through a graded series of sucrose (15% and 30%) before sectioning at $14 \mu\text{m}$. To detect sialic acid, we used biotinylated *Maackia amurensis* lectin II (Mal II; Vector Laboratories, Burlingame, CA; $100 \mu\text{g/mL}$) followed by Cy3-conjugated streptavidin ($1 \mu\text{g/mL}$). To detect integrin we used anti-integrin α V mAb

(Transduction Laboratories; $1 \mu\text{g/mL}$) followed by Cy3-conjugated goat anti-mouse ($1 \mu\text{g/mL}$). Detection of CD46 was achieved using mouse anti-human CD46 ($20 \mu\text{g/mL}$; clone MEM-258; Serotec) followed by Cy3-conjugated goat anti-mouse ($1 \mu\text{g/mL}$). To detect the extracellular region of CAR, we used rabbit anti-CAR (1:2000; gift of Jeff Bergelson, University of Pennsylvania) followed by Cy3-conjugated goat anti-rabbit ($1 \mu\text{g/mL}$). We used two antibodies to detect Müller cells. We used rabbit anti-glial fibrillary acidic protein (anti-GFAP; Novus Biologicals, Littleton, CO) followed by Cy3-conjugated goat anti-rabbit ($1 \mu\text{g/mL}$). We also used mouse anti-CRALBP (Abcam, Cambridge, MA) followed by Cy3-conjugated goat anti-mouse ($1 \mu\text{g/mL}$). CRALBP staining was performed on $20\text{-}\mu\text{m}$ frozen retinal sections, whereas all other staining was performed on $14\text{-}\mu\text{m}$ sections. All negative controls were obtained by omission of the primary antibody.

Statistical Analysis

Except where otherwise stated, experiments were performed in duplicate at least three times. Error bars represent the SD from the mean. Where appropriate, significance was calculated using Student's *t*-test with the exception of the stereological data, for which the Scheffer method was used to determine the coefficient of error.

RESULTS

Adenovirus Vectors

To compare adenovirus tropism in the retina, we investigated the performance of four different adenovirus vectors, each of which expressed GFP under the control of a chicken β -actin promoter/CMV enhancer/rabbit globin intron. We have previously demonstrated that this promoter yields significantly greater transgene expression in all layers of the retina and RPE relative to the CMV promoter.¹⁴ In each vector, the transgene expression cassette was cloned in the antisense orientation with respect to the deleted E1 region. Each vector backbone was based on Ad5. Two of the vectors were modified either by replacement of the Ad5 fiber with that of Ad35 or Ad37, and in the third vector the RGD (Arg-Gly-Asp) motif in the Ad5 penton base was deleted (Fig. 1A). Hereafter each vector is referred to as Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD, respectively. To confirm expression and incorporation of the modified fibers, denatured samples of purified virus preparations were immunoblotted using an antibody raised against the first 17 amino acids at the N terminus of the Ad5 fiber. The immunoblots correlated with the predicted sizes according to amino acid sequence analysis for the fiber monomers of the denatured samples of Ad5 (64 kDa), Ad5/F35 (35 kDa), and Ad5/F37 (38 kDa; Fig. 1B). Trimerization of the fibers was observed in the samples that were not denatured for Western blot analysis and also correlated to the predicted sizes for Ad5 (192 kDa), Ad5/F35 (105 kDa), and Ad5/F37 (114 kDa).

Ad5/F35 Vector

Before examination of the tropism of each of the above vectors in the retina, we confirmed that each vector binds its respective receptor in cell culture. To examine whether our novel Ad5/F35 vector was redirected from CAR to CD46, we infected murine HEPA 1c1c7 cells with either Ad5 or Ad5/F35. The MOI for each vector was adjusted to attain a GFP-positive cell population of approximately 10%. For Ad5, this was achieved at an MOI of 20, whereas for Ad5/F35 an MOI of 750 was necessary for the same transduction frequency. When murine HEPA 1c1c7 cells were pretransfected with a cassette expressing human CD46, infection by the Ad5/F35 virus increased by nearly threefold compared with pretransfection with a control plasmid that expressed LacZ. In contrast, a decrease in infection by the Ad5 virus was observed when cells were pretrans-

FIGURE 4. After subretinal injection, Ad5ΔRGD has more robust expression in the retina and RPE than do Ad5, Ad5/F35, and Ad5/F37. (A) Flat-mounts showing area of transduction. (B) The percentage of the eyecup, as measured by ImageJ, transduced by each virus varies only slightly. (C) The intensity of GFP expression, measured as pixel intensity using ImageJ, from Ad5ΔRGD is greatly increased over Ad5, Ad5/F35, and Ad5/F37. (D) qRT-PCR analysis of GFP expression reveals that the Ad5ΔRGD virus is able to express at higher levels in the retina than Ad5, Ad5/F35, or Ad5/F37. (E) qRT-PCR of GFP expression shows that in the RPE, relative to Ad5, both Ad5/F35 and Ad5/F37 are reduced, whereas Ad5ΔRGD is slightly elevated. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

fectected with human CD46 relative to lacZ (Fig. 2A). We also noted that the process of transfection alone (with a control plasmid) was sufficient to enhance infection by both viruses, but transfection with CD46 had a significantly greater effect on Ad5/F35 than on Ad5.

Ad35 fiber binding has previously been shown to occur through the short consensus repeat 1 (SCR1) and 2 (SCR2) regions of CD46.²⁰ To examine the binding of Ad5/F35 to SCR1/2, we preincubated murine HEPA 1c1c7 cells with the antibody MEM258, which is known to bind SCR1. Rabbit anti-sheep IgG was used as a nonspecific control. As described, cells were also pretransfected with either a human CD46 or a lacZ-expressing plasmid. We found that whereas there was no significant change in Ad5 infection after blocking of CD46 with MEM258, Ad5/F35 infection was reduced by more than two-fold (Fig. 2B). Detailed characterization of the Ad5/F37 virus used in this study, and specifically its ability to use sialic acid as a receptor, has been described by us previously.¹⁸

Previous studies have shown that CD46 is expressed on human retinoblastoma (Y79) cells, allowing for efficient transduction of these cells by Ad5/F35 viruses.¹⁵ We confirmed that Y79 cells express CD46 and sialic acid along with moderate amounts of CAR but very little α_v integrin by immunohistochemistry (Fig. 3A). Y79 cells were infected at an MOI of 500 with each recombinant virus for 24 hours and were analyzed for GFP expression by FACS. The proportion of GFP-expressing cells for Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD was determined to be $10\% \pm 1\%$, $31\% \pm 2\%$, $2\% \pm 1\%$, and $12\% \pm 1\%$, respectively (Fig. 3B). Collectively, these data suggest that our

recombinant Ad5/F35 construct binds CD46 and efficiently transduces cells that express CD46 on their surfaces. We determined that sialic acid is also expressed on Y79 cells, yet Ad5/F37 did not transduce Y79 cells efficiently, perhaps because Y79 cells express very little integrin for which Ad37 viruses have been shown to have a high affinity.²¹ Interestingly, Ad5 and Ad5ΔRGD had nearly identical transduction efficiency in Y79 cells.

In Vivo Studies

To study the kinetics of transgene expression from each of the viral constructs in the retina in vivo, 10^9 viral particles were injected into the subretinal space of 6-week-old C57BL/6J mice. Six days later, the eyes were harvested, flat mounted, and quantitated for GFP fluorescence using ImageJ software. Diffusion of the virus through the retina was measured as the total GFP-positive surface area relative to the surface area of the total eyecup. The transduced area for each virus varied slightly and, for Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD, was determined to be $9\% \pm 1\%$, $5\% \pm 1\%$, $6\% \pm 1\%$, and $9\% \pm 2\%$, respectively (Figs. 4A, 4B). The mean GFP pixel intensity for Ad5, Ad5/F35, Ad5/F37, and Ad5ΔRGD was 12 ± 1 , 5 ± 0.4 , 8 ± 1 , and 61 ± 4 relative units, respectively (Figs. 4A, 4C). To differentiate gene expression levels between retina and RPE, quantitative RT-PCR was performed separately on retina and RPE/choroid. We found that relative to Ad5, transgene expression from Ad5ΔRGD was *increased* 12 ± 3 -fold in the retina, whereas transgene expression from Ad5/F35 and Ad5/F37 *decreased*

FIGURE 5. Fluorescence microscopy of transverse sections of retinas infected with Ad5, Ad5/F35, Ad5/F37, and Ad5 Δ RGD show that though all transduce the RPE, each has a distinct tropism for certain cell types in the retina. (A) Ad5 transduces a small portion of photoreceptors and scattered Müller cells. (B) Ad5/F35 only transduces the RPE with no detectable GFP evident in the retina. (C) Ad5/F37 transduces mainly Müller cells in the retina (in addition to RPE). (D) Ad5 Δ RGD transduces mostly photoreceptors but also transduces scattered Müller cells. (E) In some regions of the retina, Ad5 Δ RGD-transduced photoreceptors show GFP in the cytoplasm only in the ONL and in inner and outer segments. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

9 ± 1 -fold and 3 ± 1 -fold in the retina, respectively (Fig. 4D). Although Ad5 Δ RGD was originally designed to have reduced tropism for RPE and, hence, potentially to redirect viral tropism toward photoreceptors, we found that relative to Ad5, transgene expression from Ad5 Δ RGD-transduced RPE increased 11 ± 7 -fold (Fig. 4E). Hence, higher transduction of photoreceptors might have been achieved in part by greater infection by the Ad5 Δ RGD virus overall in the retina. This is surprising given that an important component of adenovirus entry, the integrin binding RGD domain in the penton base, is deleted in this virus. In contrast, transgene expression in Ad5/F35- and Ad5/F37-infected RPE was reduced 160 ± 38 -fold and 4 ± 2 -fold, respectively (Fig. 4E).

To identify the specific cell types in the retina infected by each virus, we prepared frozen retinal sections from animals injected in the subretinal space. As expected, GFP fluorescence was clearly visible in the RPE of all injected animals. However, transduction of different cell types in the retina was also noted. As described previously, Ad5 infected a small number of photoreceptor cells (Fig. 5A). However, contrary to previous studies, our Ad5/F35 or Ad5/F37 virus did not significantly infect photoreceptors, at least to the extent detectable by observation of direct GFP fluorescence. Whereas Ad5/F35 infected only the RPE, Ad5/F37 infected primarily the Müller

cells in the retina (in addition to the RPE) (Figs. 5B, 5C), based on cellular morphology. Müller cell transduction was further confirmed by costaining with antibodies against glial fibrillary acidic protein (GFAP) and cellular retinaldehyde-binding protein (CRALBP; Fig. 6). GFP was readily detectable in photoreceptor cell bodies and inner and outer segments in the retina transduced with Ad5 Δ RGD with occasional Müller cell transduction. However, there was some variability in the GFP expression pattern within the ONL with Ad5 Δ RGD; in some cases sporadic GFP-positive cell bodies had a cytoplasmic expression pattern and in other cases perinuclear GFP was observed in the ONL. In both cases, inner and outer segments were also GFP positive (Figs. 5D, 5E).

Retinal Cell Layer Most Efficiently Transduced by Ad5 Δ RGD Is the Outer Nuclear Layer

We found that Ad5 Δ RGD has the greatest transgene expression in the retina relative to Ad5, Ad5/F35, and Ad5/F37 and that it transduces a number of cell types. To determine the proportion of transduced cells in the ONL compared with the INL, we took a semiquantitative approach involving a modified stereological technique (Stereoinvestigator; Micro Brightfield). Briefly, serial sections were taken through the entire quadrant

FIGURE 6. Müller cells are transduced by Ad5/F37. (A) Ad5/F37-transduced retinal sections labeled with an antibody against CRALBP. A gradient of CRALBP expression, with reduced expression toward the center of the region of injection was observed. Downregulation of genes involved in the phototransduction cascade has been observed previously³¹ after subretinal injection. (B) Ad5/F37-transduced retinal sections labeled with GFAP antibody.

of the eye showing GFP expression. A stereological grid was placed over the retina of every tenth section of the ROI, and the total number of GFP-positive cells was counted and compared with the total number of cells for both ONL and INL. We found that of the $52,739 \pm 0.04$ total cells in the ONL, $11,507 \pm 0.1$ cells (22%) were transduced (Figs. 7A, 7C). In the INL there were $12,816 \pm 0.06$ total cells, and of those 1381 ± 0.3 (11%) were transduced (Figs. 7B, 7C). Transduced cells of the INL tended to occur in discrete patches and likely corresponded to Müller cells, whereas those in the ONL were evenly distributed (Figs. 7B, 7D).

Photoreceptor-Specific Transgene Expression from Ad5 Δ RGD Vectors

Having demonstrated that Ad5 Δ RGD vectors can transduce retinal cells more effectively than Ad5, Ad5/F35, or Ad5/F37, we sought to investigate the possibility of achieving photoreceptor-specific transgene expression from adenovirus vectors. Previously, we demonstrated photoreceptor-specific transgene expression *in vivo* using adenovirus vectors containing a 4.7-kb murine rod opsin promoter.¹⁴ However, transgene expression from such a promoter was achieved only in discrete patches across the retina. Hence, we generated an adenovirus vector (Ad5sRhoGFP Δ RGD)-expressing GFP regulated by 257 bp from the 5' conserved enhancer/promoter region of the mouse rod opsin gene (Fig. 8A). This promoter has previously been shown to be photoreceptor specific in the context of adeno-associated virus.²² A total of 10^9 Ad5sRhoGFP Δ RGD viral particles were injected into the subretinal space of adult C57BL/6J mice and examined 14 days after injection in frozen sections. Substantial GFP expression restricted to the ONL that contained primarily the photoreceptor cell bodies was detectable (Fig. 8B). In addition, we observed significant levels of GFP in photoreceptor inner and outer segments.

DISCUSSION

The viability of adenovirus as a gene therapy vector for ocular disease has recently been demonstrated in two clinical trials, one delivering PEDF for the treatment of age-related macular degeneration and another delivering thymidine kinase for the treatment of retinoblastoma.^{4,5} Currently, 221 mutations have been implicated in retinal degeneration (<http://www.sph.uth.tmc.edu/retnet/disease.htm>). Many of these proteins are critical for proper functioning of the phototransduction cascade and are expressed exclusively within photoreceptors. One of the major drawbacks to the use of adenovirus in the eye is its inefficient transduction of photoreceptor cells. Three adenoviral vectors containing modification to the Ad5 capsid proteins have been previously described to yield enhanced photoreceptor transduction.^{14–16} Here, for the first time, we directly compare Ad5, Ad5/F35, Ad5/F37, and Ad5 Δ RGD using the same promoter, transgene expression cassette, injection, and detection techniques. Our results indicate that each vector has a distinct tropism for particular cell types of the retina. Our results differ from earlier reports that indicated Ad5/F35 and Ad5/F37 have enhanced transduction of photoreceptors relative to Ad5.

It has previously been shown that Ad5/F35 transduces the RPE and photoreceptors.¹⁵ In our study, Ad5/F35 exclusively transduced the RPE and with 160-fold less efficiency than Ad5. There are, however, differences between these studies. We used the CBA promoter, whereas earlier investigators used the CMV promoter. However, we have shown previously that, unlike the CMV promoter, the CBA promoter allowed us to observe Ad5 transduction of photoreceptors. We did not amplify the GFP signal through the use of an antibody. However, in our studies, Ad5 expression was observed without the need for a GFP antibody. Therefore, Ad5/F35 appeared to be less efficient at transducing photoreceptors than Ad5.

We have shown that, similar to the Ad5/F35 used in previous studies, Ad5/F35 more efficiently transduces cells that express human CD46 than Ad5,¹⁵ Ad5/F37, or Ad5 Δ RGD. The need for a higher MOI for Ad5/F35 relative to Ad5 to achieve the same level of transduction in HEPA 1c1c7 cells is further evidence of a redirection of tropism from the Ad5 receptor and can possibly be explained further by previously published observations of low expression of CD46 in murine hepatocytes.²³ Although mice do carry a CD46 gene, some report that expression is restricted to the testes,²⁴ whereas others indicate expression by photoreceptors.¹⁵ We were unable to obtain a commercially available antibody for mouse CD46 but did test several tissues from mice for expression by quantitative RT-PCR using primers to mouse CD46. Our data agree with reports that mouse testes have the most CD46 and that levels are greatly reduced in liver, retina, and RPE/choroid (data not shown). A murine homologue to human CD46, crry, has been shown to have a similar expression pattern and to perform a comparable role in the complement system.²⁵ However, it is not known whether Ad5/F35 is able to bind crry. Expression in the retina of both crry in mice and CD46 in humans is most abundant in the RPE, where it is restricted to the basal and lateral surfaces of the RPE cells.²⁶ Because Ad5/F35 is injected into the subretinal space, it would be exposed to the apical side of the RPE, limiting access to crry. This could be another explanation for less efficient transduction of the RPE by Ad5/F35.

Aside from the RPE, we observed Ad5/F37-mediated GFP expression in Müller cell processes at the inner and outer limiting membranes and possibly the inner retinal neurons. This is in contrast to what has been found previously¹⁶ in which both the RPE and the photoreceptors were transduced. This could be attributed to several differences in our approach:

FIGURE 7. Sections through the region of transduction used for modified stereology to determine portions of cells transduced in the ONL and INL. (A) Numbers of transduced cells relative to the total number of cells in the ONL. (B) Numbers of transduced cells relative to total number of cells in the INL. (C) 22% of the cells in the ONL were transduced, whereas 11% were transduced in the INL. (D) Fluorescent micrographs used to determine cells transduced in the ONL/INL.

(1) We used the CBA promoter whereas earlier investigators used the CMV promoter. (2) There was a single amino acid difference between the F37 fiber used in our study and that of earlier studies (ours has a threonine at position 10, but previous studies have an aspartic acid). (3) Our mode of injection was subretinal compared with the previously used intravitreal delivery. However, it would be surprising that viral diffusion would bypass the photoreceptors to be taken up by Müller cells after subretinal injection. We have seen the same pattern of expression after injection with an Ad5/F17 virus¹⁴; both were classified as subgroup D adenoviruses. It has been shown that Ad37 binds to sialic acid.¹⁵ Although sialic acid is present throughout the retina, it does not appear to be preferentially expressed at higher levels on Müller cells (data not shown). It has also been shown that Ad37 has a higher binding affinity to integrin than Ad5.²¹ This could explain our observation of reduced transduction of Y79 cells by Ad5/F37, which do express sialic acid but very little α_v integrin.

It has been shown that Müller cells express four integrin subunits (α_1 , α_2 , α_3 , and β_1).²⁷ It would be interesting to see

whether this combination of integrin and sialic acid expression on Müller cells preferentially enhances transduction for Ad5/F37 over the other vectors tested. Müller cells have been shown to become phagocytic when foreign particles are injected into the eye.²⁸ Therefore, another possible explanation could be that Müller cells are activated by Ad5/F37 and then phagocytose the virus.

Our Ad5 Δ RGD construct was designed to eliminate integrin binding with the intention of reducing viral uptake by the RPE, diverting the virus to the juxtaposed photoreceptors. To this end we did see significantly elevated expression in photoreceptors relative to all the other vectors tested. More surprisingly, we observed an increased amount of RPE transduction relative to Ad5. This indicated that, at least in the context of the RPE, integrin binding may not be critical for Ad5 uptake. Aside from increased photoreceptor transduction compared with Ad5, we also observed a different pattern of expression within the cells. In some cases the entire photoreceptor cell body, along with the inner/outer segments, contained GFP. In other regions GFP expression in the cell body, along with the inner/

FIGURE 8. Structure of the Ad5sRhoGFPΔRGD construct. (A) A 257-bp mouse opsin enhancer/promoter (sRho) driving expression of an EGFP cassette was inserted into the Ad5ΔRGD vector in an antisense orientation into the deleted E1 region. (B) Fluorescent micrograph of transverse section through retina 14 days after subretinal injection of 10^9 viral particles indicates robust expression restricted to only the photoreceptors.

outer segments expressing GFP, appeared to be cytoplasmic. A possible explanation for the two different patterns of expression could be a result of the amount of GFP expressed by the cell. The GFP transgene used in our study does not have a nuclear localization signal and we do not, therefore, have reason to believe that it enters the nucleus with high efficiency. It is possible that nuclear uptake and binding of GFP are concentration dependent and that exclusive perinuclear localization occurs in those photoreceptors containing fewer viral particles per cell. That all four vectors have distinct tropism in the retina yet infect the RPE suggests a possible unknown mechanism for viral uptake by the RPE. One role of the RPE is to phagocytose the outer segments of photoreceptors.²⁹ This may be one explanation for how any modified adenovirus vector enters the RPE independently of its tropism in the retinal layers.

By placing GFP expression under the control of a 257-bp enhancer/promoter of mouse opsin (sRho), we were able to drive robust expression that was restricted to the photoreceptors. The pattern of expression was consistent with that seen using adeno-associated virus vectors, with the entire cell body and the inner/outer segments containing GFP.²¹ However, it is not consistent with that reported for nonviral gene delivery vectors in which the inner segments lack GFP expression.³⁰ To our knowledge, this is the first report of robust photoreceptor-specific transgene expression from an adenovirus vector.

To summarize, our data suggest that either changing the fiber or deleting the RGD domain in the penton base can be sufficient to enhance adenovirus transduction of distinct cell types in the retina. We show that Ad5/F35 targets the RPE, Ad5/F37 targets the RPE and Müller cells, and Ad5ΔRGD targets the RPE, photoreceptors, and some Müller cells. Finally, we show that robust expression can be restricted to the photoreceptors by using a 257-bp mouse opsin promoter in the

Ad5ΔRGD construct. These data are relevant for the design of adenovirus-based gene transfer vectors to target specifically those cell populations affected in the various retinopathies.

References

- Hazin R, Freeman PD, Kahook MY. Age-related macular degeneration: a guide for the primary care physician. *J Natl Med Assn.* 2009;101:134–138.
- Smith AJ, Bainbridge JW, Ali RR. Prospects for retinal gene replacement therapy. *Trends Genet.* 2009;25:156–165.
- Kellner U, Kraus H, Heimann H, Helbig H, Bornfeld N, Foerster MH. Electrophysiological evaluation of visual loss in Muller cell sheen dystrophy. *Br J Ophthalmol.* 1998;82:650–654.
- Chevez-Barrios P, Chintagumpala M, Mieler W, et al. Response of retinoblastoma with vitreous tumor seeding to adenovirus-mediated delivery of thymidine kinase followed by ganciclovir. *J Clin Oncol.* 2005;23:7927–7935.
- Campochiaro PA, Nguyen QD, Shah SM, et al. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. *Hum Gene Ther.* 2006;17:167–176.
- Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A.* 1996;93:13565–13570.
- Kumar-Singh R, Yamashita CK, Tran K, Farber DB. Construction of encapsidated (guttated) adenovirus minichromosomes and their application to rescue of photoreceptor degeneration. *Methods Enzymol.* 2000;316:724–743.
- Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci U S A.* 2001;98:13282–13287.
- Henry LJ, Xia D, Wilke ME, Deisenhofer J, Gerard RD. Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in *Escherichia coli*. *J Virol.* 1994;68:5239–5246.
- Bergelson JM, Cunningham JA, Droguett G, et al. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science.* 1997;275:1320–1323.
- Wickham TJ, Mathias P, Cheresch DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell.* 1993;73:309–319.
- Gaggar A, Shayakhmetov DM, Lieber A. CD46 is a cellular receptor for group B adenoviruses. *Nat Med.* 2003;9:1408–1412.
- Arnberg N, Edlund K, Kidd AH, Wadell G. Adenovirus type 37 uses sialic acid as a cellular receptor. *J Virol.* 2000;74:42–48.
- Cashman SM, McCullough L, Kumar-Singh R. Improved retinal transduction in vivo and photoreceptor-specific transgene expression using adenovirus vectors with modified penton base. *Mol Ther.* 2007;15:1640–1646.
- Mallam JN, Hurwitz MY, Mahoney T, Chevez-Barrios P, Hurwitz RL. Efficient gene transfer into retinal cells using adenoviral vectors: dependence on receptor expression. *Invest Ophthalmol Vis Sci.* 2004;45:1680–1687.
- Von Seggern DJ, Aguilar E, Kinder K, et al. In vivo transduction of photoreceptors or ciliary body by intravitreal injection of pseudotyped adenoviral vectors. *Mol Ther.* 2003;7:27–34.
- Fallaux FJ, Kranenburg O, Cramer SJ, et al. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther.* 1996;7:215–222.
- Cashman SM, Morris DJ, Kumar-Singh R. Adenovirus type 5 pseudotyped with adenovirus type 37 fiber uses sialic acid as a cellular receptor. *Virology.* 2004;324:129–139.
- He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A.* 1998;95:2509–2514.
- Sakurai F, Murakami S, Kawabata K, et al. The short consensus repeats 1 and 2, not the cytoplasmic domain, of human CD46 are crucial for infection of subgroup B adenovirus serotype 35. *J Control Release.* 2006;113:271–278.

21. Mathias P, Galleno M, Nemerow GR. Interactions of soluble recombinant integrin α v β 5 with human adenoviruses. *J Virol*. 1998;72:8669–8675.
22. Khani SC, Pawlyk BS, Bulgakov OV, et al. AAV-mediated expression targeting of rod and cone photoreceptors with a human rhodopsin kinase promoter. *Invest Ophthalmol Vis Sci*. 2007;48:3954–3961.
23. Inoue N, Ikawa M, Nakanishi T, et al. Disruption of mouse CD46 causes an accelerated spontaneous acrosome reaction in sperm. *Mol Cell Biol*. 2003;23:2614–2622.
24. Sakurai F, Kawabata K, Koizumi N, et al. Adenovirus serotype 35 vector-mediated transduction into human CD46-transgenic mice. *Gene Ther*. 2006;13:1118–1126.
25. Li B, Sallee C, Dehoff M, Foley S, Molina H, Holers VM. Mouse Crry/p65: characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. *J Immunol*. 1993;151:4295–4305.
26. Yang P, Tyrrell J, Han I, Jaffe GJ. Expression and modulation of RPE cell membrane complement regulatory proteins. *Invest Ophthalmol Vis Sci*. 2009;50:3473–3481.
27. Guidry C, Bradley KM, King JL. Tractional force generation by human Muller cells: growth factor responsiveness and integrin receptor involvement. *Invest Ophthalmol Vis Sci*. 2003;44:1355–1363.
28. Mano T, Puro DG. Phagocytosis by human retinal glial cells in culture. *Invest Ophthalmol Vis Sci*. 1990;31:1047–1055.
29. Finnemann SC. Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. *EMBO J*. 2003;22:4143–4154.
30. Farjo R, Skaggs J, Quiambao AB, Cooper MJ, Naash MI. Efficient non-viral ocular gene transfer with compacted DNA nanoparticles. *PLoS One*. 2006;1:e38.
31. Rattner A, Toulabi L, Williams J, Yu H, Nathans J. The genomic response of the retinal pigment epithelium to light damage and retinal detachment. *J Neurosci*. 2008;28:9880–9889.