Adeno-Associated Virus Encoding Green Fluorescent Protein as a Label for Retinal Pigment Epithelium

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Purpose. To determine whether transduction with adeno-associated virus encoding green fluorescent protein (AAV-GFP) is useful for labeling transplanted retinal pigment epithelial cells (RPE).

Methods. Transduction was performed by infection of confluent or subconfluent cultured feline RPE or by subretinal injection. Cells transduced in vitro were analyzed to determine label stability over time and label conservation with cell division. RPE transduced in vivo were harvested at 5 weeks for transplantation or immunohistochemical detection. Two cats received subretinal injections of harvested cells and were killed at 3 or 7 days.

Results. In vitro transduction of confluent RPE resulted in stable GFP fluorescence for at least 3 months. There was a marked decline in fluorescence after cell division. Nonconfluent transduced cells conserved label after cell division but showed a marked decline in the number of cells, due to cell death. In vivo transduction resulted in a high level of labeling, allowing labeled cells to be harvested and transplanted. Transplanted cells were detected immunohistochemically. Photoreceptor labeling was detected over areas containing a high density of transplanted, labeled RPE derived from cells transduced in vivo. Possible light toxicity to transduced RPE was observed.

Conclusions. AAV-GFP–labeling of confluent cultured RPE and RPE in situ can be used to identify transplanted RPE, with some reservations. Cell division may cause dilution of the label, and release of cell contents into the subretinal space may cause label transfer to photoreceptors. Exposure to light of transduced cells should be limited. (*Invest Ophthalmol Vis Sci.* 2003;44:772–780) DOI:10.1167/iovs.02-0091

Age-related macular degeneration (AMD) is the leading cause of legal blindness among persons more than 55 years of age in the United States.[1–4] Most patients with AMD lose vision as a result of choroidal new vessel (CNV) growth from the choriocapillaris through Bruch's membrane into the subretinal space. Retinal pigment epithelium (RPE) transplantation has been proposed as a possible adjunctive treatment for AMD and some retinal dystrophies involving the RPE.[5] Studies performed on Royal College of Surgeons (RCS) rats, in which the RPE are dysfunctional, show that transplanted RPE can rescue photoreceptors and restore useful vision.[6–16] Despite encouraging results in animal models of retinal disease, RPE transplantation in humans has not been successful thus far,[17–22] and additional experimental work is needed to refine the technique. Experimental studies, particularly in animals with healthy RPE, are difficult to interpret, because of the inability to differentiate transplanted from native RPE cells.

Ideally, a marker for RPE transplantation should have the following characteristics: long-term stability, no transfer of marker to native cells by diffusion into the subretinal space or release of marker after cell death, preservation with cell division, and no long-term toxicity or interference with cell metabolism. One of the earliest and easiest methods used to identify transplanted RPE cells was pigmentation (i.e., transplantation of pigmented RPE cells into recipients who had no pigmented RPE).[6,12,23–28] Although this method may be suitable for short-term studies, release of pigment into the subretinal space[26] and subsequent phagocytosis of pigment by native RPE cells[29,30] prevents unambiguous identification of the transplanted cells. In addition, RPE cells lose their pigment with cell division.[31,32] Studies by Wang et al.[24] show that at 3 days, transplanted RPE microaggregates show some signs of proliferation, as evidenced by positive Ki-67 labeling and the presence of mitotic cells. Labeling with carbon particles[33,34] or with fluorochromes[8,30,35] can present the same problems.

To overcome the problems associated with using pigmentation or fluorochromes as the only marker of the transplanted cell, some investigators have used an additional label, such as BrdU[36–38] or tritiated thymidine.[30,39–41] However, these labels are incorporated only by dividing cells and therefore are not entirely satisfactory for studies involving transplantation of freshly isolated RPE cells.

For allogeneic transplantation, delivery of female cells into male recipients, and vice versa, can be performed with markers that permit specific identification of transplanted female or male cells.[24,42] A major hurdle to this approach is immune rejection of cells and the inability to detect a large percentage of the transplanted cells. Y-chromosome labeling can identify approximately 47% of RPE cells in male tissue sections,[24] and Barr body staining permits identification of approximately 58% RPE cells in female tissue.[43]

This labeling strategy, although feasible for short-term allogeneic transplants, cannot be applied to long-term studies of transplanted allogeneic RPE unless the animals are immunosuppressed.[28,34,42] Animal studies to assess the viability of autologous transplants must use a different strategy for cell labeling.

Recently, virus vectors carrying a marker such as β-galactosidase[44,45] or green fluorescent protein (GFP)[46,47] have been used to label transplanted RPE cells. In addition, subretinal virus injection can label RPE successfully.[48,49] Such labeling approaches enable the investigator to identify transplanted RPE

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cells that have been harvested in a peripheral biopsy[30,39,50] and used for autologous or allogeneic transplants. GFP offers the advantage that one can use a fundus camera equipped with fluorescein filters for in vivo monitoring of transplanted cells.[46–48] In addition, antibodies to GFP are available[46] that allow identification of labeled cells by immunohistochemistry.

In the present study, we assessed the efficacy of adeno-associated virus (AAV) containing the cDNA encoding GFP for labeling RPE, both in culture and in situ. AAV is an appropriate vector for labeling RPE cells in vivo because, unlike retroviral vectors,[45,46] AAV can effectively transduce nondividing cells[51] and is stable. AAV, as measured by reporter gene expression, has been demonstrated in RPE cells for up to 1 year after subretinal injection.[52] In addition, AAV has been shown to cause minimal inflammatory response[48] and has no toxic effects on the retina after subretinal delivery.[53,54] To determine whether AAV-GFP is an appropriate marker for cultured RPE cells, we studied the long-term stability of AAV-GFP in transduced cells and the preservation of label after cell division. In vivo studies (i.e., subretinal injection of AAV-GFP) were performed to identify time of peak labeling, to determine the optimal time to harvest the transfected, labeled RPE, to determine the efficiency of in vivo RPE labeling, and to demonstrate the feasibility of identifying labeled RPE after transplantation.

METHODS

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Virus Preparation: AAV-GFP

The AAV-GFP virus was prepared by packaging the recombinant DNA into AAV-2 particles by complementation as described.[55,56] The transgene cassette contained enhanced cDNA coding green fluorescent protein (EGFP; Clontech, Palo Alto, CA) under control of a cytomegalovirus (CMV) promoter. The recombinant virus was purified through three successive centrifugations in CsCl gradients.

The number of virion DNA particles was determined by slot blot analysis and was found to be 10^{13} particles/mL. Titer of the purified virus was determined by infectious unit (IU) assay on 84 to 31 cells, an E1/E4-complementing cell line derived from 293 cells and defined as infectious units per milliliter. The titer of the purified virus was 1.6×10^{11} IU/mL.

In Vitro AAV-GFP Transduction of Cat RPE

Isolation and Culture of RPE. Domestic shorthaired cats, 6 to 12 months of age and weighing 2.0 to 5.0 kg, were killed with an intravenous overdose of pentobarbital sodium (200 mg/kg) after sedation with ketamine (20 mg/kg) and xylazine (0.5 mg/kg). Feline RPE cells were isolated according to the protocol of Stramm et al.[57] Briefly, after whole-eye storage overnight in Pucks Saline F, RPE cells were collected from posterior segments after incubation in 0.25% trypsin in Ca^{2+}- Mg^{2+}-free Pucks Saline F at 37°C for 45 minutes. Cells were collected in complete DMEM (GibcoBRL, Grand Island, NY) containing: 15% fetal bovine serum (FBS), 300 μg/mL L-glutamine, 2.5 μg/mL amphotericin B, 0.05 mg/mL gentamicin, and 1 ng/mL bFGF and stored on ice. The RPE cells were centrifuged at 1000 rpm for 5 minutes, resuspended in 2 mL of complete medium, and plated in a 35-mm tissue culture dish. Cultures were incubated in a humidified incubator (Forma Scientific, Marietta, OH) containing 10% CO_2-90% air. Medium was changed every other day. Once confluent, the cells were passaged at a 1:4 dilution with 0.25% trypsin/0.1% EDTA. Cultures continued to be refed with complete medium every other day.

AAV-GFP Transduction. First-passage confluent cat RPE cells were incubated for 2 hours at 37°C, 10% CO_2-90% air with 3% FBS in DMEM containing approximately 1×10^{10} purified AAV-GFP particles/mL.[55] At the end of the incubation period, the volume of medium in each dish was adjusted to render a final concentration of 15% FBS in complete DMEM. Control dishes were incubated with the same medium containing the vehicle only in place of the AAV-GFP. All cultures were refed every other day with complete medium.

Experimental Design for In Vitro Studies. To determine the stability of GFP expression, transduced confluent cultures from three different cell lines were labeled and maintained in culture for 3 months, along with nontransduced control cultures of the same cell line. Cultures were photographed at week 1, 2, 3, 4, 6, 7, 9, and 12 after transduction. To determine GFP expression after cell proliferation, confluent transduced cultures were passaged 1:2 or 1:3. These cultures were photographed at day 1 and week 4 after passage. With a microscope (Axiovert 135; Carl Zeiss, Thornwood, NY), phase contrast and fluorescence photographs of four designated fields were taken before inoculation and at specified time points for all experimental conditions; 35-mm images were scanned for analysis (Duoscan flatbed scanner and FotoLook 2.09.04 software; AGFA, Orangeburg, NY) with Photoshop (ver. 5.0; Adobe, San Jose, CA).

AAV-GFP Transduction In Vivo

Domestic short-haired cats (age 6–12 months, weight 2.0–5.0 kg) underwent surgery. After sedation with ketamine (20 mg/kg) and xylazine (0.5 mg/kg), each animal was intubated and anesthetized with isoflurane. The pupil was dilated with 1% tropicamide. The animal was placed under a surgical microscope (URBAN; Storz, St. Louis, MO). Globe exposure was increased by lateral canthotomy, and the superonasal and temporal quadrants were exposed by 240° conjunctival peritomy. Pars plana incisions were made 4 mm posterior to the limbus with a 20-gauge blade (V-lance; Alcon Surgical, Fort Worth, TX) in both superior quadrants and inferotemporally. An infusion cannula (3 mm in length) was introduced through the inferotemporal sclerotomy and secured with a 4-0 silk mattress suture. Balanced saline solution (BSS; Alcon Surgical) was infused into the vitreous cavity. A light pipe and vitrectomy probe (Ocutome; Alcon Surgical) were introduced through the superior sclerotomies, and a contact lens was applied to the cornea. After pars plana lensectomy and core vitrectomy, the infusion solution was changed from saline solution to saline solution without Ca^{2+} to facilitate retinal detachment without damage to the RPE. The infusion fluid was again changed after 20 minutes to saline solution containing calcium. A 39-gauge cannula (Storz) attached to a 50-mL syringe was positioned against the pigmented area of the retina by the surgeon. A syringe pump (SP 100I; World Precision Instruments, Sarasota, FL) injected saline solution (40 mL/h) to create a controlled retinal detachment approximately 4 mm in diameter. A 30-gauge cannula connected to a 100 μL syringe (Hamilton, Reno, NV) was used to inject AAV-GFP (20 μL, 1×10^{13} particles/mL) slowly into the subretinal space through the retinotomy. Sclerotomies, conjunctival incisions, and the canthotomy were closed with 7-0 nylon sutures (Vicryl; Ethicon, Somerville, NJ). Dexamethasone sodium phosphate (1.2 mg) and gentamicin (12 mg) were injected subconjunctivally, and the eye was dressed with 1% chloramphenicol, 0.3% tobramycin, 0.1% dexamethasone, and 1% atropine ophthalmic ointment.

Initial studies were performed to determine the time of peak expression of GFP. Fundus photographs using fluorescein filters were taken to monitor expression of GFP. Four animals were observed at 1-week intervals for up to 8 weeks after injection. These studies showed peak expression at 5 weeks, and expression lessened at 6 weeks or more (Fig. 1). Because in vitro studies suggested the possibility that light from serial photography may cause RPE toxicity (see the Results section), seven animals were monitored at 5 weeks only, to confirm expression of GFP immediately before they were killed for histology and immunocytochemistry studies, cell culture, or transplantation.

Experimental eyes from two animals were harvested specifically to isolate transduced cells for culture. Of the five remaining eyes included in this study, three were killed and processed for analysis by histology and immunocytochemistry, and two served as donors for RPE transplantation experiments.

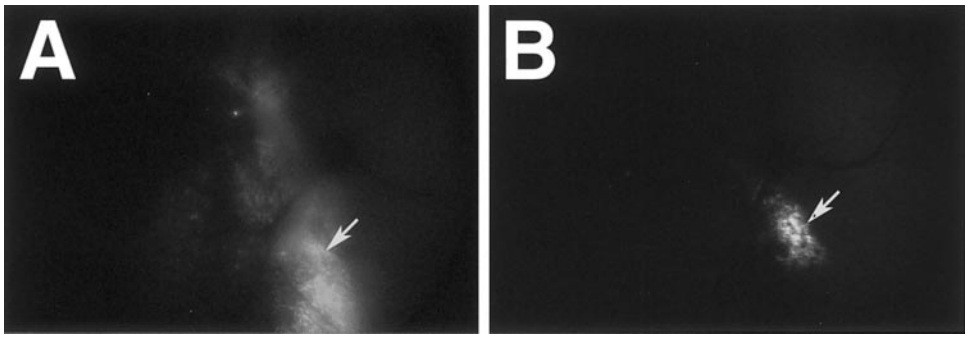

FIGURE 1. An eye transduced with AAV-GFP and photographed at 1-week intervals. AAV-GFP green fluorescence peaked at week 5 after transduction (**A**) and decreased at week 7 (**B**). No visible fluorescence was seen at week 8 (not shown). *Arrows*: GFP fluorescence.

Transplantation of AAV-GFP–Transduced RPE

AAV-GFP–transduced RPE were prepared by isolating a full thickness biopsy consisting of retina, RPE, and choroid from the donor eye. The biopsy, which was approximately 3 × 3 mm^2, was incubated in collagenase type IV (0.04 mg/mL) for 1 hour at 37°C. After incubation, labeled RPE cells (monitored by green fluorescence with an inverted microscope) were separated from the choroid and retina. RPE microaggregates were formed by triturating harvested RPE cells in 25 μL DMEM. Labeled cells not used for transplantation were seeded onto uncoated culture dishes.

A hydraulic RPE debridement was performed as described previously.[58] Briefly, after pars plana lensectomy and vitrectomy, a 33-gauge cannula was introduced through a sclerotomy and placed on the surface of the retina overlying the tapetum. Forced injection of saline solution (through an attached syringe) simultaneously created a localized retinal detachment and removed native RPE in the center. A 30-gauge cannula attached to a syringe (Hamilton) was introduced into the subretinal space through the original retinotomy, and 5 μL of RPE cells isolated from the 3 × 3 mm^2 biopsy (approximately 50,000 cells) were injected under the detached retina. The sclerotomies, conjunctiva, and canthotomy were closed, and the eye was dressed as described earlier. Three or 7 days after surgery, the eye was harvested and processed for analysis by histology and immunocytochemistry.

Tissue Processing. Animals were injected intravenously with an overdose of sodium pentobarbital (200 mg/kg) after sedation with ketamine (20 mg/kg) and xylazine (0.5 mg/kg). Eyes were enucleated immediately and fixed in ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 to 60 minutes to harden the tissue. The anterior segment of the eye was then removed, and the posterior eyecup was immersed in fresh fixative at 4°C for 16 hours.

A piece of tissue approximately 5 × 5 mm^2 was trimmed from the specimen and dehydrated in ascending ethanol after it was rinsed in 0.1 M phosphate buffer. The tissue then was cleared in butanol and embedded in diethylene glycol disterate (DGD, Polysciences Inc., Warrington, PA) with the retina facing up in a cryomold (Miles Laboratory Inc., Elkhart, IN). Two-micrometer-thick sections were cut on an ultramicrotome (Ultracut UCT; Leica Microsystems Inc., Exton, PA), transferred to slides (ProbeOn Plus; Fisher Scientific, Pittsburgh, PA), and baked at 55°C overnight.

Groups of consecutive slides were selected from one end of the bleb to the other end, separated by approximately 200 μm. The consecutive slides were stained in 0.125% toluidine blue or treated with anti-GFP antibody, respectively.

Immunocytochemistry. Selected sections were dewaxed and rehydrated. Sections were incubated in 2% normal goat serum and 0.5% bovine serum albumin in PBS for 45 minutes at room temperature to block nonspecific labeling. Primary antibody (rabbit anti-GFP 1:2000; Molecular Probes, Eugene, OR) was applied to sections overnight at 4°C followed by rinsing with PBS for 30 minutes with three changes. Sections were incubated in linker solution (biotinylated anti-mouse-rabbit polyclonal antibody) from a kit (LSAB2 Kit; Dako Corp., Carpinteria, CA) for 15 minutes at room temperature and rinsed with PBS for 15 minutes with three changes. Sections were then incubated in alkaline phosphatase-conjugated streptavidin for 15 minutes at room temperature and rinsed for 15 minutes with PBS. 5-Bromo-4-chloro-3-indoxyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT; Dako Corp.) was used as the phosphatase substrate. After incubating in BCIP/NBT for 5 minutes at room temperature, sections were dehydrated and permanently mounted.

Detection of AAV in RPE by Polymerase Chain Reaction

DNA was extracted from nuclear and cytoplasmic fractions of confluent (15 weeks after inoculation, $n = 1$) and nonconfluent (15 weeks after inoculation, $n = 1$, and 6 days after inoculation, $n = 1$) RPE cultures transduced with AAV-GFP. Similarly, DNA was extracted from corresponding control, nontransduced cells. DNA extraction was performed with a kit (DNeasy Tissue; Qiagen, Valencia, CA). Polymerase chain reaction was performed with primers specific to the cytomegalovirus (CMV) promoter region of the rAAV and a recombinant *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The primer sequences were: forward primer, 5′-ACATCAATGGGCGTGGATAGCG-3′, and reverse primer, 5′-TCAATGGGGCGGAGTTGTTACGAC-3′. The expected product size was 135 bp. PCR reaction conditions were as follows: 94°C for 2 minutes and 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with extension for 10 minutes at 72°C. PCR products were separated by 1.5% agarose gel electrophoresis and the bands photographed.

RESULTS

In Vitro AAV-GFP Transduction of Feline RPE Cells

Transduction in cell culture after incubation in AAV-GFP was observed within 48 hours. The percentage fluorescence in AAV-GFP–transduced cultures was stable for 3 months within each cell line (Figs. 2, 3). The mean percentage of fluorescence of all cell lines at week 1 was 78.38% ± 5.15%; at week 6, 79.03% ± 4.70%; and at week 12, 78.26% ± 9.24% ($n = 4$). The change in percentage of fluorescent cells with time in each cell line was subjected to a one-way analysis of variance (ANOVA). None of the cell lines showed a statistically significant change in percentage of fluorescent cells at the three different time points studied (1, 6, and 12 weeks; P2 cell line: $P = 0.97$; K cell line: $P = 0.3$; F5 cell line: $P = 0.11$; and Y-cell line: $P = 0.07$). We did not observe morphologic differences between transduced cell lines and the nontransduced control at the time points studied (Fig. 2). Patches of cell death began to occur by 12 weeks in some fields of the AAV-GFP–transduced cell lines, corresponding to the areas receiving light stimulation from

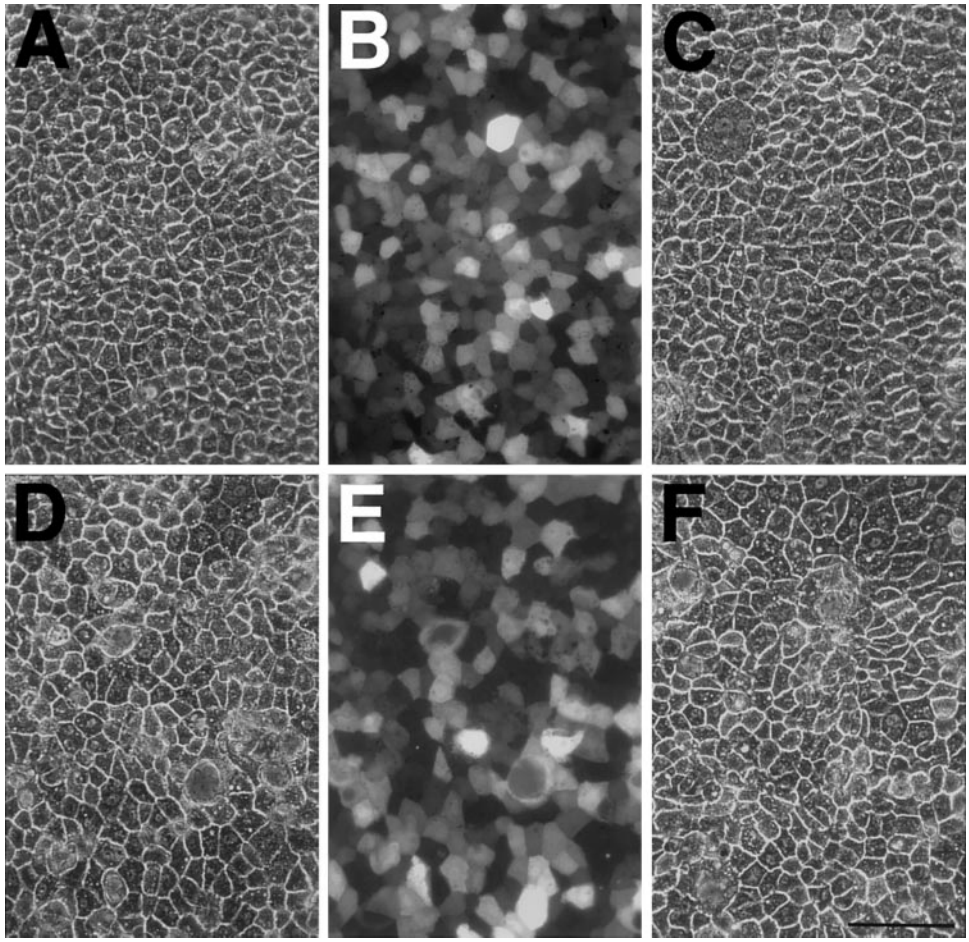

FIGURE 2. AAV-GFP fluorescence in confluent cultured RPE cells. (**A**) Bright-field and (**B**) fluorescence images 1 week after AAV-GFP transduction. (**C**) Bright-field image of control cells at 1 week. (**D**) Bright-field and (**E**) fluorescence images of the same fields as (**A**) and (**B**) 12 weeks after AAV-GFP transduction. (**F**) Bright-field image of control cells (same field as **C**) at 12 weeks. Scale bar, 50 μm.

photography (Fig. 4). Additional areas of cell death occurred in photographed fields of the AAV-GFP–transduced cultures after the termination of the 3-month study.

Four confluent cell lines, which were passaged to assess fluorescence stability with cell division, showed an initial labeling percentage of 83.6%, 81.2%, 71.9%, and 83.4% (mean, 80.05% ± 5.49%). One field of each cell line was counted at the beginning of the study. The expression of GFP decreased after the passage of the cell cultures (Figs. 5, 6). The percentage of fluorescence after a 1:2 splitting of the original cultures was 20.36% ± 2.19% (n = 2) at 4 weeks. After a 1:3 splitting of the original cultures, the percentage of fluorescence was 13.44% ± 1.52% (n = 2) at 4 weeks. Because the cells were still undergoing cell division, they appeared larger and not quite as compact as the cells before passage. Once these split cultures became confluent, the percentage of small, compact fluorescent cells remained stable for up to 11 weeks.

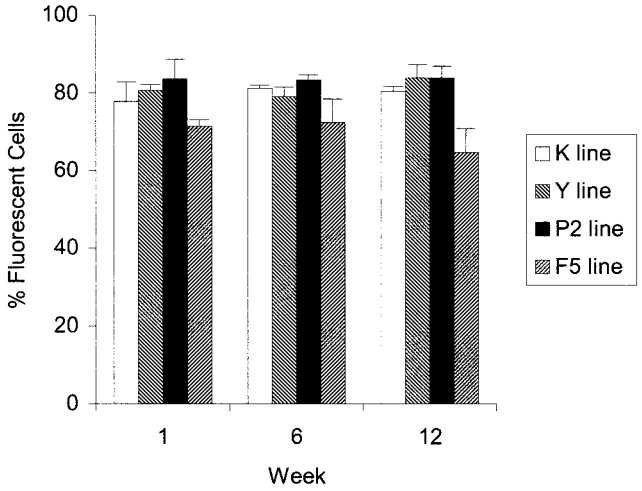

FIGURE 3. Percentage of fluorescent AAV-GFP–transduced RPE cells. Confluent cultures of four RPE cell lines were transduced with approximately 10^{10} AAV-GFP particles/mL. The percentage of fluorescent cells within each cell line was not statistically significantly different between time points.

Based on the results of these studies, additional studies were performed to determine whether the fluorescence is more stable after transduction of nonconfluent cultures. Confluent feline RPE cultures (passage 1) from two cell lines were trypsinized and passaged at a 1:6 dilution. Four fields (0.14 mm^2 each) were photographed before transduction and post-transduction days 1 and 22. The four fields were of initial densities ranging from 10 to 112 cells/field in control areas and 21 to 185 cells/field in dishes to be transduced. At day 1, control cell density ranged from 89 to 281 cells/field, and transduced cell density ranged from 25 to 250 cells/field. At 22 days after transduction, control cell density ranged from 249 to 394 cells/field, whereas transduced cell density decreased to 8 to 90 cells/field. At day 1, 59% of the RPE were fluorescent in one dish, and 72% were fluorescent in the other dish. At day 22, 89% of the RPE cells were fluorescent in one dish, and 55% were fluorescent in the other dish.

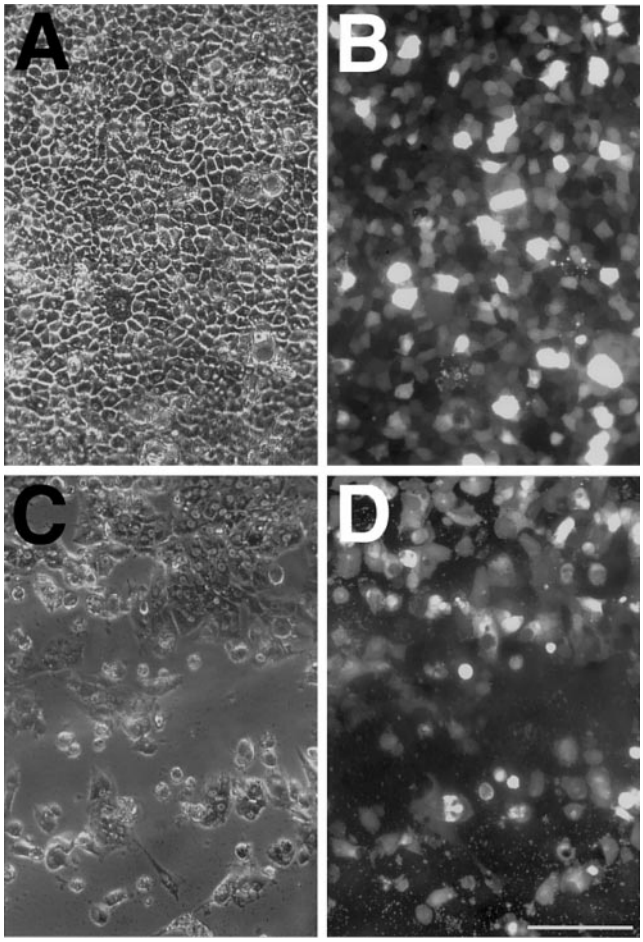

FIGURE 4. Photoxicity of AAV-GFP–transduced RPE cells. (**A**) Bright-field and (**B**) fluorescence images 3 weeks after AAV-GFP transduction. (**C**) Bright-field and (**D**) fluorescence images 12 weeks after AAV-GFP transduction (same fields as **A** and **B**). Areas of cell death were observed in the photographed field at week 12. Scale bar, 80 μm

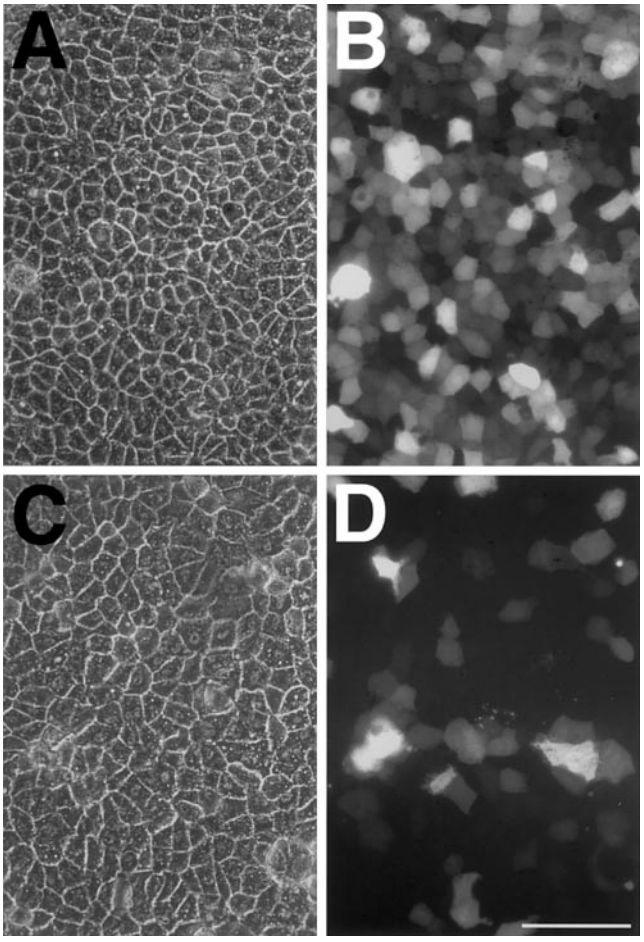

FIGURE 5. AAV-GFP fluorescence of feline RPE cells after passage (1:2 split). (**A**) Bright-field and (**B**) AAV-GFP fluorescence images of transduced cells just before passage. (**C**) Bright-field and (**D**) AAV-GFP fluorescence images 4 weeks after passage of transduced culture shown in (**A**) and (**B**). Scale bar, 50 μm.

Histology and Immunocytochemistry following In Vivo AAV-GFP Transduction

Fundus photographs with fluorescein filters showed intense green fluorescence throughout the retinal bleb in one eye (Figs. 7A, 7B), whereas only punctate or no green fluorescence was present in the two other eyes (Figs. 7C, 7D) at 5 weeks after subretinal injection.

In all eyes with intense or punctate green fluorescence, anti-GFP antibody labeled the cytoplasm of most, if not all, RPE cells in the area of the localized retinal detachment (Fig. 8). In the eye with no green fluorescence, anti-GFP labeled approximately 80% of the RPE. The RPE cells varied in their fluorescence intensity. Some photoreceptors were also labeled in the inner and outer segments and cytoplasm. Very little, if any, labeling was observed in the inner nuclear layer (INL) and ganglion cell layer. The retina in the area of the detachment appeared well preserved, with irregular photoreceptor outer segments in both eyes and some displaced INL nuclei in one of three eyes (Fig. 8A).

Culture of Harvested In Vivo AAV-GFP–Transduced Cells

Cells transduced with AAV-GFP in vivo, harvested, and then cultured showed decreased GFP fluorescence with cell division (Fig. 9).

Transplantation of AAV-GFP–Transduced RPE

In the area of localized retinal detachment and RPE transplantation, fundus photographs showed a large pigmented patch in

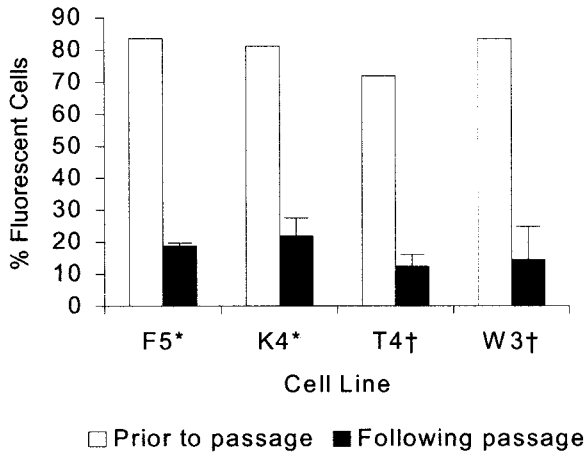

FIGURE 6. Percentage of fluorescent AAV-GFP–transduced cells after passage. Cells were passaged 1:2* or 1:3† and analyzed after 4 weeks in culture.

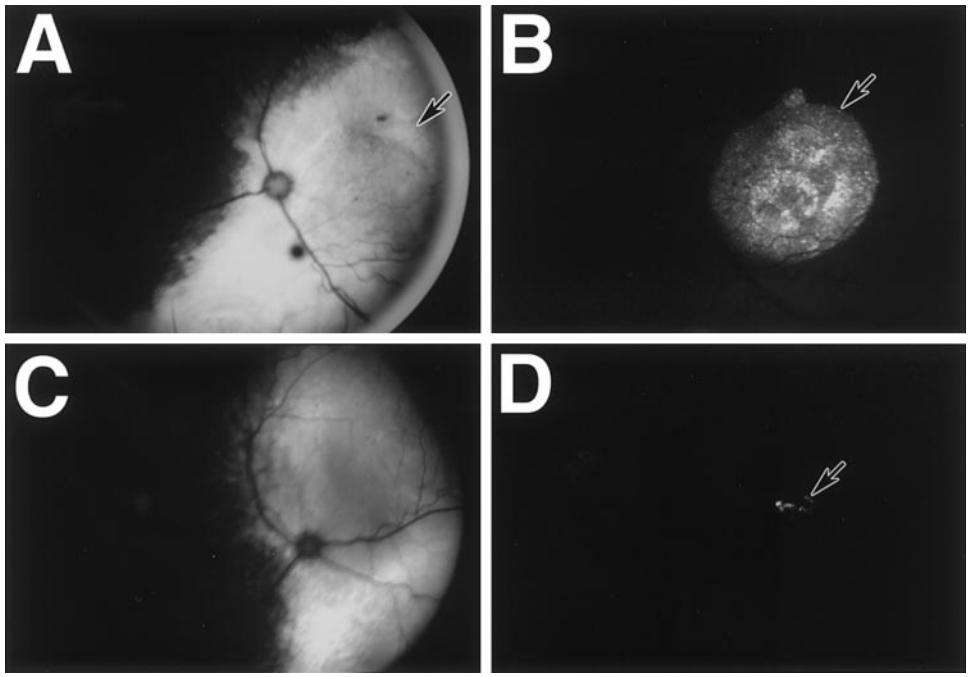

FIGURE 7. Fundus photographs of two eyes 5 weeks after subretinal AAV-GFP transduction. (**A**, **B**) and (**C**, **D**) were from the same eye. (**B**) and (**D**) were viewed with fluorescein filters. In both eyes, AAV-GFP was injected under the superior nasal retina (**A**, *arrow*). (**B**, **D**, *arrows*) GFP green fluorescence was intense in one eye (**B**), whereas only punctate fluorescence was present in the other eye (**D**).

the 3-day survival transplant whereas only a small, pigmented patch was present in the 7-day survival transplant. In vivo examination with fluorescein filters showed no green fluorescence in either transplant area.

Histologically, in the 3-day survival transplant, large pigmented RPE multilayers were seen in the subretinal space. GFP antibody labeled some but not all cells in the RPE multilayer. In the 7-day survival transplant, a patch of pigmented multilayered RPE was present (Fig. 10). Anti-GFP labeled most of the pigmented RPE cells within the patch with variable intensity. RPE monolayers at the bleb edge and some cells attached to Bruch's membrane within the pigmented RPE multilayers were not labeled (Fig. 10). Light labeling was also observed focally in

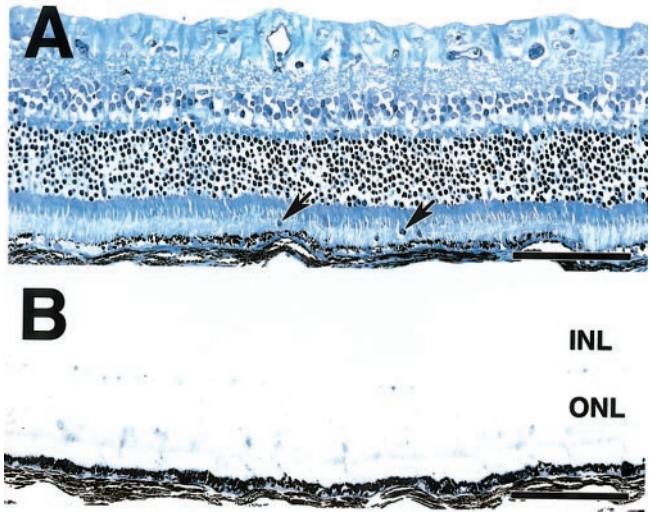

FIGURE 8. (**A**) Toluidine blue staining of the same eye as shown in Figure 7**C** and 7**D**. Retina over the injection appeared well preserved with few displaced ONL nuclei (*arrows*). (**B**) GFP labeling in a consecutive slide of the same eye. RPE cells were labeled in the cytoplasm with variation of labeling intensity. Some photoreceptors were also labeled in the inner and outer segments and cytoplasm. Very little, if any, labeling was observed in the INL. Scale bars, 100 μm.

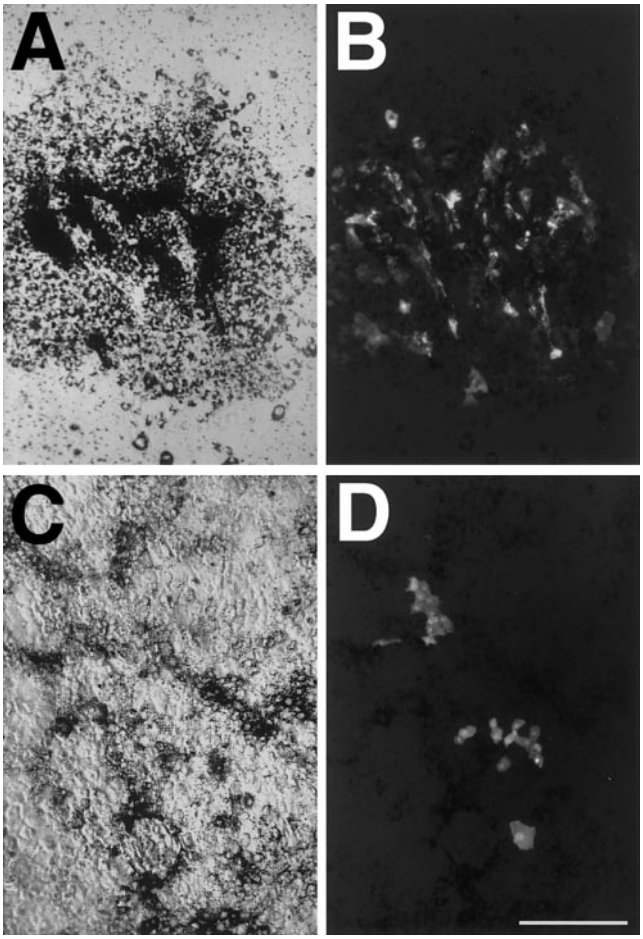

FIGURE 9. RPE cells isolated 5 weeks after a subretinal injection of AAV-GFP. (**A**) Bright-field and (**B**) fluorescence images 4 days after isolation. (**C**) Bright-field and (**D**) fluorescence images 4 months after isolation. Scale bar, 160 μm.

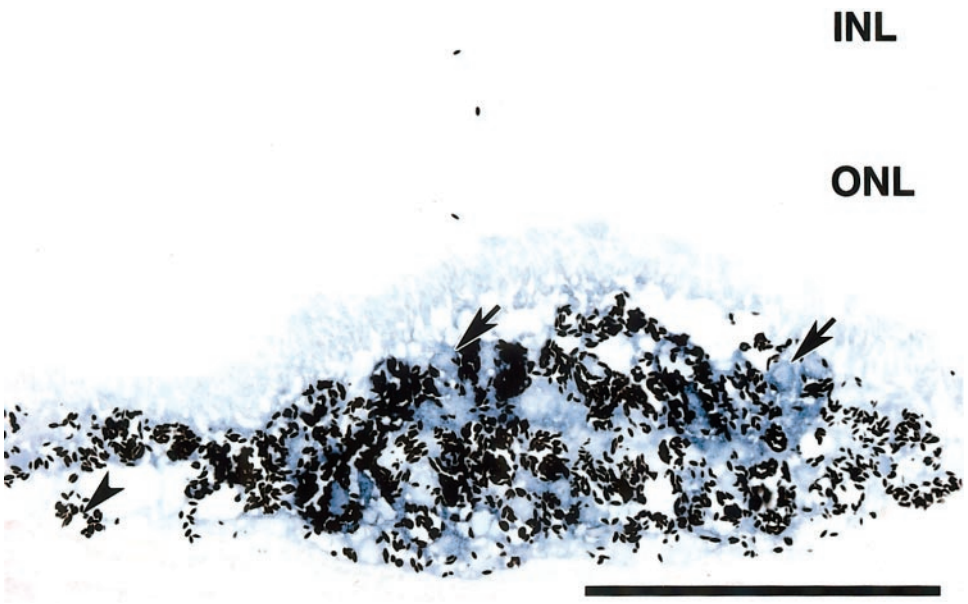

FIGURE 10. AAV-GFP–tranduced RPE transplant treated with anti-GFP antibody 7 days after transplantation. GFP labeling was present in the cytoplasm of some cells (*arrows*) in the pigmented RPE multilayer. Labeling intensity varied in different cells. A GFP-negative cell (*arrowhead*) was seen on Bruch's membrane. Scale bar, 100 μm.

photoreceptor inner and outer segments over areas of high density RPE in both transplants. In both eyes, retina over transplants was fairly well preserved except that in one area over the large RPE multilayer present in the 3-day survival transplant, disorganized outer nuclear layer nuclei were seen. No inflammatory cells were observed at either time point.

Detection of AAV by PCR

AAV was detected in the cytoplasmic and nuclear fractions of both confluent (Fig. 11, lanes 7 and 8) and nonconfluent (Fig. 11, lanes 1, 2, 4, and 5) transduced cells as judged by the presence of the 135-bp band in the PCR products. Control RPE cultures were negative for presence of AAV (Fig. 11, lanes 3, 6, and 9).

DISCUSSION

Previous studies have shown that recombinant AAV effectively and stably transduces RPE in vivo.[48,51,53,55] These in vivo studies showed that RPE are highly permissive to AAV-mediated gene transfer, and reporter gene expression (e.g., *LacZ* or *GFP*) in transduced RPE can be detected for up to 1 year after subretinal injection.[52] In a study by Grant et al.,[53] reporter gene expression of RPE cells in some areas approached 100%. In the present study, we showed that transduced RPE can be harvested after in vivo transduction with AAV-GFP, and these cells can be identified after transplantation (Fig. 10). In addition, our studies show that monitoring GFP expression in vivo using GFP fluorescence can underestimate the number of cells transduced. In an animal in which only punctate labeling was observed with in vivo fluorescence imaging, we observed a high percentage of cells (close to 100%) showing positive anti-GFP labeling histologically (compare Figs. 7D, 8B). Thus, immunohistochemical detection of GFP is a more sensitive method to identify labeled transplanted cells.

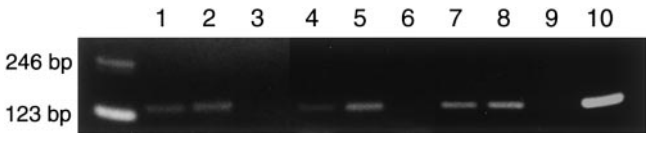

FIGURE 11. PCR products from the nuclear and cytoplasmic fractions of confluent and nonconfluent AAV-GFP–transduced retinal pigment epithelial cells. *Lanes 1* and *2*: nonconfluent transduced cells 6 days after inoculation; *lanes 4 and 5*: nonconfluent transduced cells, 15 weeks after inoculation; *lanes 7* and *8*: confluent transduced cells 15 weeks after inoculation; *lanes 3*, *6* and *9*: nonAAV–transduced control cultures; *lane 10*: AAV virus. *Lanes 1*, *4*, and *7*: nuclear fraction; *lanes 2*, *5* and *8*: cytoplasmic fraction. *Far left lane*: marker.

In preliminary in vivo studies monitoring GFP fluorescence after subretinal AAV-GFP injection, we observed decreased GFP fluorescence after repetitive (weekly) fundus photographs and fluorescence examination. The animals showed a marked decline in fluorescence after peak expression at 5 weeks. These results and results of long-term cell culture studies showing loss of cells at 3 months after repeated photography (Fig. 4) lead us to conclude that one should refrain from exposing AAV-GFP–transfected RPE to repeated light exposure. Light-induced cell loss may result from absorbance of light by the RPE. In a study reported by Bennett et al.,[54] monkeys receiving AAV-GFP subretinal injections and photographed weekly showed areas of RPE atrophy at 6 months after injection. In addition, at the termination of these long-term studies, only occasional RPE cells were labeled. Reducing light exposure by using an SLO may not be enough to prevent cell loss. Lai et al.[46] followed GFP-labeled RPE transplants weekly with SLO examination. Their studies showed disappearance of transplant fluorescence starting at 2 weeks. As suggested in studies by Lai et al.,[46,47] the loss of fluorescence may be due to rejection of the cells, but based on our findings, some of the loss of fluorescent cells may be due to light toxicity. Because our nontransduced cultures, which were photographed at the same time intervals as the AAV-GFP–transduced cells, did not show cell death, we conclude that light stimulation per se is not causing this phenomenon. It is possible that AAV transduction alone confers light sensitivity, but we suspect that the GFP label mediates this effect.

In vitro transduction studies of confluent cat RPE showed that by monitoring reporter gene expression, one can detect labeling of approximately 80% of RPE cells for up to 3 months. In addition, in vivo transduction by subretinal injection can lead to effective labeling of RPE. However, this expression appears to be stable only if the cells are not proliferating. This result indicates either inactivation of the promoter or absence of integration into the host genome.[59] Because our PCR studies showed viral presence in the cytoplasm (Fig. 11), nonintegra-

tion appears to be a likely explanation for the decrease in detectable label after cell division. In contrast to our findings, Lai et al.[59,60] added AAV-GFP to an 80% confluent culture of an established human RPE cell line (407A), and, after selection of neomycin resistant/GFP clones, the cells showed stability of the label after more than 24 passages. These cells are near triploid[61] but retain many in vivo RPE characteristics. In a further study, using three different human RPE cell lines, Lai et al.[59] showed transduction efficiencies up to approximately 80% and demonstrated stable, random viral integration into the host chromosome. They hypothesized that perhaps the stability of their transductions is due to integration of the viral genome into the host in proliferating cells as well as the fact that they used selective pressure (neomycin resistance) to select clones expressing the viral genome. Our studies with transduction of nonconfluent cells suggests viral integration into the host genome occurs because we observe fluorescence in cells after initial cell division at posttransduction day 1. However, we noted a marked decrease in cell number 22 days after AAV-GFP transduction. It may be that AAV-GFP transduction alters the ability of the cells to proliferate. Also, the decrease in cell number may indicate that some of the cells die after incorporation of AAV-GFP. Cells exposed at low levels of confluence never became confluent, although many of the remaining cells were brightly fluorescent.

The observation that some photoreceptors became GFP-positive in the outer and inner segments in both 3- and 7-day survival transplants (Fig. 10) indicates that GFP can be transferred from transfected to nontransfected cells. The observation that label transfer was present in the photoreceptors focally and only above areas of high RPE density (Fig. 10) indicates that the transfer of label is not due to simple diffusion from the transplanted cells. Rather, the label transfer may be due to local areas of cell disruption within the transplant. Based on the studies by Wang et al.,[24] the percentage of TUNEL-positive transplanted feline RPE cells at days 1, 3, and 7 after transplantation is less than 1%, and the percentage of darkly staining (possibly necrotic) nuclei is 2.89% to 6.25%. Because these cells were found within the areas of RPE multilayers, they could contribute, through cell death and liberation of AAV-GFP, to the focal labeling of photoreceptor inner and outer segments in the present study.

In conclusion, AAV-GFP can be an effective marker for transplanted RPE cells with some limitations. Transduction of confluent cultures of RPE cells may not be stable and can result in loss of label with subsequent cell division. The loss of GFP fluorescence will result in an underestimation of the number of transplanted cells. However, based on the studies by Wang et al.,[24] the number of proliferating transplanted cells ($<10\%$) may be minor compared with the number of cells transplanted. Histologic tissue analysis may permit more sensitive detection of label in this setting. Ideally, transduction of proliferating cultures rather than confluent cultures may be better suited for transplantation studies due to stable viral genome integration into the host.[59] However, transduction of nonconfluent RPE cells may also present a limitation, in that integration into the host DNA may interfere with cell survival and/or further cell division. In vivo transduction of RPE cells through subretinal injection of AAV-GFP is very effective and can allow GFP fluorescence or immunohistochemical identification of transplanted, uncultured RPE cells. However, in vivo monitoring of GFP fluorescence should be performed with caution to limit light toxicity to the RPE cells.

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