Surgical Removal of Internal Limiting Membrane and Layering of AAV Vector on the Retina Under Air Enhances Gene Transfection in a Nonhuman Primate

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PURPOSE. To determine if the surgical removal of the internal limiting membrane (ILM) in nonhuman primates (NHPs) will result in safe and effective transfection of adeno-associated viral (AAV2) vectors using green fluorescent protein (GFP) as a reporter.

METHODS. Six Macaca fascicularis NHP eyes underwent vitrectomy, ILM peel with layering of 1.7 × 10^13 genome copies per milliliter of AAV2-GFP under air. Four control eyes underwent only vitrectomy and pooling under air. The intensity and area transfected was quantified in vivo with fundus autofluorescence (FAF) imaging. NHPs were euthanized 16 weeks postsurgery and immunohistochemical analysis assessed GFP expression at the cellular level.

RESULTS. There was a larger area of fluorescence in ILM peeled eyes then in non-ILM peeled eyes (50.7 [33.1–58.4] pixel^2 versus 5.1 [0.6–7.6] pixel^2, P < 0.01). The intensity of fluorescence was also higher in ILM peeled eyes (10.3 [2.2–18.5] vs. 1.9 [0.6–4.4], P = 0.05). Non-ILM peeled eyes displayed fluorescence confined to the foveal center. Histological sections showed colocalization in the Müller cell layer, ganglion cell layer, and photoreceptor cell layer in the ILM peeled eyes. Non-ILM peeled eyes GFP expression was only in the ganglion cell layer in three eyes and was confined to the immediate vicinity of the fovea.

CONCLUSIONS. ILM appears to be the predominate barrier to AAV transfection. An efficacious and safe method of AAV2 gene delivery, taking into account the potential need for repeat treatments, appears to be the surgical removal of ILM and layering of AAV under air.

Keywords: adeno-associated vector, gene, inner limiting membrane

Gene therapy mediated by adeno-associated viral (AAV) vectors has shown great potential in the treatment of a wide range of diseases. Recombinant AAV as a genetic transfer vehicle has several inherent characteristics that afford advantages over other gene delivery systems. First, its nonpathogenic nature generates only mild immune responses with no evidence of toxicity. Second, it also has the ability to transfect a wide range of tissue with sustained, long-term expression of the therapeutic phenotype. Finally, its relative ease of modification allows for targeted gene expression in specific cells, making it ideal for bespoke gene therapy.1

AAV vectors have proven to be very useful in gene therapy for diseases in the eye with minimal side effects. Preclinical and early human trials using AAV-mediated gene therapy have shown promising results in the treatment of ocular diseases, such as Lebers congenital amaurosis, choroideremia, X-linked retinoschisis,7–6 and glaucoma.7–5

A novel application for AAV gene therapy in the eye is the treatment of neovascular AMD (nAMD). nAMD is a common cause of vision loss in the elderly and with an aging population, this proportion is likely to rise.10 The current gold standard of treatment is the administration of intravitreal injections of anti-VEGF agents to suppress neovascularization and its associated complications.11–14 This therapy has proven to be effective in the stabilization and improvement of vision; however, it comes with a significant treatment burden. Treatment posology dictates frequent intravitreal injections of anti-VEGF agents that may be required over many years to maintain vision and to control the disease process.15,16 Gene therapy, with its ability to provide longer, sustained protein expression, appears to be a promising alternative solution. Previous studies report the use of soluble FMS-like tyrosine kinase-1 (sFLT-1), a naturally occurring VEGF inhibitor, coupled with an AAV delivery vector for sustained VEGF inhibition in the retina, hence negating the need for frequent anti-VEGF injections.17–19

In AAV gene therapy, the effective expression of the transgene is contingent on the integration of the genetic material into the host genome of terminally differentiated and nondividing cells. In the context of AAV-mediated gene therapy for retinal diseases, this has previously been achieved via a
subretinal injection to ensure close proximity of the vector with the cells that require transfection. This method has been proven in animal models and early clinical trials to effectively transfect the RPE cells\(^4\) and the photoreceptors.\(^{20,21}\) This method of delivery, while effective, has several shortcomings. It is a technically challenging procedure to perform and transfection is confined to the area of subretinal bleb raised. Intravitreal injections of AAV gene constructs have been studied; however, levels of transfection have been shown to be markedly reduced as compared with subretinal delivery and is largely confined to the foveal area.\(^{22–24}\)

Because of the potential that AAV-mediated gene therapy has in the treatment of multiple ocular conditions, there is a need to develop a practical, robust, and safe method of delivery. In this study, we aimed to demonstrate that the relatively safe and technically simple, time-tested procedure of internal limiting membrane (ILM) peeling after trans pars plana vitrectomy combined with the direct pooling of AAV on the peeled retina surface intraoperatively under air would allow for a safe, effective, and improved method of transfection. We performed the study on nonhuman primates (NHPs), which most closely resemble the human retina, with AAV2-mediated transfection with green fluorescent protein (GFP) used as a reporter.

**METHODS**

**Nonhuman Primates**

Five adult *Macaca fascicularis* NHPs were used, each weighing approximately 6 kg with ages ranging from 3 to 11 years. All eyes including retina were examined and ascertained as normal at baseline. All animal procedures conformed to the adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the SingHealth standard for responsible use of animals in research, from whom ethical approval was obtained (approval number 2016/SHS/1227). The research performed was carried out in the SingHealth Experimental Medicine Centre, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

**AAV Preparation**

The AAV vector and gene payload was obtained commercially (Vector Biolabs, Burlingame, CA, USA). Briefly, AAV vectors were packaged and purified by standard methods. The virus used contained both AAV serotype 2 capsid and inverted terminal repeat-containing genomes packaged inside AAV2 capsid carrying enhanced GFP (eGFP). The eGFP expression was under the control of a cytomegalovirus (CMV) promoter. Physical titer of the vector was \(1.7 \times 10^{13}\) genome copies (GC)/mL. Efficacy of the AAV-GFP was confirmed by preliminary in vitro transfection of both endothelial and Müller cell lines kindly donated by S. Singal.

**Surgery**

All NHPs underwent surgery with general anesthesia provided by an expert veterinary anesthesiologist using a combination of ketamine and xylazine. A total of 10 eyes in five NHPs were studied. All eyes underwent standard 25G three-port pars plana vitrectomy under sterile operative conditions. Core vitrectomy was performed, then posterior vitreous detachment was achieved in all eyes. This was clearly evident as a ring of vitreous detached in a wave extending out from the optic disc to the equator (Fig. 1). Six eyes in five animals then underwent an ILM peel under membrane blue-DUAL visualization (Dutch Ophthalmic, USA, Exeter, NH, USA). All eyes then underwent a complete fluid-air exchange before the delivery of AAV-GFP. All eyes received 40 μL of \(1.7 \times 10^{13}\) GC/mL AAV-GFP. This was delivered via a soft tip mounted on a microinjector into the air-filled eye. The AAV-GFP was pooled over the peeled ILM region of the macula or on the normal macula in nonpeeled eyes, respectively. The animal was subsequently left in a supine position for 1 hour to maximize contact of AAV-GFP with the macula. The vitrectomy and ILM peel were performed using a Constellation (Alcon, Fort Worth, TX, USA) vitrectomy machine and operating microscope (OPMI MDO; Carl Zeiss, Oberkochen, Germany). An intraoperative optical coherence tomogram (OCT) was also available for visualization of the ILM and retina layers.

**Imaging**

Fundus color photographs and cross-section OCT of the retina were obtained. Two imaging modalities were used to ascertain the extent of GFP transfection in vivo. Fluorescent images of GFP transfection were obtained using a modified fundus camera with an excitation bandpass filter of 457 to 487 nm (FF01–472/30; Semrock, Rochester, NY, USA) and a barrier filter with a bandpass of 502.5 to 537.5 nm (FF01–520/35; Semrock). Fundus autofluorescence (FAF) images were acquired using infrared imaging by confocal scanning laser ophthalmoscopy (cSLO) (Heidelberg Retinal Angiograph [HRA]; Heidelberg Engineering, Dossenheim, Germany). All imaging was performed at baseline and weeks 2, 4, 8, and 16 (before euthanasia) after surgery for each eye.

**Histological Analysis and Immunohistochemistry**

Animals were euthanatized 16 weeks after surgery and AAV vector administration for histological analysis. The eyes were enucleated and fixed with 4% paraformaldehyde in PBS overnight at 4°C. The cornea and lens were then removed and fixed in 4% paraformaldehyde in PBS overnight at 4°C. The eyes were soaked sequentially in 10%, 20%, and 30% sucrose overnight, after which they were frozen in optimum cutting temperature compound at 80°C. 10-mm-thick sections were cut using a cryostat (HYRAX C 50, Carl Zeiss Microimaging GmbH, Germany). Hematoxylin and Eosin staining and Toluidine Blue O (TBO) staining were performed to detect the area of peeled ILM.

After blocking thin sections (7 μm) in 5% BSA in PBS containing 0.1% Triton X-100 (PBS), the sections were incubated in a humidified chamber overnight at 4°C with a rabbit anti-GFP IgG antibody (1:1000 dilution; Invitrogen, Carlsbad, CA, USA). The sections were then washed three times with PBS and incubated with Alexa 488 goat anti-rabbit IgG (1.500 dilution; Invitrogen) for 90 minutes at room temperature. To analyze GFP expression in each eye, we examined seven slides containing sections 50 μm apart from the center of the macula. Two images of corresponding regions were taken from each slide using the same camera gain and time settings.

To examine the extent of GFP localization in individual retinal cell layers and RPE, we used double immunofluorescence staining on 7-μm sections for both GFP and either calretinin (ganglion cells), glutamine synthetase (Müller cells), arrestin (cone photoreceptor cells), or RPE 65 (RPE cells).

**Imaging Analysis: In Vivo Imaging**

To compare GFP expression, we analyzed each cSLO FAF image and GFP expression image using Imagej (http://imagej.nih.gov/ij/), provided in the public domain by the National Institutes of
Health, Bethesda, MD, USA). The extent of green fluorescence of GFP imaging was determined qualitatively. For quantitative measurements cSLO, FAF imaging was used. Two metrics were used: (1) the area transfected, which was measured as pixel area of fluorescence on cSLO FAF images; and (2) intensity of fluorescence, which was determined by calculating the ratio of the mean pixel intensity of the fluorescent area over the mean pixel intensity of blood vessels of the retina. This ratio was presented as a fold increase of fluorescent intensity. A $\chi^2$ test and two-tailed $t$ test were performed to ascertain significance in proportions and means, respectively. A $P < 0.05$ was used as the level of significance. All statistical analysis was performed with R version 3.2.2.

**RESULTS**

**In Vivo Postoperative Course and Inflammatory Responses in NHP Eyes**

Study details of the 5 NHPs are summarized in Table 1. Vitrectomy and posterior vitreous detachment was achieved in all 10 eyes. One control eye had localized retinal trauma nasal to the optic disc, but this did not lead to retinal detachment. There were no further complications associated with the surgical trauma.

Inflammation was noted in four eyes (three ILM peeled eyes and one non-peeled eye). In two eyes (ILM peeled eyes, MK2445 right eye and MK5338 right eye), transient inflammation was noted at 4 weeks postsurgery, which was suppressed with the use of topical anti-inflammatory drops and subsequently resolved by 10 weeks postsurgery. In the other two eyes (one ILM peeled eye and one non-peeled eye of the same NHP, MK7048 left and right eye, respectively), inflammation was noted at 4 weeks postsurgery and was persistent despite topical treatment. Intravitreal dexamethasone, 1 mg in 0.05 mL was administered at week 8. Inflammation subsequently resolved in both eyes by week 14 with no further intervention. The resolution of inflammation was determined by clinical slit lamp biomicroscopy, which confirmed the resolution of anterior chamber and vitreous cells and flare.

**GFP Fluorescent Imaging**

FAF on cSLO was found to be more sensitive for imaging GFP transfection, detecting green fluorescence as early as 2 weeks postsurgery as compared with fluorescent imaging by the modified fundus camera, which only detected green fluores-
### TABLE 1. NHP Study Details

<table>
<thead>
<tr>
<th>NHP</th>
<th>Eye</th>
<th>Vitrectomy Without ILM Peel</th>
<th>Vitrectomy + ILM Peel</th>
<th>Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Right</td>
<td>+</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>Left</td>
<td>No</td>
<td>Yes (topical steroids)</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Right</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>Right</td>
<td>+</td>
<td>Yes (topical steroids)</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>Right</td>
<td>+</td>
<td>Yes (intravitreal steroids)</td>
<td>No</td>
</tr>
</tbody>
</table>

Both eyes of five NHPs were used in this study. Four eyes underwent vitrectomy with retinal surface pooling of AAV-GFP under air, and six eyes underwent vitrectomy and ILM peel with pooling of AAV-GFP under air. AAV-GFP was allowed to pool on the retina postsurgery with the NHP left in a supine position for 1 hour. Inflammation was noted in three eyes: two ILM peeled eyes and one non-peel eye. Inflammation in MK7048 was severe enough to warrant the administration of intravitreal dexamethasone. All inflammation resolved eventually. +, indicates procedure was performed.

### Histological Imaging

To analyze the extent of GFP expression, we obtained histological sections of each eye through the foveal area and also through the non-peeled areas of the more peripheral retina. Expression of GFP was noted in most layers of the retina in all ILM peeled eyes as compared with expression confined to the inner retinal layers around the fovea in non-peeled eyes (Fig. 3). This could explain the increase intensity of fluorescence noted in the ILM peeled eyes. Staining to identify retina cell layers and colocalization with GFP fluorescence was performed. In the ILM peeled eyes, there was evident transfection in the deeper retinal layers as seen in the qualitative analysis of the immunohistochimical sections.

In these eyes, there was colocalization in Müller cell layer (glutamine synthetase antibody stain). In the photoreceptor layer, although there was no colocalization with cone photoreceptors (cone arrestin antibody stain), adjacent cells with distinctive shape and location suggestive of rod photoreceptors expressed strong transfection. Amacrine cells (calretinin antibody stain), however, did not show any colocalization (Fig. 4).

An alternating hyper-hypofluorescent halo immediately adjacent to the ILM peeled area was noted in four of the six ILM peeled eyes on FAF (MK2445 right eye, MK5229 right eye, MK5338 right eye, and MK7048 left eye). Histology section through this area showed diffused RPE clumping (Fig. 5); however, the rest of the retina layers appeared intact with no obvious thinning and relatively normal architecture (Figs. 5, 6).

### DISCUSSION

Our results demonstrated a markedly increased efficacy of AAV-mediated GFP transfection when delivered with a combination of an ILM peel, vitrectomy and intraoperative pooling of the AAV-GFP on the retina under air compared with eyes that had no ILM peel. Improved GFP transfection was seen in terms of a large increase in area as well as intensity on multimodal in vivo imaging and this was confirmed histologically. The broad transfection area in eyes that underwent ILM peel correlated well with the area of ILM peel, whereas in eyes without ILM peel, transfection occurred only at the foveal center with minor scattered spots throughout the peripheral retina. More cell layers were transfected in ILM peel eyes as compared with non-peeled eyes as seen on histological examination. This also correlates well with the increased intensity of fluorescence noted.

Our imaging techniques in this study included the use of FAF on cSLO, which provided much more obvious evidence of transfection compared with traditional FAF systems. In this study, imaging was also obtained using

### TABLE 2. Comparison of Measures of AAV-GFP Transfection In Vivo Between Vitrectomy-Only and Vitrectomy With ILM Peel at 16 Weeks Before Enucleation

<table>
<thead>
<tr>
<th></th>
<th>Vitrectomy Without ILM Peel</th>
<th>Vitrectomy and ILM Peel</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area of HE, pixels² per 1000 (CI)</td>
<td>5.1 (0.6–7.6)</td>
<td>50.7 (33.1–58.4)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Intensity of HE pixel intensity (CI)</td>
<td>122.3 (116.9–127.7)</td>
<td>130.5 (74.1–186.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Intensity of reference fluorescence, pixel intensity (CI)</td>
<td>54.5 (36.6–71.8)</td>
<td>40.0 (28.8–51.1)</td>
<td>0.2</td>
</tr>
<tr>
<td>Ratio of intensity of HE to reference fluorescence, ratio (CI)</td>
<td>3.2 (2.2–4.3)</td>
<td>2.4 (2.1–2.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Area of histology staining, pixels² per 1000 (CI)</td>
<td>1.9 (0.6–4.4)</td>
<td>10.3 (2.2–18.5)</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Two modalities were used to compare the amount of transfection between the two groups. FAF imaging allowed for in vivo area measurement of hyperfluorescence (HF) of the retina representing the area of transfection. Intensity of fluorescence was also determined by obtaining a ratio of the mean hyperfluorescent intensity over the fluorescent intensity of a reference point (blood vessel). Histology was obtained at 16 weeks and transfected cells visualized. Area of green signifying transfection was measured and compared between the two different groups. Eyes that underwent ILM peel showed significantly more area of transfection and intensity of fluorescence on histology than those that underwent vitrectomy only.
Figure 2. Serial FAF images of eyes over time from 2 to 16 weeks. Top row shows an example of an eye that underwent vitrectomy and ILM peel (MK5229 left eye). The bottom row shows an example of an eye that underwent only vitrectomy (MK 2445 left eye). In both cases, the area of fluorescence does not change throughout the course of the study, with maximal transfection already present at 2 weeks.

Figure 3. Comparison of the extent of transfection between corresponding peeled and non-peeled eyes of the same NHP. Top row shows histology of the fovea region of four eyes that underwent ILM peel with 4',6-diamidino-2-phenylindole (DAPI) and GFP expression. The bottom row shows the corresponding other eye that did not have ILM peel. The level of GFP expression in the peeled eyes is much more evident than in non-peeled eyes. From left to right, MK2445, MK5538, MK792, and MK7048.
traditional FAF systems, which used the addition of excitation and barrier filters on a fundus camera. FAF images acquired by cSLO on the HRA system uses an excitation wavelength of 488 nm and a barrier filter with a cutoff at 500 nm, which blocks the excitation wavelength and allows transmission only of the autofluorescent light. The advantage in the cSLO-acquired FAF images is the ability to overcome the low-intensity signal of traditional FAF systems and the lens interference. In addition, the HRA system uses image averaging, in which a series of FAF images are captured and combined to reduce background noise, increase contrast, and improve the quality of the image captured. Although these features of the FAF cSLO system allowed for more subtle transfection to be appreciated, quantification of the intensity of the fluorescence image between subjects could not be reliably performed because of the intrinsic image manipulations for improved contrast.

**FIGURE 4.** Immunohistochemistry staining. *Top row:* Calretinin (CR)-staining amacrine cells. The corresponding DAPI with GFP showing no colocalization with amacrine cells (*white arrowheads*). *Middle row:* Glutamine synthetase (GS) staining Müller cells and processes. DAPI and GFP show distinct Müller cell-expressing GFP that colocalizes well (*white arrowheads*). *Bottom row:* Cone arrestin (CS) staining cone photoreceptors. DAPI and GFP show no colocalization with cone photoreceptors; however, distinct rod-like photoreceptors show green expression on DAPI and GFP, which corresponds to the appropriate location of rods cells (*arrows*). Magnified view of GFP-expressing cells in the photoreceptor layer suggestive of rod photoreceptors (*inset*) (CR stain: MK2445, GS stain: MK5338, CA stain: 7048).
Hence, the level of intensity of fluorescence reported in our findings was a ratio of the average hyperfluorescence over the background fluorescence level of the same image. This relative increase of fluorescent intensity allowed for a quantitative interimage comparison and also allowed us to track the change in intensity over time.

This study reports two aspects we consider pertinent to the delivery of AAV-mediated gene therapy in the eye. The implications of our findings are important, as this form of therapy may be applicable to a wide range of therapies for ocular diseases, including genetic retinal disease, angiogenic disease, and glaucoma. First, it reveals the predominate nature of the ILM as a barrier to transfection, and second, we describe and demonstrate an effective and practical method for AAV-mediated gene therapy delivery.

The ILM appears to be the predominate barrier to AAV transfection as compared with other possible ocular barriers, such as the vitreous gel or a dilution effect on the injected dose. The ILM is a basement membrane that lies between the vitreous and retina. It has been shown to be a physical barrier to AAV transfection in prior studies using small animal and NHP models in which increased transfection of AAV-GFP was noted after the ILM was enzymatically digested,25,26 as well as in genetically modified rodent eyes with ILM compromise. 27

The other barrier to transfection is postulated to be the vitreous itself. Two mechanisms of action are postulated to contribute to the barrier function of the vitreous. First, compared with the subretinal space, which is a relatively immune-privileged area, the vitreous cavity has antibodies present that can result in the neutralization of AAV.28 Second, the dilution of AAV within the vitreous humor and its gel structure has also been suggested to result in poorer transfection of the retina.29

The design of our study allowed a comparison of purely the effect of the ILM as a barrier to transfection. All eyes underwent vitrectomy, hence removing the barrier properties...
of the vitreous. AAV was also pooled directly on the retina surface in an air-filled eye intraoperatively in both vitrectomized-only eyes and those with vitrectomy and ILM peel to ensure maximal contact and dose concentration. Despite these measures, the amount of transfection in the non-peeled eyes was similar to previous reports after intravitreal injections of AAV without vitrectomy, in which only a ring of transfection occurred around the fovea with minor scattered transfection in the peripheral retina. 

The transfected cell types seen on both histological and cSLO imaging were confined in these cases with ILM intact to the inner retinal ganglion cell layer. In contrast, the ILM peeled eyes in our study showed transfection of deeper retinal layers, including Müller cells and Henle nerve fiber layer, when examined within the area of the peel. Within the photoreceptor layer, cone photoreceptors did not appear to be transfected; however, rod photoreceptors as inferred by their well-defined shape and location in proximity to cone cells showed strong transfection. These findings are consistent with a prior study, including the inference on rod transfection, on the efficacy and safety of AAV2 versus AAV8 transfection.

In this study, it was difficult to comment on the transfection of the RPE. Due to its inherent autofluorescent nature, examination of the RPE stain with a GFP filter showed positive colocalization regardless of the degree of transfection, hence we were unable to ascertain a reliable comparison between ILM peeled and control eyes. In addition, the limitation of AAV2 and CMV promoter may also restrict the type of cells transfected.

In addition to showing the predominate function of ILM as a barrier to transfection, we also demonstrated a surgically simple and safe technique of vector delivery. Traditionally, AAV delivery is via a subretinal injection. This technique can be technically challenging and is complicated by the fact that subretinal injection is carried out in disease-compromised retinas, adding to the difficulty of the procedure. The subretinal injection induces a bleb of fluid where the AAV vector resides in an iatrogenic retinal detachment. Prolonged presence of subretinal fluid has been shown to affect eventual retinal function despite eventual anatomical reattachment.

The other possible mechanism of damage could result from the direct toxic effects from the therapeutics injected. Even a subretinal injection of an iso-osmolar balanced salt solution, a relatively inert and harmless compound, has been shown to cause mild photoreceptor outer-segment damage over time.

Other strategies for vector delivery to circumvent the barrier properties of the ILM have shown promising results. One such method involves sub-ILM injections as opposed to subretinal injections. However, this may also prove to be even more technically challenging than a subretinal delivery. The advantage of sub-ILM injection is that there is minimal disruption to the anatomy of the eye and consequently less chance of iatrogenic damage. The disadvantage of this technique is that the ILM is left intact and repeated treatments will likely entail repeated complex surgery. AAV gene therapy may result in long-term expression of the transgene; however, the therapeutic effects are unlikely to last forever and may still require repeat treatments at later dates. In the animal model studies exploring the efficacy of sFLT for long-term anti-VEGF inhibition, a biological effect was noted to last up to 8 months in mice and 17 months in NHPs; however, expression beyond these time points is unknown. This may be particularly relevant in the treatment of nAMD, in which repeated gene therapy may be required to maintain therapeutic levels of anti-VEGF in an ongoing disease process.

The ideal method of vector delivery would still be via intravitreal injection without complex surgical intervention. This method of administration is technically simple and can be safely performed in the clinic setting. Studies have explored the delivery of AAV-mediated gene therapy vector via intravitreal injection; however, results have so far been disappointing and fail to show widespread transfection in terms of area and depth. An alternative approach has been to develop novel AAV variants that are able to partly overcome the barriers in the eye and achieve efficacious transfection through all layers of the retina. Specifically, AAV2-7m8 and AAV2 (quadY-FL-VTV) have shown promise in transfection of deep retinal layers of the eye in rodent models. However, although more effective than prior AAV variants, these novel variants still display only patchy transfection when used in NHP models. Nonetheless, due to the therapeutic potential, especially in the treatment of nAMD, there is great interest both in the research and commercial space in developing a novel AAV variant that can effectively transfect deep retinal cells via intravitreal injection. These variants, however, are still in the early preclinical stages and require further research and proof of concept in early human trials.
In our study, we present the results of transfection using a simple safe and familiar method. Vitrectomy with ILM peel is a well-proven technique in the surgical treatment of vitreoretinal interface abnormalities, such as epiretinal membrane, and full-thickness macular hole.\textsuperscript{39,40} This procedure is routine for vitreoretinal surgeons and requires minimal to no change to the technique when used for AAV-mediated gene therapy. This affords the practical advantage of delivering gene therapy with a procedure in which vitreoretinal surgeons are well versed, and it is well tolerated by patients. Our technique also involves the direct pooling of AAV on the peeled retina under air for an hour, which we suggest is as important a step as the ILM peel to achieve effective transfection. The fluid-air exchange during surgery and subsequent air fill postsurgery is also a well-recognized and common surgical step with air commonly left in the eye postoperatively as a tamponade during vitreoretinal surgery in humans with no known damage to the retina.\textsuperscript{41–43} Taken together, we postulate that because the ILM barrier is completely removed over the whole posterior pole, any further repeated treatments may require only an intravitreal injection of AAV gene product with or without fluid/air exchange and supine positioning for 1 hour.

The importance of the direct “pooling under air” technique should not be underestimated, as evidenced by a similar study undertaken by Takahashi et al.\textsuperscript{44} who compared transfection in eyes pretreated with either a vitrectomy and ILM peel or vitrectomy only, with the key difference in that AAV-GFP was administered as a delayed intravitreal injection as compared with the immediate and direct pooling of AAV under air during surgery in our study. Their study did not demonstrate obvious in vivo transfection after 16 weeks in either group; however, histological imaging revealed some transfection through the deep retinal cell layers at the corresponding peeled areas versus patchy and reduced transfection in vitrectomy-only eyes.\textsuperscript{44} We postulate that the delayed intravitreal injection of AAV resulted in a weaker transfection efficacy as compared with our technique of direct “pooling under air.” The difference in methodology between our study and that performed by Takahashi et al.\textsuperscript{44} adds to the further understanding of the natural barriers of transfection in the eye. The removal of both the ILM and vitreous with direct contact of AAV-GFP on peeled retina resulted in transfection, which was seen by in vivo imaging as early as 2 weeks posttreatment. Compared with the results presented by Takahashi et al.,\textsuperscript{44} our findings suggest that the barrier properties of the vitreous itself and dilution effect in the vitreous cavity should also not be underestimated. We postulate that these differences in efficacy may also be due to the dilution effect of the AAV-GFP in the fluid-filled vitreous cavity despite a prior vitrectomy. There also could be a reaccumulation of antibodies within the vitrectomized eye, which could neutralize AAV and reduce transfection. Furthermore, in a vitrectomized eye, there also could be a reaccumulation of antibodies within the vitrectomized eye, which could neutralize AAV and reduce transfection. Furthermore, in a vitrectomized eye, which could neutralize AAV and reduce transfection. Furthermore, in a vitrectomized eye, which could neutralize AAV and reduce transfection.

In conclusion, we have demonstrated that the ILM is the predominate barrier to transfection after vitreous injection of AAV gene constructs. We have also described a practical and relatively simple method of AAV gene delivery, taking into account the potential need for repeat treatments. Although most studies on this subject focus on the therapeutic effects of various transgenes, this study addresses the practical aspects of therapeutic delivery in this promising field. The shortcomings of this study include the lack of functional testing postsurgery. Further studies also should be carried out to explore the effects of immune response on AAV-mediated gene therapy. However, this technique has been proven to be safe in both animal models and clinical use. Further studies should combine the herewith described techniques and therapeutic transgenes to assess the therapeutic efficacy with AAV-mediated gene therapy.

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References


