Plasma-Mediated Transfection of RPE

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ABSTRACT

A major obstacle in applying gene therapy to clinical practice is the lack of efficient and safe gene delivery techniques. Viral delivery has encountered a number of serious problems including immunological reactions and malignancy. Non-viral delivery methods (liposomes, sonoporation and electroporation) have either low efficiency in-vivo or produce severe collateral damage to ocular tissues.

We discovered that tensile stress greatly increases the susceptibility of cellular membranes to electroporation. For synchronous application of electric field and mechanical stress, both are generated by the electric discharge itself. A pressure wave is produced by rapid vaporization of the medium. To prevent termination of electric current by the vapor cavity it is ionized thus restoring its electric conductivity. For in-vivo experiments with rabbits a plasmid DNA was injected into the subretinal space, and RPE was treated trans-sclerally with an array of microelectodes placed outside the eye. Application of 250-300V and 100–200 μs biphasic pulses via a microelectrode array resulted in efficient transfection of RPE without visible damage to the retina.

Gene expression was quantified and monitored using bioluminescence (luciferase) and fluorescence (GFP) imaging. Transfection efficiency of RPE with this new technique exceeded that of standard electroporation by a factor 10,000. Safe and effective non-viral DNA delivery to the mammalian retina may help to materialize the enormous potential of the ocular gene therapy. Future experiments will focus on continued characterization of the safety and efficacy of this method and evaluation of long-term transgene expression in the presence of phiC31 integrase.

<u>Keywords:</u> gene therapy, RPE, electroporation, cavitation

1. INTRODUCTION

Retinal degeneration diseases such as the age-related macular degeneration and a family of diseases known as retinitis pigmentosa involve a number of genetic causes (summarized at RetNet: http://www.sph.uth.tmc.edu/retnet/) that affect the photoreceptor cells or the retinal pigment epithelium (RPE) layer that supports them. Gene therapy represents potentially a powerful approach to treating retinal diseases [1]. Indeed, a number of studies have used viral vectors for gene delivery to photoreceptor cells [2-4] and RPE cells [5-7]. Despite these successes, viral vectors suffer from many drawbacks: they can be immunogenic, toxic, and, most notably for adeno-associated virus, have limited carrying capacity8. Moreover, viral vectors are difficult and expensive to manufacture, and randomly-integrating viruses have led to insertional mutagenesis9. Consequently, interest in non-viral gene delivery has grown. Chemical methods, such as liposomes, are often inefficient and toxic to cells [10]. Electroporation is effective for nearly all cell types and has become widely adopted as the method of choice for hard-to-transfect cells [11]. Electroporation is useful both for large molecules such as plasmid DNA and small molecules, but the mechanism of electrotransfer seems to be different. Whereas very short (μs) pulses are sufficient for small molecule delivery, efficient DNA delivery typically requires longer (20-50 ms) pulses [11]. The major disadvantage of electroporation is the high rate of cell death, which is considered an unavoidable consequence of the technology itself [12]. This limitation is especially significant for transfection of tissues in situ. Damage may occur by loss of cytoplasmic materials from the cell due to prolonged permeabilization of the cell membrane and also as a result of thermal damage [13,14]. Amaxa biosystems (Köln,

Germany) offers more efficient transfection technology, however, these methods are available for a limited number of cell types and, while improved, still lead to a high rate of cell death [15].

Sonoporation (the use of ultrasound) has recently been demonstrated for DNA [12,16], as well as for small molecules [17-20], and is thought to affect cellular membranes through a mechanism of acoustic cavitation involving the creation and collapse of gas bubbles [21]. Optimized in a muscle cell line, sonoporation has been reported to be relatively inefficient (transfection of ~2-4%) and somewhat toxic (viability of ~60-80%) [12]. Some models propose that mechanical stress can also cause formation of pores in cellular membranes [22].

In this investigation, we sought to optimize electroporation parameters for gene transfer to RPE in a larger animal model in order to overcome the limitations of conventional electroporation seen in our previous study using rats23. We begin with a model system, chorioallantoic membrane (CAM), and move to rabbits as a model organism, which have large eyes and allow for more elaborate surgical techniques that are more readily translated to the clinic. We developed a new technology for non-viral gene transfer that uses a combination of high electric field and mechanical stress, called plasma-mediated transfection. We evaluate efficacy and safety of this technique in the rabbit retina.

2. MATERIALS AND METHODS

Electroporation protocols

The luciferase expression plasmid pNBL224 has been used. The pMax vector was obtained from Amaxa biosystems (Köln, Germany).

For conventional electroporation, a pair of platinum electrodes was constructed having width 1 mm and length 4 mm and separated by 4 mm. Initial electroporation experiments used a variety of settings. Optimized parameters were: A high voltage square-wave pulse lasting 250 µs at +150 V, followed immediately by a low voltage square-wave pulse lasting 5 ms at –5 V. 50 such cycles were applied at 1 Hz. These parameters were used in subsequent experiments with CAM and rabbits. To apply tensile stress, a 0.2 mm agarose gel was used to separate electrodes from the CAM. By pushing down on the agarose gel slice, the CAM layer was stretched by ~5%. Application of ultrasound used the settings 2.4 W/cm^2 60 kHz ultrasound for 50 seconds (Transducer US 15CB, Nakanishi Inc., Japan).

For plasma-mediated gene transfer, biphasic pulses of 250 µs per phase were applied. Experiments on CAM used a single 100 µm microelectrode 1 mm in length and a range of voltage settings from ± 250 V to ± 600 V. For rabbit RPE experiments, an electrical probe was constructed that could be inserted behind the sclera in the rabbit eye and contained an array of microelectrodes surrounded by a return electrode. The probe also contained an optical fiber illuminated with a Xe lamp (Ophthalmic Technologies, Inc., Toronto, Canada) to facilitate localization of the probe under the bleb. Rabbit experiments used the setting ± 250 V, and 5 pulses were applied at each location, with 5-15 locations treated in each eye.

Chorioallantoic membrane (CAM) gene transfer and culture

Fertilized chicken eggs (California Golden Eggs, Sacramento, CA) were incubated under development conditions until day [9-13]. The shell and shell membrane were removed to expose the CAM surface, DNA was pipetted onto the membrane, electrodes were placed in the DNA solution, and pulses were applied. To culture the tissue, the shell, shell membrane, and CAM were carefully cut with scissors ~1 cm below the first cut, so that the treated area remained intact and the eggshell ring formed a scaffold to support the CAM. The tissue was washed twice for 15 minutes in 2x antibiotic/antimicotic (Invitrogen), rinsed with PBS (Invitrogen), and cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% fetal bovine serum.

Animal studies

Initial experiments measuring luciferase activity were performed with 1.8-2.0 kg Dutch Belt rabbits, and all subsequent experiments used 3.5-4.5 kg New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, TN). All experimental protocols were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Administrative Panel on Laboratory Care (A-PLAC) at Stanford University.

Subretinal injection and electroporation in vivo

Animals were anesthetized with 40 mg/kg ketamine (Vedco, St Joseph, MO), 5 mg/kg xylazine (Vedco), and 0.02 mg/kg glycopyrollate (Baxter, Deerfield, IL). 1% atropine (Alcon Laboratories, Fort Worth, TX), 2.5% phenylephrine (Alcon), 0.5% tetracaine (Alcon), and 2.5% methylcellulose gel (Akorn, Buffalo Grove, IL) were applied to eyes. A corneoscleral limbus-based conjunctival peritomy was performed to allow electrode access to the back of the eye, and space was opened by separating blunt-tipped tenotomy scissors. A 25-gauge trocar-cannula (Alcon) was

pushed through the sclera 3.5 mm from the corneoscleral limbus at a steep angle to avoid the lens, and the trocar was removed leaving the cannula in place.

For the subretinal injection, a 1 ml tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) connected to 10 cm tubing ending in a 30-gauge needle (Becton Dickinson) that had been blunted and polished was loaded with DNA and air bubbles removed. The needle was guided through the cannula until just above the retina. A pulse of DNA was injected, consisting 50-100 µl of 1 µg/µl DNA in balanced salt solution (BSS), forming a subretinal bleb. Electrodes were placed through the peritomy. The lamp on the electrode was used to align probe directly under the bleb and five pulses was applied. The electrodes were moved slightly and the next set of pulses was applied to cover the bleb. The sclerotomy and peritomy were closed with 7-0 vicryl sutures (Ethicon, Somerville, NJ). Following surgery, animals received atropine eyedrops, 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).

Evaluation of gene transfer

Bioluminescence imaging was performed in vitro for both chorioallantoic membrane (CAM) and for freshly enucleated rabbit eyecups 24 hours after gene transfer. The posterior eyecup was prepared and the neural retina was left in place. Drops of luciferin substrate (30 mg/ml) were applied to the tissue. After 10 minutes, bioluminescence was measured with the IVIS 200 (Xenogen, Alameda, CA). After the initial measurement, the neural retina was removed, luciferin was re-applied directly to the RPE, and bioluminescence imaging was repeated.

For in vivo fluorescence imaging, animals were anesthetized and their pupils dilated as described above. Surgical microscope equipped with a 465-495 nm blue excitation filter and 518-557 nm green emission filter was connected to a Retiga 1300 digital camera (QImaging, Burnaby, Canada). Retina was photographed using the Mini Quad VIT contact lens (Volk).

Electroretinograms

Animals were anesthetized and their eyes dilated as described above. Electrodes were made by fixing a silver reference electrode to a standard JET electrode (LKC Technologies Inc., Gaithersburg, MD). ERGs were measured in response to a flash from bilaterally placed Model 1621 full-field Grass stimulators (Grass-Telefactor, West Warwick, RI). Stimulation light intensity was measured as 1.5 cd·s/m^2. Animals were placed in a Faraday cage, and light adaptation measurements were taken after two minutes of full-field exposure to LEDs of 20 cd/m^2 and averaged over 60 flashes at 1 Hz. After 45 minutes of dark adaptation two recordings were taken 45 seconds apart, and the response was averaged over the two recordings. Recordings were filtered and amplified with a SR560 amplifier (Stanford Research Systems, Sunnyvale, CA) and recorded on a TDS 3034 digital oscilloscope (Tektronix Inc., Beaverton, OR). Data was analyzed in MATLAB (Mathworks Inc, Natick MA), where data was smoothed with the spline function and adjusted to agree 7 ms after the stimulus to negate the stimulus artifact. Data was evaluated by comparing the A and B-wave amplitudes for treated and untreated eyes.

Histology

Animals were euthanized by intravenous injection of 85 mg/kg Beuthanasia-D (Schering-Plough, Kenilworth, NJ). Eyeballs were enucleated and histology was prepared as described25. Briefly, eyes were placed immediately into fixative containing 2.5% glutaraldehyde and 1% paraformaldehyde in Sorensons phosphate buffer containing 1.5% sucrose and 1 mM MgSO4, pH 7.4. The tissue was fixed overnight and washed with 0.1 M Sorenson's phosphate buffer containing 1.5% sucrose, dehydrated in a series of methanol and acetone, and embedded in Eponate 12/DMP-30 embedding resin (Ted Pella, Redding, CA). One micron sections were cut with a Reichert OMU-3 and stained with toluidine blue for light microscopy.

Frozen sections were prepared for fluorescence microscopy as described [26]. Tissue was fixed immediately in freshly prepared 4% paraformaldehyde in Sorensen's phosphate buffer, pH 7.4, for 24 hours. The tissue was cryoprotected with 30% sucrose in Sorensen's phosphate buffer, embedded in Optimal Cutting Temperature compound (Sakura Finetechnical Co., Tokyo, Japan), and sectioned with a Leica Ultracut E cryostat. Fluorescence microscopy was performed on frozen sections with an Axioplan 2 fluorescent microscope (Zeiss), an auto-fluorescence reducing GFP filter cube (Chroma), and the AxioVision software (Zeiss).

3. RESULTS

In our previous study [23], were electroporation electrodes have been placed on cornea of both eyes, significant damage was observed in the anterior structures of the eye, such as corneal wounds and cataracts. In our current study electrodes were placed behind the sclera, thus keeping them away from the sensitive anterior structures and closer to the retinal cells that were the targets of DNA transfer. In this design, the electrodes are separated from the

targeted RPE cells only by the sclera and choroid. Photoreceptor cells are at a greater distance over the area of the induced retinal detachment associated with sub-retinal injection of DNA.

Electroporation protocols that have been optimized for other tissues such as muscle [11] have used a train of pulses for DNA delivery, each pulse having a short (μs), high voltage phase followed by a long (ms), low voltage phase. Optimization accounts for many parameters, including electrodes size and spacing, the number of pulses, short pulse duration and amplitude, long pulse duration and amplitude, and polarity of the pulses.

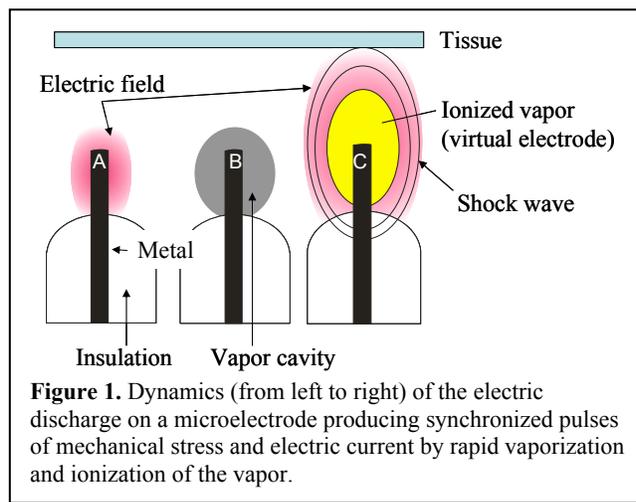### *Optimization of electroporation in chorioallantoic membrane*

Since electroporation protocols vary for different tissues, our initial experiments were aimed at a better understanding and optimization of pulse parameters for RPE transfection. We started optimization with a model tissue, the chorioallantoic membrane (CAM) from the developing chicken egg, which has been used to model retinal tissue for surgical techniques [27,28]. CAM is a live, readily available, and inexpensive tissue. Its epithelial layer is uniform and has high resistance, making it a good model for RPE. In this model system, the shell and shell membrane were first removed from one end of the egg, leaving the CAM intact. 100 μg of pNBL2 plasmid DNA encoding the luciferase gene in 1.1 ml PBS were pipetted onto the CAM. Electrodes were placed in the DNA solution ~ 0.2 mm from the CAM surface and pulses were applied. The tissue was then cultured and assayed for luciferase bioluminescence.

These optimization experiments demonstrated the best DNA transfer with the least amount of damage when multiple pulses were applied, and when a short (μs) high-voltage pulse was followed by a long (ms) low-voltage pulse. Specifically, the optimized parameters were a 250 μs, 150 V phase, followed by a 5 ms, 5 V phase in the same polarity. Optimal results were achieved with 50 cycles applied at 1 Hz. When electrodes were placed in contact with CAM, luciferase expression levels were $~10^6$ photons/s. At distances of 0.2 mm, however, much lower levels of signal (10^4 photons/s) were observed in each treated area. We reasoned that the higher expression with direct electrode contact could be either due to 1) the target cells experiencing a higher electric field due to the closer proximity of the electrodes, or 2) the pressure of the electrodes on the CAM surface causing the CAM to stretch, which contributed to DNA transfer. To test whether tissue stretching affected DNA transfer, we placed a 0.2 mm layer of 1% agarose gel between the CAM and the electrodes. The resistance of the agarose gel was similar to saline and to sclera/choroid. Slight pressure was applied to the electrodes in order to stretch the CAM by ~5%. Electroporation with these settings yielded luciferase expression ~20-fold higher than at the same distance but without pressure.

We next asked whether another source of mechanical stress, ultrasound, could be used in combination with electroporation to enhance DNA transfer. Application of 2.4 W/cm^2 60 kHz ultrasound for 50 seconds, a regime similar to what was used to transfect a muscle cell line [12] led to luciferase signal levels lower than 10^4 photons/s (data not shown). In combination, however, ultrasound and electroporation yielded luciferase expression $> 10^7$ photons/s, ~1000-fold above electroporation alone, suggesting that sources of mechanical stress in combination with high electric field could enhance the efficiency of DNA transfer.

Plasma-mediated transfection in chorioallantoic membrane

When sufficiently high voltage is applied, a mechanical stress wave synchronized with a pulse of electric current can be produced by the electric discharge itself [28]. This occurs through rapid vaporization of conductive medium with a short (microseconds) pulse of current. Fast expansion and collapse of the transient cavitation bubble produces stress and tensile waves in the surrounding medium [29]. When the vapor bubble is formed, however, it disconnects the electrode from the conductive physiological medium, thus terminating the pulse of electric current. Conductivity can be restored by ionizing the vapor cavity, which can be achieved at sufficiently high voltage (Figure 1). This phenomenon has been utilized for tissue fragmentation and dissection [28,30,31]. We hypothesized that a similar regime, where high voltage is used as a source of both the high electric field and mechanical stress, could achieve efficient DNA transfer. This hypothesis was tested by using CAM in a protocol similar to that used for the conventional electroporation experiments. Using a 50 μm wire microelectrode of 1 mm in length, we applied a series of

Figure 1. Dynamics (from left to right) of the electric discharge on a microelectrode producing synchronized pulses of mechanical stress and electric current by rapid vaporization and ionization of the vapor.

symmetric biphasic pulses, each phase being 250 µs in duration 600 V in amplitude. The microelectrode was scanned over a 4 mm x 4 mm area and approximately 50 pulses were applied. The resulting luciferase expression was ~10^9 photons/s, 10,000-fold higher than levels seen with conventional electroporation alone.

Plasma-mediated transfection in the retina

Having observed efficient DNA delivery in a model tissue with electroporation plus ultrasound and with the plasma-mediated electric discharges, we proceeded to test these protocols for transfection of rabbit RPE in vivo. A rabbit received a subretinal injection of ~75 µg pNBL2. Electrodes and an ultrasonic transducer were placed behind the eyeball, and the settings for electroporation plus ultrasound as described above were applied. During application, the retina became opaque in places, indicating areas of severe acute retinal damage. After 24 hours, animals were examined and the posterior eyecups were assayed for luciferase activity. Low levels of luciferase expression were observed over a small area.

For plasma-mediated transfection, a probe with an array of three horizontal microelectrodes of 50 µm in diameter and 1.5 mm in length, surrounded by a return electrode, was constructed. To facilitate alignment of the probe, inserted behind the sclera, to the subretinal bleb containing DNA, the end of the probe was illuminated with an embedded optical fiber. Another design of the probe contained nine microelectrodes of 50 µm in diameter that protruded above the plane of the probe by 100 µm. The face of the probe containing the microelectrode array was 3 mm x 3 mm. The probe was placed behind the sclera, aligned to the site of the bleb, and 5 biphasic pulses of 250 µs/phase in duration and of ± 250 V in amplitude were applied in 5 locations, covering the area of the subretinal bleb. Since the target RPE cells were separated from the electrodes by about 1 mm – the thickness of the sclera and choroid, electric field they experienced was about 2.5 kV/cm. After 24 hours, treated and control eyes were enucleated and the posterior eyecups were prepared leaving the neural retina intact. With both probe designs, abundant luciferase activity (5 x 10^6 photons/s) was observed over the area of subretinal injection. The neural retina was removed, and luciferase activity was measured separately for the neural retina alone and the remaining eyecup. The bulk of the signal was detected in the eyecup containing RPE, suggesting that the measured activity was due to DNA transfer to RPE cells rather than cells of the neural retina. Similar treatment with intravitreal administration of DNA did not yield detectable luciferase activity (data not shown).

To further investigate the retinal cell types that were transfected with the avalanche method, rabbits received pMax plasmid expressing a green fluorescent protein, and were evaluated by in vivo fluorescent fundus photography after 48 hours. Fluorescence was strongly visible in animals treated with the avalanche method (Figure 2), but not when treated with injection alone (data not shown).

Figure 2. Combined fundus photo of a rabbit eye demonstrating expression of GFP gene at the transfection site (pointed by the arrow).
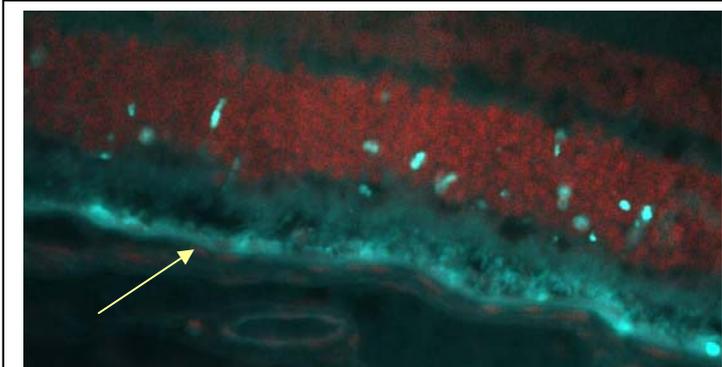

Figure 3. Histology of the rabbit retina demonstrating GFP expression (green) primarily in the RPE (pointed by the arrow). Sample was also stained with Propidium Iodide to visualize location of the cell nuclei in the retina (red).

When pMax was used, the quantity and spatial distribution of fluorescence was variable, with some experiments

resulting in signal that was lower or restricted to a smaller area within the bleb. For localization of signal, frozen sections were made from treated and control eyes and evaluated by fluorescence microscopy. Transgene expression was visible in the RPE cell layer over most of the area under the bleb (Figure 3). In some areas, particularly around the edges of the bleb, fluorescence was also present in the outer nuclear layer of the photoreceptor cells.

Safety of the plasma-mediated transfection

Safety of the plasma-mediated transfection method was assessed in two ways: functionally with electroretinography (ERG) and structurally with histology. The full-field ERG is a pan-retinal measurement, therefore a wide area of the retina should be treated in order to observe any potentially damaging effects. To test sensitivity of the full-field ERG one animal was treated with long pulses that caused coagulation of the retina. 30 spots were administered, covering > 1/3 of the central posterior pole. In this animal, both, the dark-adapted and the light-adapted ERG responses for the treated eye decreased by a factor of 4, to 25% of their original values and in comparison to the untreated eye.

Three animals were then treated with similar placement of 30 spots of the treatment regime, 5 biphasic pulses of 250 µs/phase at ± 250 V. To control for the mechanical effects of the eye manipulations, one animal was sham-treated by performing a similar procedure, but applying no voltage. All animals were followed by weekly ERG recordings for 4 weeks post-operatively. For treated, sham-treated, and untreated animals, ERG responses were found to be within the normal range of variability [32]. After 4 weeks, the eyes were collected for histology, which also showed no difference between the treated and untreated eyes.

4. DISCUSSION

This study describes a new approach for the delivery of plasmid DNA to retinal cells, including RPE and, to a limited extent, photoreceptors. We used an array of microelectrodes placed behind the eyeball to produce a localized electric field in proximity to the cells targeted for transfection, but far from the sensitive anterior structures of the eye. Unlike previous studies which have used electrodes on the corneal surface and electroporation across the head of the animal [23,33], the approach worked well with a large animal model and has potential for clinical translation. Subretinal administration is far more effective in reaching the targeted cell types in the retina than less invasive techniques (e.g., intravitreal injection). We acknowledge, however, that subretinal injection is more invasive and carries more clinical risk than intravitreal injection since a retinal detachment is required. We did not observe any complications associated with placing electrodes on the back of the eye and we believe that these methods would be well tolerated by patients.

Using chorioallantoic membrane as a model tissue, we observed that tensile stress applied by tissue stretching or by ultrasound, dramatically enhanced transfection efficiency. Based on this finding we developed a new transfection method called plasma-mediated transfection, which uses a high electric field to produce synchronized pulses of electric current and mechanical stress. This new method differs from conventional electroporation in several ways. First, it uses microelectrodes rather than large electrodes. Second, the avalanche method relies on ionization of the vapor cavity to deliver the electric field and mechanical stress, whereas arc production is considered detrimental in conventional electroporation. These settings result in much higher electric fields: 1-3 kV/cm in the avalanche method, as opposed to 0.1-1 kV/cm with conventional settings. Third, the regime uses pulse durations of 250 µs per phase, whereas conventional electroporation requires pulse durations of 5-100 milliseconds for efficient DNA transfer [11]. Because the pulse is short and biphasic, little or no muscle movement occurs when pulses are delivered, which is desirable for both precision and patient comfort. Because of these properties, plasma-mediated transfection may be a preferred alternative to conventional electroporation for some applications.

Increase in efficiency of electroporation under tensile stress could occur because the lipid bilayer becomes less stable and thus more susceptible to permeabilization by the electric field. Such a hypothesis is consistent with previous work showing that membrane stretching destabilizes the lipid bilayer and may contribute to pore formation22. It is possible that tensile stress not only reduces the threshold of electroporation, but also increases its safe dynamic range, i.e., the range of settings that cause poration but not irreversible cellular damage. The strong enhancement of permeabilization that was observed with microsecond plasma-mediated electrical discharge warrants additional studies exploring the mechanisms involved.

In the rabbit retina, conventional electroporation plus ultrasound caused severe retinal damage and did not result in efficient DNA delivery. The avalanche method, by contrast, did deliver DNA efficiently, and the signal was localized predominantly to RPE cells. One reason for localized transfection could be that tight junctions between the RPE cells result in high resistance, and thus the voltage drop across the RPE is higher than across other tissue layers. In

addition, cells of the neural retina were further removed from the electrodes by the subretinal bleb itself and therefore experienced lower current densities. This explanation was further supported by the observation that at the edges of the bleb, where neural retina was closer to the electrodes, some photoreceptors were also transfected. Penetration depth of the electric field into the tissue can be controlled by the geometry of the electrode array, and a different geometry may be more optimal for efficient DNA transfer to photoreceptors.

In initial experiments, the plasma-mediated treatment did not cause damage to the retina, as assessed by ERG recordings and histology. We acknowledge, however, that the toxicity profile may be different in the presence of DNA. However, any such toxicity would be localized to the site of the injection, a relatively small area of the retina, and would therefore not be detectable by a full-field ERG. In experiments where DNA was subretinally injected and plasma-mediated transfection was used, animals showed no signs of damage by fundus photography or histology. One experiment that used significantly higher settings for plasma-mediated transfection resulted in visible damage (data not shown). In future studies, it will be important to determine the thresholds of damage in the retina as well as in other tissues of interest in the presence of DNA and with a long follow-up.

One major shortcoming of non-viral DNA delivery is transient expression of the transgene. A number of recent studies, however, have shown that the integrase from bacteriophage φC31 confers genomic integration of plasmid DNA and long-term expression in mammalian cells in a variety of contexts [23,34-40], including RPE cells [23]. Used together, plasma-mediated transfection and φC31 integrase would be a powerful combination for long-term non-viral gene therapy.

Many potential applications exist for plasma-mediated transfection. Although the scope of this study was limited to plasmid DNA, the method is likely to be effective for less bulky molecules as well, such as proteins, mRNA, siRNA, and small molecules. This method is amenable to a variety of cell types. In addition to efficient DNA delivery to CAM and rabbit RPE cells, we have also successfully transfected HEK293 cells in situ and rabbit conjunctival fibroblast explants. Follow-up experiments will test other desirable targets for gene therapy, including skeletal and cardiac muscle, liver, and skin. It is also possible to mount the microelectrode on a catheter and apply it surgically, with local injection of DNA followed by the plasma-mediated transfection. Future experiments in the eye will focus on treatment of animal models of retinitis pigmentosa.

ACKNOWLEDGEMENTS

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