Transduction of Photoreceptors With Equine Infectious Anemia Virus Lentiviral Vectors: Safety and Biodistribution of StarGen for Stargardt Disease

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Submitted: February 14, 2013
Accepted: April 18, 2013

Citation: Binley K, Widdowson P, Loader J, et al. Transduction of photoreceptors with equine infectious anemia virus lentiviral vectors: safety and biodistribution of StarGen for Stargardt disease. *Invest Ophthalmol Vis Sci*. 2013;54:4061–4071. DOI:10.1167/iovs.13-11871

Purpose. StarGen is an equine infectious anemia virus (EIAV)-based lentiviral vector that expresses the photoreceptor-specific adenosine triphosphate (ATP)-binding cassette transporter (ABCA4) protein that is mutated in Stargardt disease (STGD1), a juvenile macular dystrophy. EIAV vectors are able to efficiently transduce rod and cone photoreceptors in addition to retinal pigment epithelium in the adult macaque and rabbit retina following subretinal delivery. The safety and biodistribution of StarGen following subretinal delivery in macaques and rabbits was assessed.

Methods. Regular ophthalmic examinations, IOP measurements, ERG responses, and histopathology were carried out in both species to compare control and vector-treated eyes. Tissue and fluid samples were obtained to evaluate the persistence, biodistribution, and shedding of the vector following subretinal delivery.

Results. Ophthalmic examinations revealed a slightly higher level of inflammation in StarGen compared with control treated eyes in both species. However, inflammation was transient and no overt toxicity was observed in StarGen treated eyes and there were no abnormal clinical findings. There was no StarGen-associated rise in IOP or abnormal ERG response in either rabbits or macaques. Histopathologic examination of the eyes did not reveal any detrimental changes resulting from subretinal administration of StarGen. Although antibodies to StarGen vector components were detected in rabbit but not macaque serum, this immunologic response did not result in any long-term toxicity. Biodistribution analysis demonstrated that the StarGen vector was restricted to the ocular compartment.

Conclusions. In summary, these studies demonstrate StarGen to be well tolerated and localized following subretinal administration.

Keywords: lentivirus, gene therapy, photoreceptors

Stargardt disease (STGD1) is the most common form of inherited juvenile macular degeneration and is usually diagnosed within the first 2 decades of life. It leads to progressive irreversible loss of central vision and delayed dark adaptation and is inherited as an autosomal recessive trait.

The gene for STGD1, *ABCA4*, encodes for a member of the ABC transporter family.[1] Hundreds of mutations in *ABCA4* are known to cause impaired vision and blindness in STGD1 and in several other visual disorders including cone–rod dystrophy and autosomal recessive retinitis pigmentosa.[2,3] Individuals heterozygous for *ABCA4* mutations may also have a higher risk of developing age-related macular degeneration.[4]

The large *ABCA4* gene contains 50 exons, encodes for a ~250 kDa single-chain glycoprotein, and is susceptible to frequent mutations.[5] Expression of the ABCA4 protein is restricted to retinal rod and cone photoreceptors, where it is localized to the rim and incisures of photoreceptor (PR) outer segment disks, present at a molar ratio of approximately 1:120 with rhodopsin.[6,7] ABCA4 uses the energy of ATP hydrolysis to move a diverse set of substrates across the photoreceptor outer segment disk membrane.[8,9] The natural substrate of ABCA4 is believed to be retinylidene-phosphatidylethanolamine (N-retinylidene-PE), a precursor of potentially toxic bis-retinoid compounds, the best known of which is N-retinylidene-N-

retinylethanolamine (A2E). In advanced STGD1, the buildup of A2E lipofuscin causes atrophy of the RPE cells followed by degeneration of PRs especially in the region of the macula, which eventually results in loss of vision.[6]

There are currently no approved therapies for Stargardt disease. However, as the pathophysiology of the disease has been attributed to mutations within the *ABCA4* gene, an appropriate route to preventing or halting disease progression is through gene therapy.

Using gene therapy to introduce the normal *ABCA4* gene to PR cells in STGD1 patients would result in sustained levels of the active protein at the site of disease. Because of the chronic nature of this disease and the large size of the *ABCA4* cDNA (6.8 kb), lentiviral vectors that can accommodate larger inserts (8–9 kb) and deliver long-term gene expression are well suited to treat STGD1.

StarGen is a nonreplicating, nonhuman recombinant lentiviral vector based on EIAV. The wild-type EIAV virus causes a self-limiting anemia in horses and is nonpathogenic to humans.[10] StarGen is delivered subretinally to introduce the normal human *ABCA4* gene to cells of the retina including PR as well as RPE cells, thereby restoring normal cellular function and attenuating vision loss associated with STGD1. We have previously shown proof-of-concept for this approach, whereby an EIAV vector-expressing human *ABCA4* corrects the disease phenotype in an *ABCA4*$^{-/-}$ knockout mouse model of Stargardt disease.[11]

Here we show that EIAV-CMV-GFP reporter vector is able to transduce both rod and cone PRs in addition to the RPE with high efficiency in the adult macaque following subretinal administration. The toxicity, biodistribution and shedding characteristics of StarGen were examined over 6 months following a single subretinal injection in rabbits and macaques and the data show that the vector is safe, well-tolerated and localized in the site of administration.

Materials and Methods

Animals

All the studies involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The reporter gene expression study followed a single subretinal injection of the EIAV-CMV-GFP vector in one male and one female Rhesus macaque (4–5 years old). Each animal received a subretinal dose of 100-μL EIAV-CMV-GFP vector (3.4×10^7 transducing units per eye [TU/eye]) in the left eye, whilst the right eye was treated with 100-μL control formulation buffer.

The safety and biodistribution studies were performed on adult male and female (2–3 years old) Rhesus macaques (*Macaca mulatta*) and adult male and female (4.5–5.5 months old) Dutch-belted rabbits. All animal studies were in accordance with the US Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations. In both species, the right eye was treated either with StarGen at a dose of 1.4×10^6 TU/eye in the rabbit and 4.7×10^5 TU/eye in the macaque or control buffer in a total volume of 100 μL, whilst the left eye remained untreated (Table).

For the rabbit study, groups of equal numbers of male and female rabbits (76 total) were euthanized at five interim time points on days 3, 8, 29, 92, and 183 to collect tissues, and gross pathology and histologic examination, together with urine and blood chemistry analysis, of a wide variety of tissues was performed to determine signs of toxicity.

For the macaque study, equal numbers of male and female macaques (24 in total) were euthanized after either 3 months or 6 months, and a full macro- and microscopic examination was performed on a wide variety of tissues. A full clinical chemistry and urine analysis was also performed on samples obtained at the 3- and 6-month time points.

The assessment of toxicity against StarGen in both rabbits and macaques was based on mortality, clinical signs, body weight, and qualitative food consumption. In addition, regular in-life assessments of StarGen toleration were performed by slit-lamp biomicroscopy, indirect ophthalmoscopy, recording of IOP by Tono-Pen Vet applanation tonometry together with ERG and fundus photography by a masked American College of Veterinary Ophthalmologists (ACVO) board-certified veterinary ophthalmologist. Anterior chamber and vitreous cell scores and aqueous flare were assigned by using an estimate of cells/field of the focused slit-lamp beam and determining an inflammation score as follows: 0, no inflammation (no cells); +1, very slight (5–25 cells); +2, slight (25–50 cells); +3, moderate (50–100 cells); and +4, severe (>100 cells).

Biodistribution Evaluation

StarGen biodistribution and shedding was assessed in tear swabs, saliva, plasma, white buffy coat, and vitreous fluid and in multiple tissue samples collected for qPCR analyses in rabbits and macaques as previously described.[27]

In both rabbits and macaques, blood (buffy coat and plasma) samples and tear and saliva swabs were collected and analyzed by qPCR pretreatment and on days 3, 5, 8, 15, 29, and 92 of the dosing phase.

Vector Production by Transient Transfection

The EIAV lentiviral vector system used to produce the StarGen and the EIAV-CMV-GFP vector consists of a transient transfection of HEK293T cells, with three plasmids comprising the vector genome coding for ABCA4 or GFP, the codon optimized *gag-pol,* and the VSV-G envelope using Lipofectamine 2000 CD (Life Technologies Ltd., Paisley, UK) according to the manufacturer's instructions (Fig. 1). For the EIAV-CMV-GFP vector cell culture, supernatants were harvested and vector was concentrated 2000-fold by centrifugation (6000*g* at 4°C for 18 hours, followed 50,000*g* at 4°C for 90 minutes).

The StarGen vector used for the GLP toxicology studies was produced in a manner analogous to the Good Manufacturing Practice (GMP) grade clinical vector in which the virus-containing supernatant was harvested and the virus purified and concentrated as previously described.[27] The StarGen batch used in these studies was labeled IH44, and the titer of 1.1×10^7 TU/mL was determined as previously described.[27]

Additional safety and purity specific assessments were carried out on StarGen IH44 as previously described to ensure the vector was suitable for use in the GLP toxicology studies.[27]

Subretinal Injection

In the reporter gene expression study in the Rhesus macaque, subretinal injections of the EIAV-CMV-GFP vector or control buffer (100 μL/eye) were performed using the RetinaJect apparatus (SurModics, Inc., Eden Prairie, MN) as previously described.[27] The macula was the target site for the subretinal injections. Both eyes from each animal were harvested on day 15 for histologic analysis of GFP reporter gene expression.

For the GLP studies in rabbits and macaques, the dosing apparatus consisted of a 41-gauge (G) needle connected to a micro-extension tube and a 1-mL Luer lock syringe. The apparatus was prefilled with PBS, and a small air bubble was

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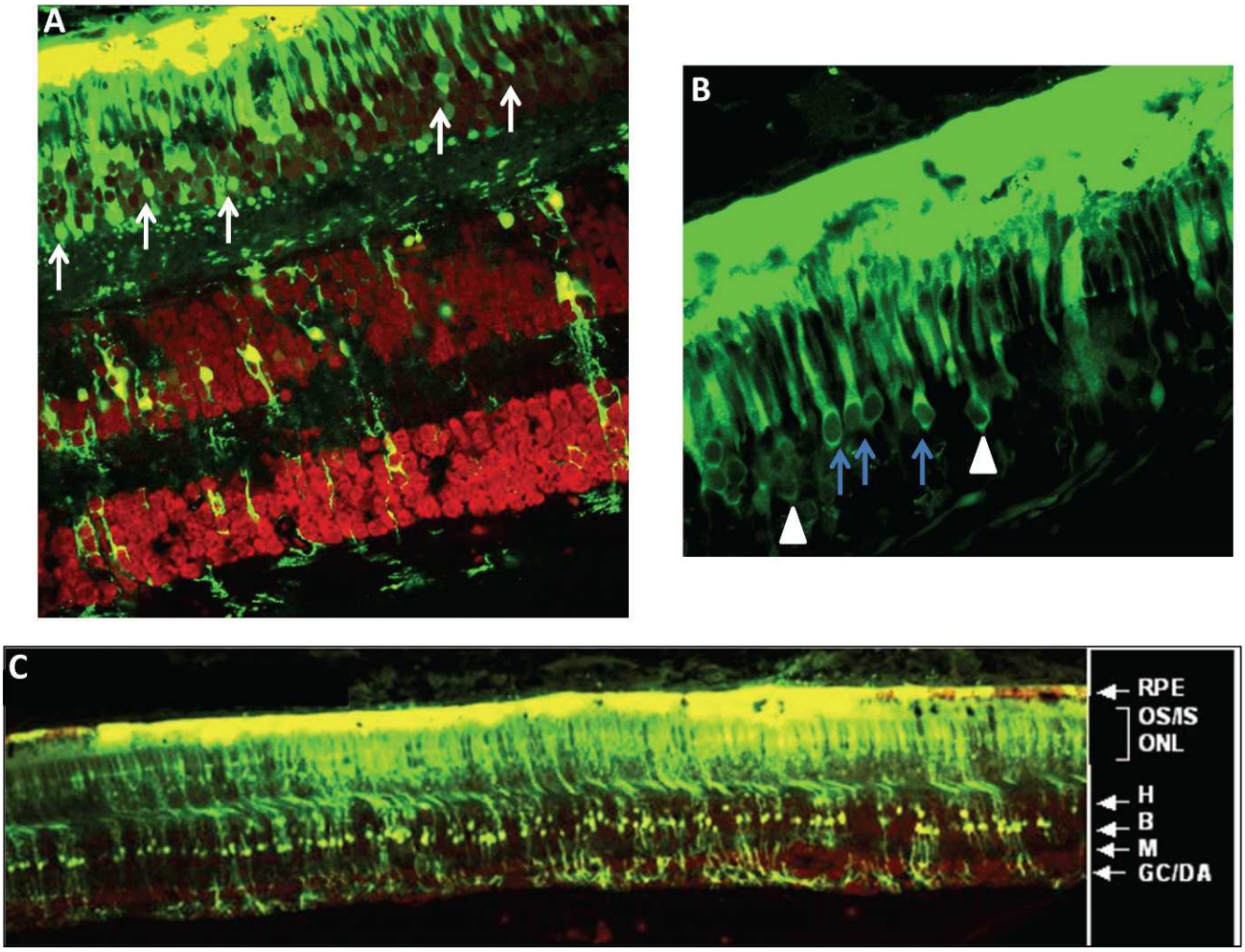

FIGURE 1. EIAV-CMV-GFP transduction of Rhesus macaque retina. At 14 days postsubretinal administration of the EIAV-CMV-GFP vector, GFP expression was visible in the retina in the region of the subretinal bleb. (**A**) The majority of the GFP expression was present in the RPE cells and the PR cells; *white arrows* denote GFP-positive PR cell bodies. (**B**) GFP-positive PRs included both the rods (*arrow head*) and cones (*arrow*). Transduction of other cells in the inner retina could also be observed, including horizontal cells (H), bipolar cells (B), amacrine cells (A), Mueller cells (M), and ganglion cells (GC). OS/IS, outer segment/inner segment; ONL, outer nuclear layer.

created in the needle hub prior to filling with 100-µL test or control article to minimize loss of vector in the dead volume of the apparatus. A light pipe was inserted transconjunctivally near the limbus using a 25-G cannulation system. For the subretinal injection, the conjunctiva was opened and an insertion site opened using a V-lance to allow insertion of the wider barrel. Following injection, the 25-G port was removed, and the opening for the dosing needle sutured. In both species, the dosing region was inferolateral or inferomedial to the optic nerve.

Electroretinography

Electroretinogram recordings were performed pretreatment and in week 13 (day 87) and week 26 (day 174) of the study in both rabbits and macaques. Animals were dark adapted for at least 30 minutes prior to ERG recording following sedation. A mydriatic agent and 0.5% proparacaine were applied prior to the test. Eyelids were retracted using a lid speculum, and a contact lens electrode was placed on the surface of each eye. A needle electrode was placed cutaneously under each eye and on the head, posterior to the brow. Carboxymethylcellulose (1%) drops were applied to the interior surface of the contact lens electrodes prior to placing them on the eyes. Both eyes were evaluated concurrently using a Ganzfeld dome. Each ERG occasion consisted of the following series of scotopic single flash stimuli: –30 dB single flash, average of 5 single flashes, 10 seconds between flashes; –10 dB single flash, average of 5 single flashes, 15 seconds between flashes; and –0 dB, average of 2 single flashes, 120 seconds between flashes. Next, the animals were adapted to background light at ~25 to 30 cd/m^2 for a period of 5 minutes, followed by an average of 20 sweeps of photopic white flicker at 1 Hz, then 20 sweeps of photopic flicker at 29 Hz.

Ocular Histologic Endpoints

At the end of the macaque gene-transfer study, the eye globes were removed and punctured carefully three times using an 18-G needle to ensure good penetration of the chilled 4%-paraformaldehyde fixative (4°C, 18 hours). Retinal sections were obtained over the region of the subretinal bleb, and GFP expression in the retina was observed via fluorescence and confocal microscopy.

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Table. Study Design for 6-Month Toxicology Studies in Dutch-Belted Rabbits and Rhesus Macaques

Group	Dose Level TU/Eye, Right	Article	Number of Animals/Group, M/F	Euthanasia Time Points* D3 M/F	D8 M/F	D29 M/F	D92 M/F	D183 M/F
Dutch-belted rabbits								
1	—	Formulation buffer	19/19	3/3	3/3	3/3	7/7	3/3
2	1.4×10^6	StarGen vector	19/19	3/3	3/3	3/3	7/7	3/3
Rhesus macaques								
1	—	Formulation buffer	6/6				3/3	3/3
2	4.7×10^5	StarGen vector	6/6				3/3	3/3

M/F, male/female.
* Days 3, 8, 29, 92, and 183.

On days 92 and 183 of the GLP study, eye tissues were fixed and preserved in neutral-buffered 10% formalin. The retina/choroid tissue was paraffin-wax embedded and cross-sectioned in the region of the subretinal bleb and stained with hematoxylin and eosin. At least five sections were evaluated as follows: two sections in the area of the bleb, at least one section through the optic nerve, and at least one section on either side of the optic nerve outside of the bleb region.

Immunogenicity Assay

Blood was collected from all rabbits and macaques at baseline and on days 8, 15, 29, 92, and 183 for the detection of potential immune responses against StarGen. Antibody responses against StarGen, including the envelope protein (VSV-G) or the transgene product ABCA4 and other EIAV-vector components, were initially assessed by semiquantitative ELISA in the rabbit and by Western blotting in the rhesus macaque serum samples.

Rabbit anti-VSV-G peptide antibody was used to construct an ELISA standard curve (Sigma, St. Louis, MO). For Western blotting, cell lysates prepared from the negative and the StarGen-positive control cell pellets were separated by SDS-PAGE on 12% Tris-Glycine gels, blotted onto polyvinylidene fluoride (PVDF) and incubated with either the Rhesus macaque or rabbit test or control serum samples. After washing, the membrane was incubated with an appropriate horseradish peroxidase (HRP) conjugated secondary antibody.

Vector PCR

Real-time PCR analysis of biological samples from the GLP studies was used to measure vector persistence, biodistribution, and shedding. DNA was isolated from buffy-coat samples as previously described.[27] The LLOQ was defined in each assay using the standards.

RNA was isolated from various fluid samples (including left and right eye vitreous fluid, plasma, urine, swabs of saliva, and left and right eye tears) as previously described.[27] Average copy numbers per cell were estimated by dividing the mean number of vector sequences detected by the predicted number of cells in 1-μg genomic DNA (1.47×10^5 cells).

Results

EIAV-CMV-GFP–Mediated Gene Expression in Macaque Photoreceptors

Following subretinal delivery of the EIAV-CMV-GFP vector in the Rhesus macaque eye, green fluorescent protein (GFP) expression was limited to the region of the subretinal bleb, with expression extending across all retinal layers including the inner nuclear layer and ganglion cell layer (Fig. 1A). Toward the edge of the subretinal bleb, the GFP expression gradually became limited to the outer segments of the photoreceptor cells and the RPE cell layer. Based on cell morphology, both rod and cone cells expressed the GFP reporter gene (Fig. 1B). Although the majority of GFP expression was present in the RPE and PR cells; in sections taken toward the center of the bleb, there was transduction in the deeper regions of the retina (Fig. 1C), including horizontal cells, bipolar cells, amacrine cells, Mueller cells, and ganglion cells.

Six-Month Toxicology, Biodistribution, Shedding, and Immunogenicity Studies

The rabbit and macaque toxicologic studies were designed to examine ocular toleration to subretinally dosed StarGen vector (Table). The ocular tolerability of the rabbit and the macaque eyes to subretinally dosed StarGen was investigated in several pilot ocular studies, where it was determined that the rabbit eye tolerates a higher dose than the macaque eye (data not shown). Therefore, the dose of StarGen used in the macaque was a third of that used in the rabbit, but both doses were chosen to be close to the maximal tolerated ocular dose for that species. There were no deaths related to StarGen in either species, and there were no significant changes in body or relative organ weights compared with control animals dosed subretinally with the control buffer. Blood chemistry, red and white cell counts, and blood clotting times were unchanged at all sampling time points in all groups. In addition, there were no significant treatment-related microscopic observations in any rabbit or macaque tissues.

Clinical Ophthalmic Observations

Retinal elevation caused by the subretinal bleb formation was assessed in both species at day 3 (Fig. 2A). In the macaque eyes, very slight retinal elevation was observed in 41% of the animals in both groups on day 3, but this resolved by day 15. In the rabbit, retinal elevation was observed at a higher frequency (71%), intensity, and duration in the StarGen-treated group compared with the buffer group (44%; Fig. 2A). Resolution of the elevated retina occurred in most buffer-treated rabbits (87%) by day 8 and in most StarGen-treated rabbits (85%) by day 22. Very slight retinal elevation remained in one StarGen- and two buffer-treated rabbit eyes until the day-85 evaluation but was no longer apparent on day 176.

The IOP measurements in the rabbit and the macaque remained within the normal range for the duration of the study. However, there was a slight transient reduction in the IOP following subretinal delivery of either StarGen or buffer in the rabbit eyes compared with the contralateral uninjected eyes, which was most likely related to the dosing procedure (Fig. 2B). By day 22, the IOP measurements in all the injected and uninjected eyes were comparable. In the macaques, the IOP

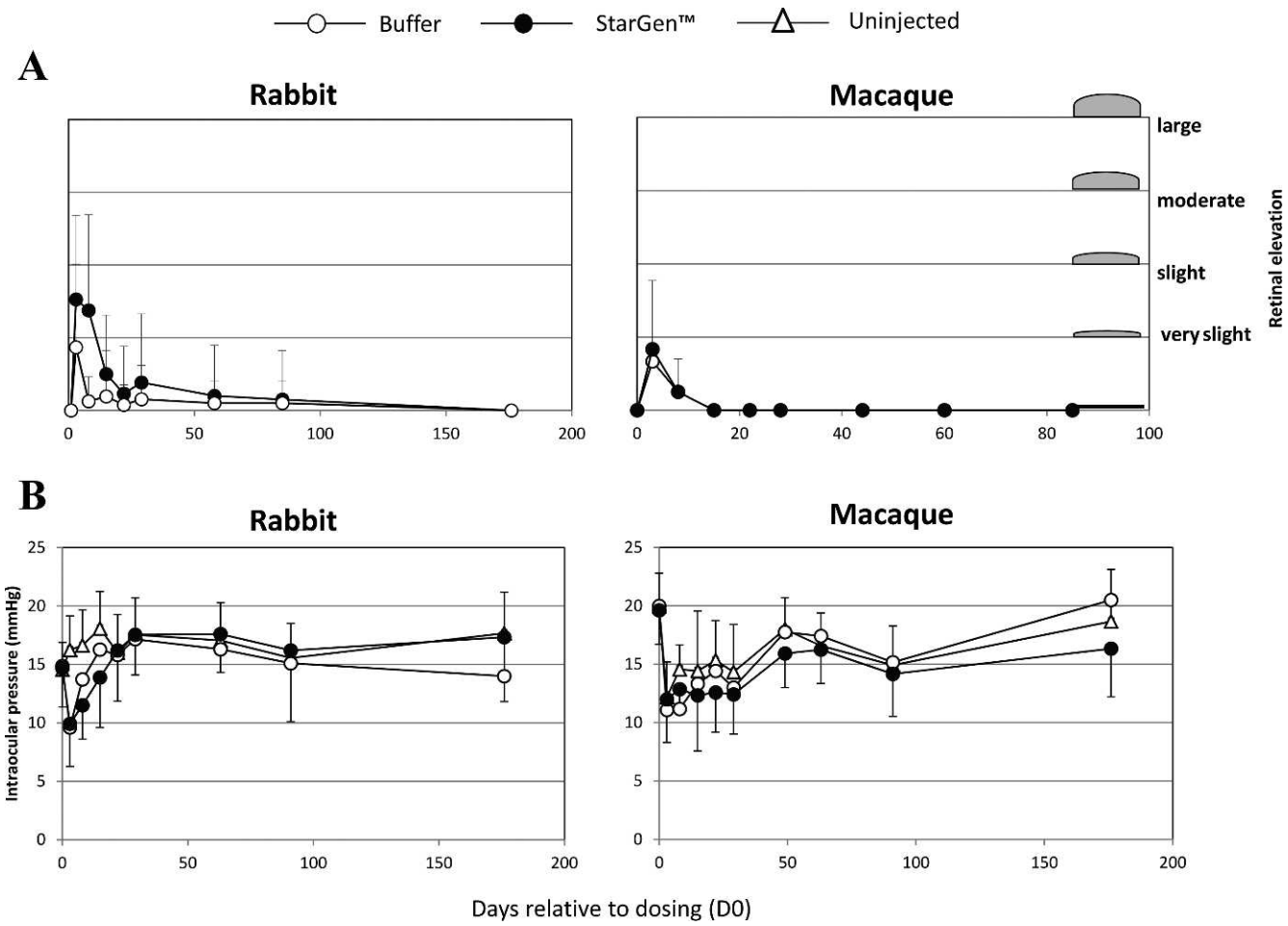

Figure 2. (**A**) Retinal elevation assessment in macaques and rabbits. The extent of the retinal elevation was assessed during the ocular examinations in both rabbits and macaques. The extent of retinal elevation in each eye following subretinal delivery was graded on a scale of very slight to severe. The mean score at each time point in both rabbits and macaques is shown ±SD. (**B**) Intraocular pressure measurements. The mean IOP measurement in rabbit and macaques at each time point for each group is shown ±SD.

measurements remained similar in all the eyes whether treated with the buffer, StarGen, or uninjected for the duration of the study.

ERGs were carried out at baseline and at weeks 13 and 26 prior to necropsy in both eyes of the rabbits and the macaques. In the rabbit, slight changes in the mean amplitude for certain scotopic flash intensities and photopic flickers were observed on days 87 and 174. These changes were related to individual animals and were not considered to be related to the subretinal administration of StarGen since they were observed in treated and untreated eyes of both groups, and there were no associated ophthalmologic or histopathologic findings (data not shown).

Regular ocular examinations revealed a transient ocular inflammation that was slightly more prominent in the StarGen-treated eyes and took longer to resolve compared with the buffer-treated eyes in both rabbits and macaques (Fig. 3). The vitreal cell score was the most prominent ocular inflammation assessment in both species; 16 rabbit and 5 macaque eyes treated with buffer had completely resolved with respect to vitreal cell–like opacities on day 85 compared with 4 eyes and 1 eye, respectively, in the StarGen-treatment group. Two macaque eyes treated with StarGen showed a very slight to moderate (+1 to +3) vitreal cell score and a very slight (+1) aqueous cell score up to day 176. Similarly, two rabbit eyes treated with StarGen showed a slight (+2) to very slight (+1) vitreal cell score for the duration of the study. The vitreal cell score in all the remaining StarGen-treated eyes resolved by the end of the study. The aqueous cell and flare scores in the rabbit eyes treated with either StarGen or buffer were largely unremarkable and resolved by day 15 and day 22, respectively.

Perhaps the most noteworthy finding during the macaque study was the development of transient, white, perivascular retinal opacities in approximately half of the StarGen-treated eyes. These perivascular retinal opacities were not located within the bleb area but rather in the periphery of the retina and appeared as white dots into or against the retina where the vessels were present. They were mostly observed on day 29 and were apparent in one StarGen-treated eye up to day 176. These opacities were entirely absent in the rabbit study. Changes in pigmentation at the level of the retina and choroid were noted in all eyes in the early weeks of the study in both rabbit and macaque eyes following subretinal administration of StarGen or buffer; these became less prominent with time (data not shown). This type of change was expected and occurred secondarily to the physical separation between the neurosensory retina and the RPE at the time of dosing.

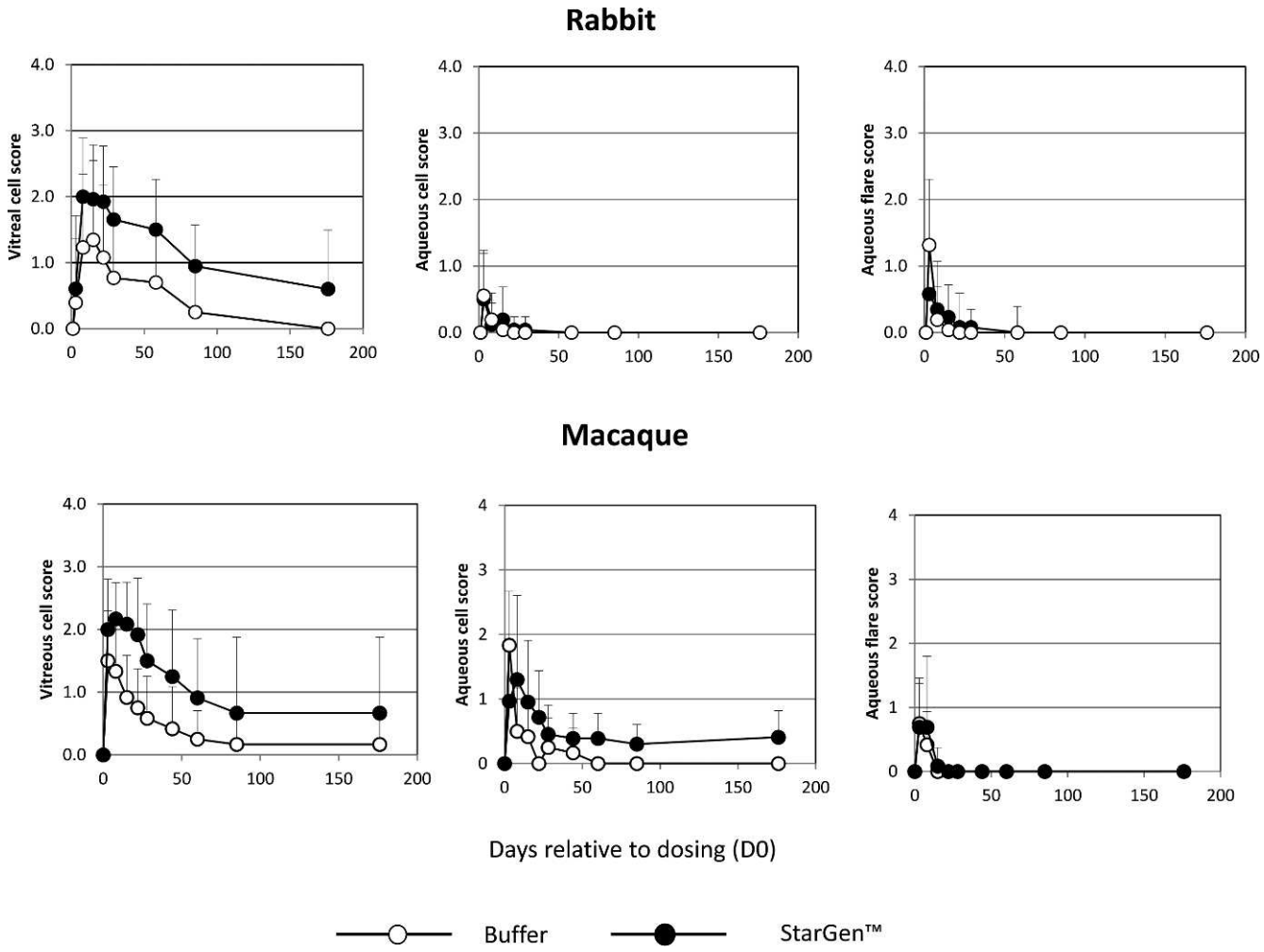

FIGURE 3. In vivo ocular inflammatory scores in macaques and rabbits. Inflammatory changes were assessed by slit-lamp biomicroscopy and indirect ophthalmoscopy. The clinical changes were graded at four levels of severity: +1, very slight; +2, slight; +3, moderate; and +4, severe. The mean score at each time point in both rabbits and macaques is shown ±SD.

Biodistribution, Persistence, and Shedding of StarGen

By measuring the amount of StarGen RNA (StarGen vector particles contain two copies of the genome) or DNA (StarGen integrates into the target cell chromosome), an estimate of vector biodistribution can be made. In the rabbit toxicology study, samples from various fluids, tissues, and buffy coat were analyzed by quantitative PCR (qPCR) at different time points up to day 92 to determine the biodistribution, persistence, and shedding of the StarGen vector following subretinal delivery. The analysis of RNA extracted from plasma samples was used to assess vector particle dissemination and/or persistence of StarGen-derived EIAV ψ sequences. The same quantitative RT-PCR (qRT-PCR) assay was used to assess vector shedding from RNA extracted from swabs of saliva and tears, urine, and StarGen-treated right eye vitreous fluid. DNA isolated from various tissues and buffy-coat samples was used to assess vector biodistribution of StarGen-derived EIAV ψ sequences.

No StarGen vector particles were detected in any rabbit buffy-coat or saliva swabs. Similarly, they were not detected in the majority (4/6 and 5/6) of rabbit-eye tear swabs at day 2 and day 5, respectively, and when detected, the levels were below the lower limit of quantification (<LLOQ) for the assay in all but one animal. All swabs were negative by day 15. StarGen vector particles were detected in a few plasma samples at the early time points at a level that was below the LLOQ. Vector particles were present in the vitreous fluid of the StarGen-treated rabbit eye in all six animals at day 3, but this dropped to one animal by day 29, by which time the level was below the LLOQ.

StarGen vector DNA was primarily detected in the rabbit ocular tissues including the retina/choroid, sclera, and optic nerve tissue samples (Fig. 4), no vector DNA was detected in any of the contralateral uninjected rabbit eyes. However, whilst vector DNA was detected in at least five out of the six retina/choroid and scleral samples at each time point, it was detected in only two or three out of six of the optic nerve samples at day 3, 8, 29, and 92 with the remainder not detected or below the LLOQ. It was considered that StarGen vector DNA detected in the optic nerve in some animals was most probably attributed to imprecise dissection of the tissue, as there are no direct connections between the optic nerve and the site of injection. No vector DNA was detected in brain, gonads, liver, or spleen at any time point above the LLOQ, demonstrating that StarGen remained within the ocular compartment at this dose level and was not able to cross into the contralateral eye following a single subretinal delivery.

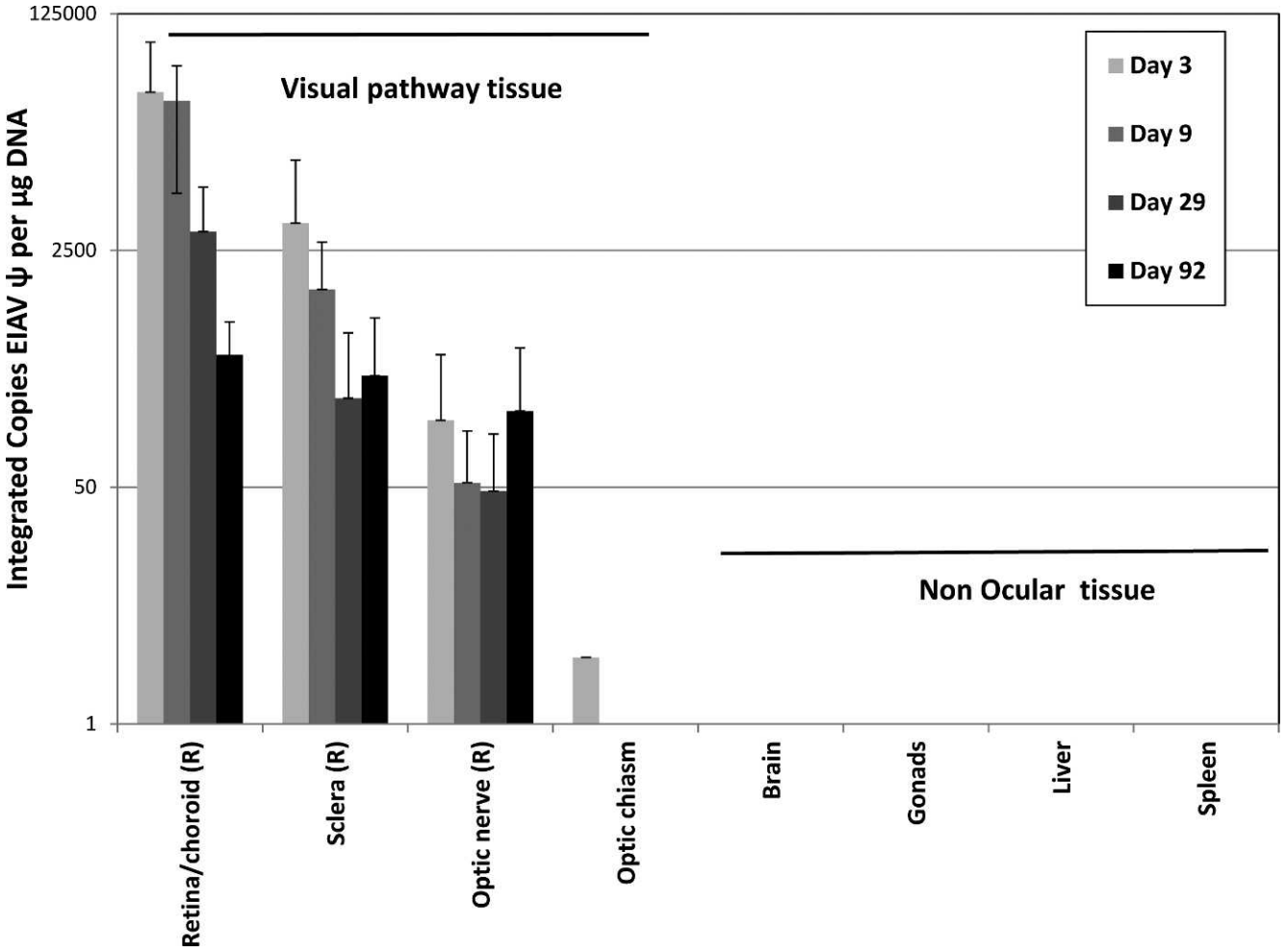

FIGURE 4. Biodistribution of StarGen in rabbits. The rabbits were dosed with either StarGen or buffer in the right (R) eye. Animals from each group were euthanized and necropsied for various organs at 3, 9, 29, and 92 days postinjection. Total DNA was prepared from each tissue, and unique StarGen-specific sequences were amplified by PCR. Copies of StarGen are reported per microgram genomic DNA. The higher level of vector DNA detected on day 92 in the optic nerve is an average of three out of the six samples that were positive, one of which was unusually high. The six retinal samples at day 92 were all positive. The means are shown ±SD for each tissue at each time point.

In the macaque study, quantifiable StarGen vector particles were not detected in saliva swabs or right-eye tear swabs at day 2 or in plasma samples at day 2, 3, or 5. Although StarGen vector particles were detected in buffy-coat samples from one animal at days 2 and 5, there was no evidence for a generalized increase in vector copy number in nucleated blood cells over time. StarGen vector DNA was not detected in most of the tissue samples at day 92 including brain, ovary, testis, spleen, optic chiasm, and right optic nerve, although nonquantifiable vector DNA (<LLOQ) was detected in the liver from two out of the six animals on day 92.

In the rabbit study, the average copy number of StarGen sequences in the target retina/choroid fell from a mean of ~0.2 copies per cell at day 3 and day 8, to 0.02 copies per cell at day 29, to 0.003 copies per cell at day 92. A similar pattern was observed in the sclera and optic nerve, although the initial number of copies per cell is significantly lower on day 3 at 0.02 and 0.001, as expected for this nontarget tissue. However, the average copy number per cell was higher in the optic nerve on day 92 compared with day 29; this reflects the unusually high level of vector DNA detected in one out of the six optic nerve samples evaluated at this time point, where we suspect imprecise tissue dissection occurred.

Immunogenicity

Immunologic assessments of serum samples from the macaque study did not show antibody responses to any StarGen vector component (data not shown). In the rabbit study, ELISA analysis of the serum samples indicated that, of the 38 rabbits that received StarGen, 21 (~55%) developed a StarGen-associated antibody response. Serum samples that tested positive by ELISA were further investigated by Western blot analysis to identify the component(s) of StarGen to which these antibody responses were directed. None of these positive serum samples showed humoral responses against the human ABCA4 protein or any EIAV component. However, antibody responses were generated against the envelope protein VSV-G in 21 (55%) animals, against the ~45 to 60 kDa human embryonic kidney (HEK) 293T-associated antigen(s) in 16 animals (~42%), and against a >250 kDa HEK293T-associated antigen in a single animal (~2.6%). The percentage of animals showing these antibody responses increased up to day 29 to day 92 and decreased by day 92 to day 183 (Fig. 5).

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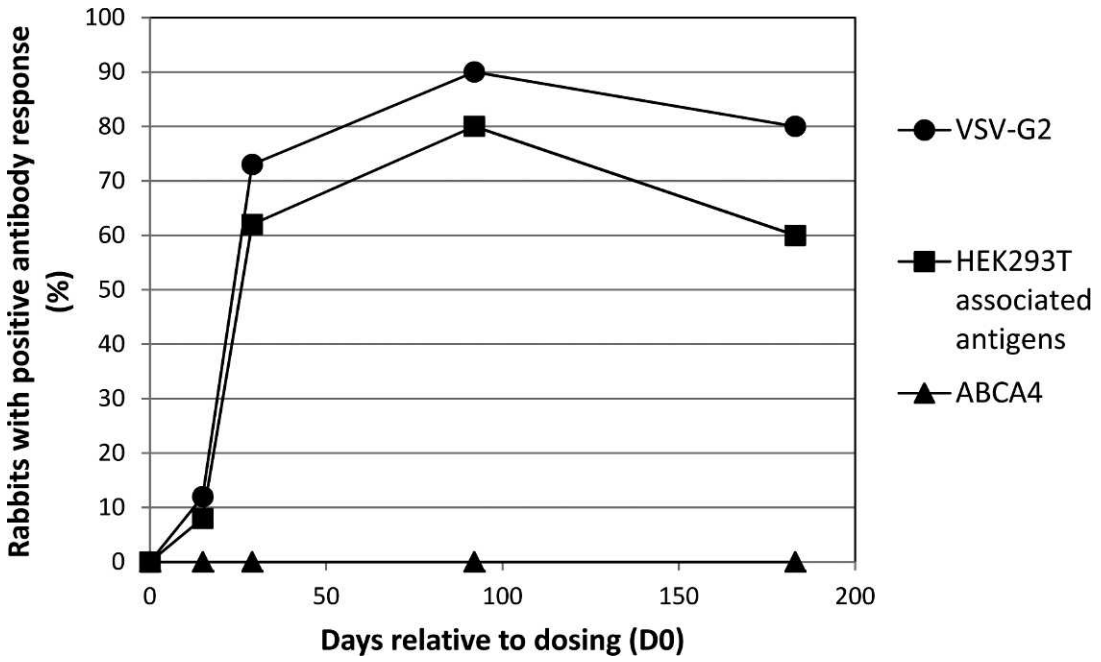

FIGURE 5. Percentage of rabbits showing an antibody response to vector-associated proteins or the ABCA4 therapeutic protein. The percentage of rabbits that showed an antibody response to the StarGen envelope protein (VSV-G2) or HEK293T cell–associated antigens or to the ABCA4 therapeutic protein is plotted for each time point following subretinal delivery of StarGen.

Ocular Histology

In the rabbit study, there were no StarGen-related microscopic findings on day 3, 8, or 29. At day 92 and day 183, slight retinal detachment was observed in a small number of buffer- and StarGen-treated eyes with a similar incidence and severity (Fig. 6). Minimal to moderate degeneration/atrophy of the retina and mononuclear cell infiltrates in the vitreous and choroid were noted in both buffer- and StarGen-treated rabbit eyes at day 183, the incidence and severity of which was slightly greater in eyes that received StarGen. These changes were interpreted to be related to the experimental procedure that was slightly exacerbated by the administration of the StarGen vector compared to the buffer.

In the macaque eyes, minimal retinal atrophy was observed after 92 days in one animal from each treatment group (Fig. 6). At day 183, slight retinal degeneration, retinal detachment, and choroidal fibrosis were observed at similar incidences in buffer- and StarGen-treated eyes. The low incidence and severity of these changes and the fact that they occurred in both buffer- and StarGen-treated eyes suggested they were related to the experimental procedure.

DISCUSSION

The eye is an ideal target for gene therapy applications; it is small, compartmentalized, and isolated from the rest of the body by the blood-retinal barrier. In addition, a number of different gene therapy vectors have been shown to effectively transduce ocular tissues, most notably lentiviral vectors and adeno-associated viral (AAV) vectors.[12] The PR cells are the most important target cell type for the treatment of inherited retinal dystrophies such as Stargardt disease.

The efficient photoreceptor transduction following subretinal delivery of AAV vectors,[13] in particular serotypes 5 and 8, has made them a popular choice for treating retinal dystrophies in independent clinical trials in individuals with Leber congenital amaurosis (LCA) resulting from a mutation in the *RPE65* gene.[14–16] However, despite their unquestionable gene transfer properties, AAV vectors have limitations when it comes to treating ocular dystrophies owing to a restricted cloning capacity of approximately 4.5 kb and potential issues associated with the presence of preexisting immunity[17,18]; although, so far this appears to be less of a problem in the immune-privileged eye.[19,20]

It is well established that subretinal administration of lentiviral vectors consistently results in efficient and stable transduction of RPE cells,[21] and we have previously demonstrated that efficient EIAV-mediated gene transfer to PRs in postnatal day 4 mice rescues the phenotype in the *ABCA4*$^{-/-}$ knockout mouse model of Stargardt disease.[11] In the current study, we used a VSV-G pseudotyped EIAV-CMV-GFP reporter vector to determine the expression profile following subretinal delivery in the adult macaque. This vector was high titre and was used undiluted to maximize the level of transduction. These data demonstrate that the EIAV vector is capable of efficient transduction of both RPE and PRs (rods and cones) in the macaque. This is in agreement with previous studies in the baboon, cynomolgus macaque, and the rabbit, which also showed RPE and PR transduction following subretinal delivery of an EIAV-LacZ vector (Kong J, et al. *IOVS* 2007;48:ARVO E-Abstract 1679; and Allikmets R, Gouras P, oral and written communication, 2004). Although GFP expression was most prominent in the RPE and PR-cell layers, toward the center of the bleb, there was expression in other cell types including horizontal, bipolar, amacrine, Mueller, and ganglion cells albeit with lower efficiency. The previously documented low efficiency of PR transduction in adult mice and macaques by lentiviruses has raised concerns as to their suitability as efficient tools for gene therapy in diseases originating from PRs, such as STGD1.[22–24] The difference between these EIAV-based data and previously reported lower efficiencies, with other lentiviral-based vectors, could be due to innate differences in the nature of the lentiviral vector itself, as well as the choice of promoter, the quality of the vector preparation, and the nature of envelope used to pseudotype the vector particles.

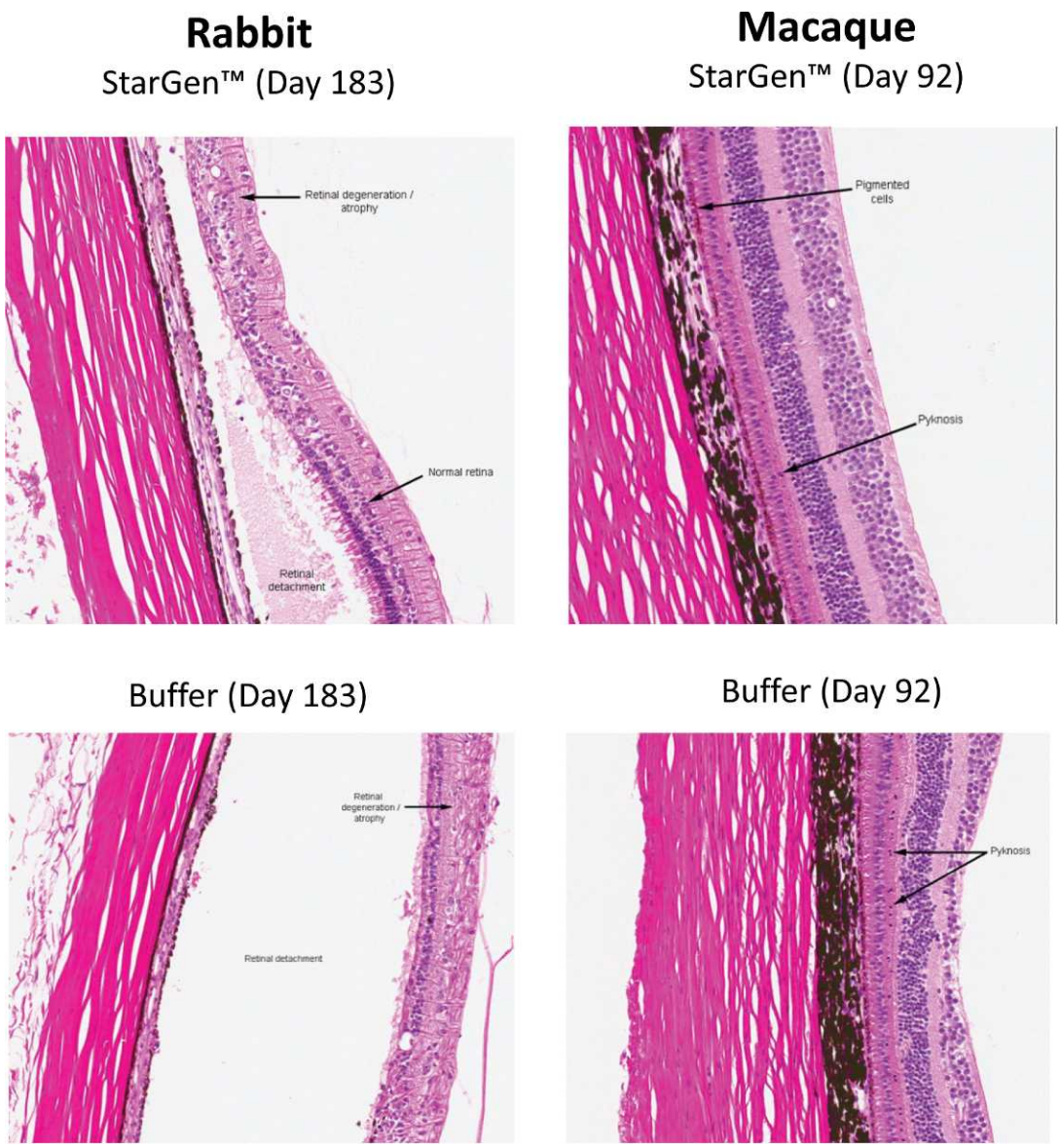

Figure 6. Rabbit and macaque retinal cross-sections. The day-183 rabbit-retinal cross-sections showed some evidence of retinal degeneration and atrophy, and in some cases the retina detached from the RPE cell layer during histologic processing. In the day-92 macaque-retinal cross-section, there is some evidence of pyknosis.

There are currently no approved treatment options for patients who have Stargardt disease. Although oral drug therapies that inhibit vitamin-A dimerization[25] and the implantation of embryonic stem cells[26] are being explored as potential treatment options, gene therapy offers an attractive approach to treat this chronic inherited condition by gene supplementation following a single subretinal administration. An EIAV-based lentiviral vector called StarGen that can easily accommodate large cDNAs such *ABCA4*, which is mutated in Stargardt patients, and is able to efficiently transduce the target PR cells, is being developed as a gene therapy treatment for these individuals.

The toleration and safety of subretinally injected StarGen was investigated in both rabbits and macaques. The studies were designed to investigate both the safety of the vector when dosed subretinally and the toxicologic risk to the target PR and nontarget cells, especially the RPE, which are efficiently transduced, following steady-state ABCA4 expression from the CMV promoter.

Following subretinal delivery of StarGen or buffer, there was an initial slight (+2) to very slight (+1) transient inflammation in the eye that was slightly greater in the StarGen-treated eyes compared with the eyes treated with buffer. In addition, other changes included retinal elevation caused by the procedure (bleb formation); variations in the pigmentation following separation of the neurosensory retina and the retinal pigment epithelium and white peripheral retinal opacities generally occurred with a slightly higher incidence, duration, and severity in the StarGen-treated groups. The peripheral white opacities, which occurred in five out of six of the StarGen-treated macaques, generally appeared on day 29 and were considered to be related to the transient ocular inflammation response that was higher in the StarGen-treated compared with the buffer-treated group. These opacities had generally resolved by day 91 (in all but one macaque), which coincided with the overall resolution of the vitreal cell score. This ophthalmologic change did not resolve into any StarGen

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treatment-related histopathologic findings at the level of the retina.

Most of these findings were not unexpected since a previous EIAV-based gene therapy vector called RetinoStat, for the treatment of "wet" AMD, elicited a similar transient inflammatory response following subretinal delivery in rabbits and macaques.[27] Similar to RetinoStat, the ophthalmologic changes in the StarGen studies did not translate into any unexpected treatment-related histopathologic or electroretinographic findings at the level of the retina, which indicates there was no cytotoxicity associated with off-target expression of ABCA4 protein in the RPE cells.

In the biodistribution and shedding study, the StarGen vector was only detected within the vector-treated rabbit ocular compartment. For the duration of the study, a small amount of EIAV-vector sequence was detected in the sclera and the optic nerve tissue sample from a subset of the animals; however, EIAV-vector sequences were never detected at quantifiable levels in the brain or optic chiasm. The reason for the positive signal in a subset of the optic nerve samples is not known but may be due to imprecise tissue dissection. The positive sclera samples are likely the result of technical difficulties in separating the retina/choroid from the collagenous sclera during processing, although the possibility that the vector transduced deeper ocular layers cannot be ruled out. Although EIAV-vector sequence was detected in two out of the six macaque liver samples, it was below the LLOQ with an estimated copy number of 1 per microgram of DNA, and there were no histopathologic findings in any of the macaque livers at the end of the study. Collectively, these data confirm the ability of the ocular anatomy to contain the majority of EIAV vector after subretinal dosing.

The qRT-PCR techniques used to detect a positive vector signal in each tissue sample in the biodistribution/toxicology study can also provide an estimate of the number of vector copies, allowing an estimate of the average copy number per tissue to be determined. The average copy number of the StarGen sequence in the target rabbit retina/choroid samples was ~0.2 at day 3 and day 8, 0.02 by day 29, and 0.003 by day 92. Although the average copy number per cell appears to reduce by 2 logs from day 3 to day 92, it must be stressed that this biodistribution/toxicology study was designed to determine the location of the vector following subretinal delivery rather than the exact copy number per tissue, and consequently, no effort was made to ensure that the subretinal bleb (i.e., the area of injection) was consistently dissected out for analysis. Therefore, this may account for part of the apparent loss of vector in the retina/choroid samples. Additionally, the highest copy number was seen at days 3 and 9, which was expected since cell entry, reverse transcription, and integration processes are complete within the first few days. Therefore, in addition to the dissection technique, the drop in copy number could be partly owing to the loss of unintegrated sequences, such as 1-long-terminal repeat (1-LTR) and 2-LTR circles over time. A study dedicated to determining the changes in copy number in the target tissue and/or the level of *ABCA4* expression would have to be carried out separately to accurately determine the reduction in copy number and the impact, if any, on therapeutic gene expression levels. Nevertheless, the biodistribution and shedding findings for StarGen, where the vector is exclusively detected in the ocular tissue samples, correlate with those observed with RetinoStat.[27]

Immunologic assessments of serum samples up to 3 months in the macaque study did not show any antibody responses to any component of the StarGen product. However, the 6-month rabbit study revealed a time-dependent increase in antibody responses to the VSV-G component of the StarGen vector and to HEK293T-associated proteins, despite there being no overt distribution of vector beyond the ocular compartment. However, there were no antibody responses detected toward the ABCA4 protein. Despite the humoral antibody response directed toward the vector envelope protein by day 92, there were no toxicologic findings in the rabbit, including in the ocular region. The reason for the differences in the immunologic responses to StarGen observed in the rabbit and macaque is unclear; the vitreal scores in both species were comparable, indicating that StarGen was well tolerated and subretinal dosing went well, with no reported injuries to the retina detected either functionally by ERG or structurally by histology. The lack of an antibody response to StarGen in the macaque study is consistent with the data generated in both rabbits and macaques following subretinal dosing of RetinoStat vector,[27] which shares many of the same features as StarGen such as the envelope. The concentrations of StarGen administered in the rabbit were approximately 3-fold higher than in the macaque study, suggesting that the vector-particle dose may be a factor involved in eliciting the antibody response in the rabbit study.

Future studies investigating the immune response after StarGen-vector delivery to both eyes on separate occasions would be informative since clinically a second dose of StarGen in the contralateral eye would likely be required in the future.

In conclusion, long-term safety studies performed in rabbits and macaques demonstrate that a single subretinal injection of StarGen is safe and well tolerated with a slight, transient cellular inflammatory response in the target eye; vector DNA sequences were confined to the ocular tissues of the StarGen-injected eye. Ocular gene therapy following subretinal delivery of RetinoStat, another EIAV-based vector, is already being evaluated in individuals with wet AMD, with no vector-related serious adverse events reported to date (ClinicalTrials.gov number, NCT01301443). In the LCA clinical studies,[14–16] in which the *RPE65* cDNA is delivered using an AAV, the safety of the subretinal intervention and the results showing stability of the improvement in visual and retinal function further support the use of gene therapy for chronic ocular diseases.[20] These preclinical data with StarGen provided support for the initiation of the first-in-man clinical trial of StarGen (ClinicalTrials.gov number, NCT01367444) in patients with Stargardt disease that is currently underway.

Acknowledgments

The authors thank Phillip Angell-Manning and the manufacturing team at Oxford BioMedica (UK) Ltd. for the production of StarGen IH44 vector.

Supported in part by funds from the Foundation Fighting Blindness, Columbia, Maryland (RA, JK, PG), National Eye Institute/National Institutes of Health Grant R24 EY019861 (RA, JK, PG), and an unrestricted grant to the Department of Ophthalmology, Columbia University, New York, New York, from Research to Prevent Blindness, Inc. Our thanks also go to Sanofi, Paris, France, for supporting this work.

Disclosure: **K. Binley**, Oxford BioMedica (UK) Ltd. (E); **P. Widdowson**, Oxford BioMedica (UK) Ltd. (E); **J. Loader**, Oxford BioMedica (UK) Ltd. (E); **M. Kelleher**, Oxford BioMedica (UK) Ltd. (E); **S. Iqball**, Oxford BioMedica (UK) Ltd. (E); **G. Ferrige**, Oxford BioMedica (UK) Ltd. (E); **J. de Belin**, Oxford BioMedica (UK) Ltd. (E); **M. Carlucci**, Oxford BioMedica (UK) Ltd. (E); **D. Angell-Manning**, Oxford BioMedica (UK) Ltd. (E); **F. Hurst**, Oxford BioMedica (UK) Ltd. (E); **S. Ellis**, Oxford BioMedica (UK) Ltd. (E); **J. Miskin**, Oxford BioMedica (UK) Ltd. (E); **A. Fernandes**, None; **P. Wong**, None; **R. Allikmets**, None; **C. Bergstrom**, None; **T. Aaberg**, None; **J. Yan**, None; **J. Kong**, None; **P. Gouras**, None; **A. Prefontaine**, None; **M. Vezina**, None; **M. Bussieres**, None;

S. Naylor, Oxford BioMedica (UK) Ltd. (E); **K.A. Mitrophanous**, Oxford BioMedica (UK) Ltd. (E)

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