Optical Modulation of Transgene Expression in Retinal Pigment Epithelium

D. Palanker[1,2], D. Lavinsky[1], T. Chalberg[3], Y. Mandel[1,2], P. Huie[1,2], R. Dalal[2], M. Marmor[2]

[1] Department of Ophthalmology, [2]Hansen Experimental Physics Laboratory, Stanford University, Stanford, CA
[3]Avalanche Biotechnologies, Inc. San Francisco, CA

ABSTRACT

Over a million people in US alone are visually impaired due to the neovascular form of age-related macular degeneration (AMD). The current treatment is monthly intravitreal injections of a protein which inhibits Vascular Endothelial Growth Factor, thereby slowing progression of the disease. The immense financial and logistical burden of millions of intravitreal injections signifies an urgent need to develop more long-lasting and cost-effective treatments for this and other retinal diseases.

Viral transfection of ocular cells allows creation of a "biofactory" that secretes therapeutic proteins. This technique has been proven successful in non-human primates, and is now being evaluated in clinical trials for wet AMD. However, there is a critical need to down-regulate gene expression in the case of total resolution of retinal condition, or if patient has adverse reaction to the trans-gene products.

The site for genetic therapy of AMD and many other retinal diseases is the retinal pigment epithelium (RPE). We developed and tested in pigmented rabbits, an optical method to down-regulate transgene expression in RPE following vector delivery, without retinal damage. Microsecond exposures produced by a rapidly scanning laser vaporize melanosomes and destroy a predetermined fraction of the RPE cells selectively. RPE continuity is restored within days by migration and proliferation of adjacent RPE, but since the transgene is not integrated into the nucleus it is not replicated. Thus, the decrease in transgene expression can be precisely determined by the laser pattern density and further reduced by repeated treatment without affecting retinal structure and function.

Key words: age related macular degeneration, gene therapy, vascular endothelial growth factor, selective laser therapy, retinal pigment epithelium

1. INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of visual loss in population older than 50 years, with 7.5 million people likely to be affected by AMD-related visual impairment in the US by 2020[1]. The current standard of care for treatment of wet AMD is frequent (often monthly) intravitreal injections of agents such as bevicizumab (Avastin), ranibizumab (Lucentis) or more recently aflibercept (VEGF-trap)[2]. These molecules inhibit VEGF signaling, thereby slowing progression of neovascularization and reducing macular edema. Frequent injections are burdensome for patients and physicians, are associated with very high cost to the healthcare system, and carry cumulative risk of ocular infection with potentially devastating consequences. In 2009, 1,270,836 intravitreal injections for eye diseases cost Medicare and Medicaid more than $2 billion, which is expected to grow rapidly as more indications are approved and the population ages. Such an immense financial burden on healthcare and patients signifies an urgent need to develop more cost-effective treatments for retinal diseases.

Gene therapy may allow viral transfection of ocular cells to create a "biofactory" that secretes therapeutic agents within the eye, eliminating the need for monthly injections. The leading program in development uses rAAV encoding for sFlt-1, which is a highly potent and naturally occurring anti-angiogenic peptide that binds and inactivates VEGF. Subretinal delivery of rAAV.sFlt-1 prevents and reverses progression of ocular neovascularization in mice and non-human primates[3], and is currently the subject of a Phase 1/2 clinical trial for wet AMD (NCT01494805).

One challenge for genetic therapy is the need to down-regulate gene expression in the case of total resolution of disease or adverse reaction to the therapy[4,5]. Several systems have been designed to address this concern[6]. Some use an inducible system, in which a small molecule such as doxycycline modulates a transcription factor regulatory domain and

keeps transgene expression on (Tet-On system) or off (Tet-Off system)[4]. Other systems involve inducers such as rapamycin[7], mifepreistone (RU-486)[8], and the ecdysone receptor from Drosophila melanogaster[9]. The most widely used systems require that a drug be taken on a constant basis to maintain expression in the "on" or "off" state[4,5]. These drugs, however, may have undesirable side effects, such as immunosuppression with rapamycin, and off-target effects of steroid hormones[8]. Systems that seem to avoid this problem, such as the Tet-On, suffer from an immune response to the gene product[10].

The primary target for genetic therapy of AMD and many other retinal diseases is the RPE, a supportive monolayer of cells underneath the neural retina. We have developed a method for down-regulation of the transgene expression in RPE that selectively destroys a designated fraction of RPE cells using a rapidly scanning laser. Microsecond pulses of light vaporize the melanosomes, and thereby destroy RPE cells while minimizing heat diffusion into the surrounding Bruch's membrane and neural retina[11]. Continuity of the RPE layer is restored by migration and proliferation of adjacent RPE cells, and the photoreceptors rapidly recover[12]. However, since with vector systems such as AAV the transgene is not replicated during cell division, the total transgene expression will decrease after the treatment. Thus selective fractional destruction of transfected cells followed by cell division may allow for precise titration of the transgene expression, controlled by the laser pattern density applied.

2. METHODS

Animals

Fifteen Dutch-belted rabbits (weight, 1.5–2.5 kg) were used in accordance with the ARVO Statement Regarding the Use of Animals in Ophthalmic and Vision Research, after approval from the Stanford University Animal Institutional Review Board. The rabbits were anesthetized using ketamine hydrochloride (35 mg/kg), xylazine (5 mg/kg), and glycopyrrolate (0.01 mg/kg) administered IM 15 minutes before the procedure. Pupillary dilation was achieved by 1 drop each of 1% tropicamide and 2.5% phenylephrine hydrochloride. One drop of topical tetracaine 0.5% was instilled in each eye before treatment.

Scanning laser system

A 532-nm laser (PASCAL; Topcon Medical Laser Systems, Santa Clara, CA) provided a beam with top-hat intensity profile. The software controlling the system was modified for rapid scanning of the laser to achieve microsecond dwell times, with a scanning speed up to 6.6 m/s. The custom graphical user interface allowed adjusting the laser power, scanning velocity, number of lines in the pattern and spacing between the lines[12].

To limit laser damage to just the RPE cells, the laser exposure time must be in the range of microseconds[11-13]. For a given target size L, the thermal confinement time $\tau=L^2/4\alpha$, where α is the thermal diffusivity of tissue. For example, for the apical melanin layer in RPE cells L≈4μm, and $\tau \approx$ 28μs in water. Microsecond exposures can be produced efficiently with a rapidly scanning continuous laser[12,14]. Exposure time is determined by the laser spot size divided by the scanning speed. For example, with a beam diameter of 100 μm and a velocity of 6.6 m/s, the exposure duration is only 15 μs.

A Mainster wide-field retinal laser contact lens (Ocular Instruments, Bellevue, WA) was used to focus the laser on the fundus, providing the retinal spot size in a rabbit eye equal to the aerial diameter. Using the line scanning software, patterns of 3 lines separated by one line width (50% coverage) were applied with 100μm spot size. Power was titrated to a level that produced damage invisible clinically and in OCT, but visible in fluorescein angiography. Further histological and TEM analysis confirmed that this level of titration produced damage limited to the RPE with no (or minimal) effect on photoreceptors' outer segments. Power ranged from 1.1 to 1.3W with scanning velocity of 6 m/s.

Viral vectors

Recombinant AAV serotype 2 expressing green fluorescent protein (GFP) under control of the CMV-IE promoter (Virovek, Inc., Hayward, CA) was produced. The rAAV.GFP was titered using qPCR and diluted to 1 x 1012 vg per mL, stored at -80ºC, and thawed immediately prior to injection. The vector titer in our study was similar to that applied subretinally in most of recent clinical trials[15].

Six eyes of six rabbits were used for subretinal injection of rAAV2.GFP. A trans-conjunctival 25-gauge trocar cannula (Alcon, Fort Worth, TX) was inserted through the sclera 3 mm from the corneo-sclerallimbus at a steep angle to avoid the lens, and the trocar was removed leaving the cannula in place. Partial posterior vitrectomy was performed and a 25G-41G extendable cannula (DORC Inc, USA) was used to inject 50 μl of fluid into the subretinal space, creating three blebs per eye, two with the viral vector and one reference bleb with vehicle alone (balanced salt solution (BSS). OCT was used to confirm the presence of subretinal bleb. After surgery, animals received atropine eye drops,

subconjunctival triamcinolone (Bristol- Myers Squibb, New York, NY), and corneal application of ointment containing bacitracin zinc (500 U/g) and polymixin B sulfate (10,000 U/g; Akorn, Lake Forest, IL). No detrimental retinal effects have been observed from the AAV or GFP expression.

In vivo imaging

To study dynamics of RPE healing after laser treatment 15 eyes with no prior surgery were submitted to the same scanning laser protocol at two months, one month, one week, three days, one day and one hour before enucleation. cSLO and OCT imaging was performed at each time point. At two months the eyes were processed for either light and transmission electron microscopy or scanning electron microscopy.

After surgery, rabbits were followed weekly with confocal scanning laser ophthalmoscopy (cSLO) autofluorescence mode and OCT (HRA2-Spectralis). One transfected bleb in each eye was randomly selected for laser and one received no laser treatment, as a control. After laser treatment at 3 weeks post injection, imaging was performed at 1 day, and then again at weekly intervals. One month after the first laser treatment, blebs were treated again with the same laser parameters, and followed for additional 3 weeks until animals were euthanized for tissue analysis.

For fluorescent imaging, animals were anesthetized and their pupils dilated. A cSLO with a 488-nm blue excitation and 500 nm green emission filter (autofluorescence mode) was used. At least five images of each bleb were captured and total brightness was quantified using ImageJ software (NIH). Data from each bleb was normalized to 21 days brightness, which represented the maximum expression before the laser treatment. The ratio of the signals from the treated and control blebs, with subtracted autofluorescent background, was calculated for each eye. Real-time eye tracking enabled averaging of multiple scans to reduce speckle noise, and ensured that the images were acquired at the same locations, improving reliability of the follow-up.

BrdU labeling

To detect the potential proliferation in the RPE and inner retinal cells, we used 5-bromo-2'-dioxyuridine (BrdU) labeling, which integrates into the DNA of dividing cells. Four eyes from four rabbits received line scanning laser using the same parameters as the treatment protocol of this study four days prior enucleation. BrdU labeling reagent (10 mL, Invitrogen, Eugene, OR) was infused slowly into an ear vein two and one day prior animal sacrifice. Eyes and control samples from small intestines were fixed in formalin, embedded in paraffin, and sectioned into 4-5 µm thick sections. Sections were then incubated with anti-BrdU antibody and Texas Red secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) following instructions of the manufacturer (Invitrogen). At least 5 sections of each sample were analyzed with fluorescent microscopy using mounting media containing DAPI.

3. RESULTS

Following laser scanning, no ophthalmoscopically visible changes have been detected, except for the visible marker burns. However, laser pattern was clearly visible in fluorescein angiography (FA), which indicates damage to RPE. Acute histology (Figure 1A) and Transmission Electron Microscopy (TEM) revealed collapsed RPE cells and minor damage to outer segments of photoreceptors but no damage to choroid or Bruch's membrane. Scanning Electron Microscopy of RPE layer at 1 day after laser demonstrated lines empty of RPE cells (Figure 1B).

Three days following laser application, the continuity of the RPE layer was reestablished. Interestingly, cells appear stretched, suggesting migration rather than proliferation, at this stage. The photoreceptor outer segments remained disorganized and edematous, but the outer nuclear layer appeared intact. After 7 days, the photoreceptor outer segments over the laser site appeared normal (Figure 1C). Some of the RPE cells filling the defect were small with prominent apical processes, suggesting both cell division and proliferation mechanisms (Figure 1D). Cell division was confirmed using a bromodeoxyuridine (BrdU) immunohistochemistry assay, which labels dividing cells, and marked nearly half of the RPE cells in the treated areas. At this time, TEM showed restoration of tight junctions and basal infoldings.

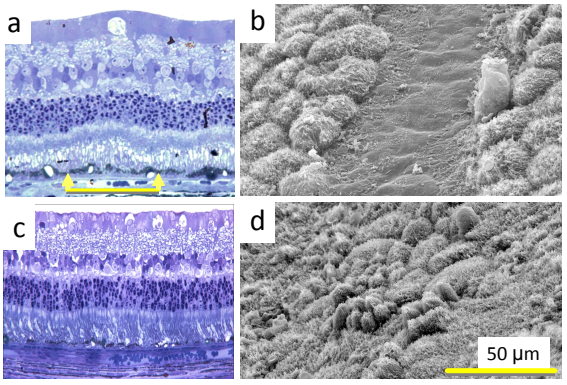

Figure 1. A. Light microscopy 1 hour after laser shows collapse of RPE cells with limited damage (swelling) in the photoreceptor outer segments; B. SEM 1 day after treatment shows an absence of RPE cells within the laser beam path. C. At 7 days LM shows normal retinal and RPE morphology, including the photoreceptor outer segments; D. SEM shows restoration of the RPE monolayer with microvilli.

To evaluate down-regulation of transgene expression, rAAV serotype 2 encoding GFP was administered to 6 pigmented rabbits. The vector was delivered via subretinal injection into two areas in each eye; balanced salt solution (BSS) vehicle was delivered to a third area as a control. Three weeks later, laser treatment was applied to one transfected bleb in each eye, with a line pattern density of 50% (i.e. laser lines were spaced one line width apart). The same areas were then retreated 4 weeks after the first laser treatment.

GFP expression was assessed using fluorescence imaging. Whereas the BSS injection site showed only slightly elevated autofluorescence, transfected areas showed uniform fluorescence throughout the bleb (Figure 2). Areas treated with laser exhibited an overall decrease in fluorescence compared to non-lasered control areas, and gaps in fluorescence were visible marking the location of the laser scans (Figure 2C). OCT showed normal photoreceptor morphology in all three areas, with outer segment markers such as the inner segment/outer segment junction line appearing intact. Histological sections of the BSS injected area 1 month after laser showed only the background autofluorescence of the RPE and photoreceptors. In contrast, the transfected control area (Figure 2B) had continuous GFP expression throughout the RPE layer. In the laser-treated area (Figure 2D), the RPE showed irregular loss of fluorescence with some cells bright and others dark, presumably from the expected pattern of selective laser treatment.

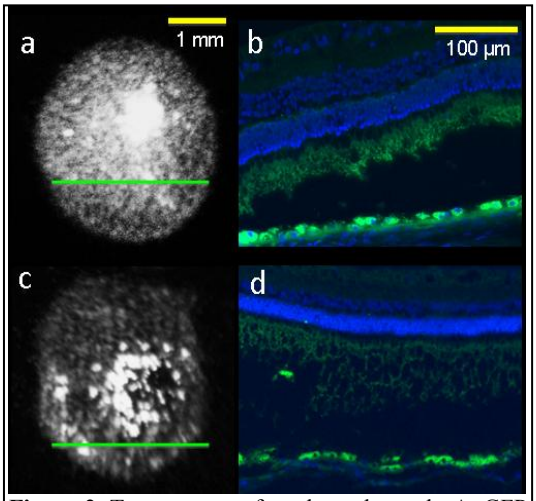

Figure 2. Top row: transfected, not lasered. A. GFP fluorescence across the transfected area in-vivo. B. Histology: Uniformly increased RPE fluorescence due to GFP expression. Bottom row: area transfected and treated with scanning laser. C. The GFP-transfected bleb treated with laser shows vertical lines of decreased fluorescence as well as reduced total fluorescence; D. Histology: Interposed areas of enhanced and decreased RPE fluorescence 1 month after laser treatment.

The time course of GFP expression and the effect of selective laser treatment on transgene expression are plotted in Figure 3. The transfected areas reached maximum fluorescence level at 3-4 weeks and remained stable throughout the follow-up period in the control (non-lasered) eyes. To allow comparison of the total fluorescent signal from transfected areas of various sizes the signals from each area were normalized to their expression at 21 days (the time of laser treatment). Figure 3b depicts the ratio of the average fluorescent signal (excluding background autofluorescence) from the laser-treated areas vs. the control (non-treated) areas. In the areas treated with line pattern of 50% density, the signal decreased by 54% within one week, and remained stable for four weeks. At that time, the same area was re-treated, again using 50% pattern density. This resulted in an additional 48% reduction, bringing the total signal to 26% of its original level, which remained stable for the last 3 weeks of the follow-up. In-vivo imaging and ultra-structural analysis with TEM and SEM after the second treatment confirmed a lack of damage to the neurosensory retina or Bruch's membrane, and a similar pattern of RPE repair as after the first treatment.

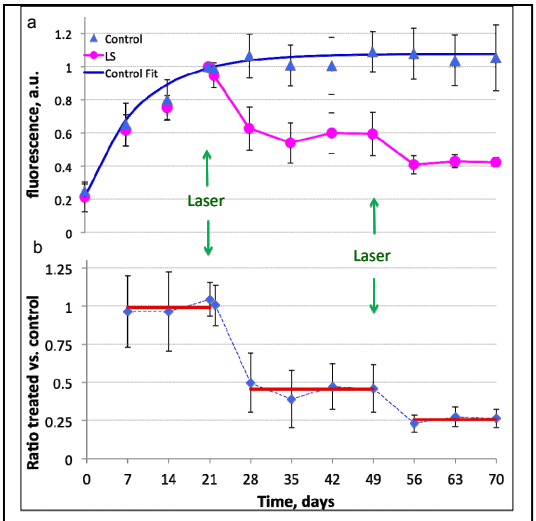

Figure 3. A. Average fluorescence of the transfected areas over time, normalized to its maximum at 3 weeks. The solid blue line is an exponential curve fit to the transfected (untreated areas) data. Retinal areas treated at 3 and 7 weeks demonstrated step reductions in fluorescence signal. B. Ratio of the fluorescent signals (excluding background fluorescence) from the lasered vs. control blebs. Solid lines show average brightness ratio over the period before the laser and after each laser treatment.

4. DISCUSSION

Clinical acceptance of retinal gene therapy would be greatly enhanced by availability of techniques to regulate transgene expression in the RPE without damage to the neural retina. The ideal system would (a) not require an inducer agent, (b) act only locally at the site of transduction, and (c) allow gradual reduction of transgene expression to a desired amount. Despite great efforts in creation of inducible gene expression systems, the developed approaches have been cumbersome and provide an incomplete solution to most clinical settings. In the present study, we have demonstrated that

rapid laser scanning can achieve these criteria, and the same area can be scanned repeatedly without any measurable adverse effect.

Following AAV-mediated gene delivery, the transgene is not replicated during cell division and as such, when stimulated to proliferate, RPE cells do not duplicate the transgene-bearing vector cassette. Therefore, selective destruction of RPE cells reduces the total amount of secreted protein even when the remaining RPE cells migrate or proliferate to restore functional continuity of the RPE layer. Selectively damaging a designated fraction of transfected RPE cells reduces the amount of expressed transgene proportionally, so that the density of the scanning laser pattern controls dosimetry of down-regulation.

Microsecond pulses can produce intracellular microbubbles around light-absorbing melanosomes leading to selective death of RPE cells, while the surrounding retinal temperature remains sublethal[11,16]. OCT imaging in human patients has demonstrated unaffected neural retina and RPE thinning at 1 hour and normal neural retina and RPE at 1 year. SRT (selective therapy of the RPE) with microsecond pulses has been tested clinically for Diabetic Macular Edema and Central Serous Chorioretinopathy (CSCR), and was shown to be safe and effective[17]. At energies corresponding to selective damage of the RPE, no immediate ophthalmoscopically visible retinal lesions are produced. Instead of using a dedicated microsecond laser, sufficiently short exposures can also be produced by rapid scanning of a continuous laser[14,18] - the method used in our study. Many retinal scanning lasers currently on the market could be adapted to perform such treatment, which will enable immediate access to this technology world-wide.

Even if the photoreceptors have been locally damaged by photocoagulation in narrow lesions, the normal anatomy of the photoreceptor layer is restored over time due to migration of the photoreceptors into the lesion[19]. Experiments with multielectrode arrays in-vitro[20] and microperimetry tests in-vivo[21] have shown that in these cases retinal sensitivity is restored as well. In our case the photoreceptors have not been destroyed, just their outer segments detach above the damaged RPE, and as soon as RPE cells are back, the outer segments become normal. Therefore, we do not anticipate any loss of retinal functionality.

If this approach is proven clinically successful, it could be directly applied to programs that utilize rAAV vectors to transfect RPE cells, including those using anti-VEGF agents for treatment of neovascular AMD, diabetic retinopathy, and macular edema. The use of chronic “biofactory” approach could alleviate the financial and organizational burden of monthly injections. The possibility of decreasing the trans-gene expression, if necessary, by laser therapy could reduce potential concerns associated with irreversibility of the gene therapy and thereby would enhance its clinical adoption.

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