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Ex vivo electroporation of retinal cells: a novel, high efficiency method for functional studies in primary retinal cultures

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Abstract

Primary retinal cultures constitute valuable tools not only for basic research on retinal cell development and physiology, but also for the identification of factors or drugs that promote cell survival and differentiation. In order to take full advantage of the benefits of this system it is imperative to develop efficient and reliable techniques for the manipulation of gene expression. However, achieving appropriate transfection efficiencies in these cultures has remained challenging. The purpose of this work was to develop and optimize a technique that would allow the transfection of chick retinal cells with high efficiency and reproducibility for multiple applications. We developed an ex vivo electroporation method applied to dissociated retinal cell cultures that offers a significant improvement over other currently available transfection techniques, increasing efficiency by five-fold. In this method, eyes were enucleated, devoid of RPE, and electroporated with GFP-encoding plasmids using custom-made electrodes. Electroporated retinas were then dissociated into single cells and plated in low density conditions, to be analyzed after 4 days of incubation. Parameters such as voltage and number of electric pulses, as well as plasmid concentration and developmental stage of the animal were optimized for efficiency. The characteristics of the cultures were assessed by morphology and immunocytochemistry, and cell viability was determined by ethidium homodimer staining. Cell imaging and counting was performed using an automated high-throughput system. This procedure resulted in transfection efficiencies in the order of 22–25 % of cultured cells, encompassing both photoreceptors and non-photoreceptor neurons, and without affecting normal cell survival and differentiation. Finally, the feasibility of the technique for cell-autonomous studies of gene function in a biologically relevant context was tested by carrying out gain and loss-of-function experiments for the transcription factor PAX6. Electroporation of a plasmid construct expressing *PAX6* resulted in a marked upregulation in the expression levels of this protein that could be measured in the whole culture as well as cell-intrinsically. This was accompanied by a significant decrease in the percentage of cells differentiating as photoreceptors among the transfected population. Conversely, electroporation of an RNAi construct targeting *PAX6* resulted in a significant decrease in the levels of this protein, with a concomitant increase in the proportion of photoreceptors. Taken together these results provide strong proof-of-principle of the suitability of this technique for genetic studies in retinal cultures. The combination of the high transfection efficiency obtained by this method with automated high-throughput cell analysis supplies the

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scientific community with a powerful system for performing functional studies in a cell-autonomous manner.

Keywords

electroporation; retina; photoreceptor; in vitro; culture; chick; gain-of-function; loss-of-function

1. Introduction

The in vitro culture of retinal cells enables researchers to study gene function and regulation in a cell-autonomous fashion and with good control of environmental factors, providing an excellent platform to complement or expand on the findings of in vivo studies (Adler, 1993; Seigel, 1999). Moreover, this culture system becomes more practical and cost effective than animal studies for the screening of drugs, soluble factors, siRNA libraries, and others, for either basic research or drug development purposes, and is particularly amenable to high-throughput assays (Vergara and Canto-Soler, 2012).

The use of the embryonic chick retina as a source of cells for culture has certain advantages over other commonly used models such as the mouse. These include the easy access to large amounts of material due to the considerable size of the eyes, and a comparatively large number of cone photoreceptors (86% of all photoreceptors in this animal are cones -Morris, 1970), which makes the chick an excellent system for studies focused on this cell type. The protocol for the culture of dissociated chick retinal cells was developed three decades ago by Ruben Adler and collaborators, and perfected and characterized by his and other groups over the following years (Adler, 2000; Adler and Hatlee, 1989; Adler et al., 1984; Adler et al., 1982; Belecky-Adams et al., 1996; Hyndman and Adler, 1982; Politi and Adler, 1986). These cultures are generally performed using embryonic day (ED) 5–8 retinas, that are dissociated and plated in low density conditions on polyornithine coated dishes. Following age-dependent cell-intrinsic developmental programs, retinal progenitors differentiate as either photoreceptor or non-photoreceptor neurons, with 55–80% of cells differentiating as photoreceptors on ED 5–6, compared with less than 30% on ED8 (Adler, 1993; Adler, 2000; Adler and Hatlee, 1989; Belecky-Adams et al., 1996; Repka and Adler, 1992; and personal observations). Moreover, photoreceptors in these cultures display many of the morphological and physiological characteristics found under in vivo conditions, such as expression of specific genes, response to light and neurotransmitters, and formation of outer segments (Adler, 1993; Saga et al., 1996; Stenkamp et al., 1993). Thus, these cultures provide a powerful experimental paradigm to investigate cell-autonomous mechanisms of photoreceptor cells, as attested by the numerous publications that have applied this system to the study of photoreceptor cell survival (Chalmel et al., 2007; Goureau et al., 1999; Hewitt et al., 1990; Leveillard et al., 2004; Lorentz et al., 2006; Nakamura et al., 2000; Paes-de-Carvalho et al., 2003; Stenkamp et al., 1993), differentiation (Fuhrmann et al., 1995; Toy et al., 2002; Yan et al., 2009), neurite outgrowth (Adler, 1982), and regulation of gene promoters (Boatright et al., 1997a; Boatright et al., 1997b; Kumar et al., 1996) among others.

Unfortunately, the lack of efficient transfection techniques allowing proper manipulation of gene expression has hampered the applicability of this powerful system. In the present article we describe a method that offers the possibility of electroporating embryonic chick retinas for dissociated cell cultures with a five-fold higher efficiency than other currently available protocols. Our optimized experimental conditions result in transgene expression in 22% of the total number of cells in culture, and 25% within the photoreceptor population specifically. Importantly, cell survival and morphological differentiation are not

compromised in the electroporated cultures as compared to unelectroporated controls. Finally, we validated the system for gain and loss-of-function studies of developmentally relevant genes by overexpressing *PAX6* through plasmid electroporation or downregulating its endogenous expression using RNA interference (RNAi). These experiments resulted in the efficient up- or downregulation of *PAX6* protein levels respectively, accompanied by a re-specification of cell fate in electroporated retinal precursors, with a decrease in the proportion of cells differentiating as photoreceptors when *PAX6* was overexpressed, and an increase in this cell type with *PAX6* inhibition. Our results attest to the value of this method as an experimental paradigm for plasmid-based gain and loss-of-function studies in retinal cell cultures.

2. Materials and supplies

2.1. Animals

All procedures were performed in accordance with the animal protocols approved by the Animal Care and Use Committee at the Johns Hopkins University. Fertilized White Leghorn chicken eggs were obtained from B&E Eggs (York Spring, PA, USA). Eggs were incubated at 37.5°C and 60% relative humidity and embryos were staged as in Hamburger and Hamilton (H&H) (Hamburger and Hamilton, 1992).

2.2. Plasmids

Two different GFP expressing plasmids were used to optimize the electroporation parameters with similar results: pEGFP-N1 (GenBank Accession #U55762; Clontech Laboratories Inc., Mountain View, CA, U.S.A.), and pCIG, which is derived from pCAGGS, modified to contain an IRES and nuclear EGFP (Megason and McMahon, 2002). For *PAX6* overexpression experiments, the chicken *PAX6* coding region was amplified according to the sequence from GenBank (NM_205066), and a Kozak consensus sequence and an HA tag were added to the 5' end. This PCR product was subsequently cloned into pCIG plasmid. For RNAi-based downregulation we used plasmids harboring a microRNA-like operon obtained from ARK-Genomics, The Roslin Institute & R(D)SVS, University of Edinburgh, UK. These were: pRFPRNAi-*PAX6A* and pRFPRNAi-*LacZ*, pRFPRNAi-*PAX6B* and pRFPRNAi-*GFP*, used to confirm the specificity of the effect. All of these plasmids contained an RFP gene driven by β -actin promoter, followed by a chick U6 promoter driving the expression of a hairpin miRNA-like operon targeting the gene of interest (for a detailed description see Das et al., 2006).

2.3. Antibodies and reagents

Antibodies used in this study were: NB600-308 anti-GFP (Novus Biologicals, Littleton, CO, USA), anti-PAX6, 7G4 anti-Visinin, and E7 anti- β tubulin (all from NICHD-funded Developmental Studies Hybridoma Bank maintained by The University of Iowa, Iowa City, IA, USA), goat anti-mouse 546 and goat anti-rabbit 488 (Molecular Probes- Life Technologies, Carlsbad, CA, USA), horseradish peroxidase-conjugated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA). Reagents, buffers and medium used in cell cultures included: Calcium-Magnesium free Hank's Balanced Salt Solution (CMF-HBSS), and HBSS (Invitrogen-Life Technologies, Carlsbad, CA, USA), Medium 199 (Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum (Invitrogen), linoleic acid BSA complex (Sigma-Aldrich), glutamine (Thermo Fisher Scientific, Pittsburgh, PA, USA), and penicillin (Invitrogen). Reagents used in histology and cytological staining included: OCT Tissue-Tek (Sakura Finetek, Torrance, CA, USA), Hoechst 33342 and ethidium homodimer (Invitrogen). Reagents used in Western blot comprised: "Complete MiniTM", protease inhibitor (Roche, Indianapolis, IN, USA), Nu-PAGE Novex 4–12% bis-tris gels (Invitrogen), casein (Vector Laboratories), and SuperSignal West Femto

chemoluminescence reagent (Thermo Fisher Scientific). For RNA extraction and real time RT-PCR we used: RNeasy mini kit (Qiagen Inc., Valencia, CA, USA), DNase I, oligo-dT primers and SuperScript III First Strand Synthesis System (Invitrogen), and IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). For plasmid preparation we used an endotoxin-free plasmid Maxi Kit (Qiagen).

2.4. Equipment

Electroporation was performed using an ECM 830 electroporation apparatus (BTX, Holliston, MA, USA), coupled to a gold tipped electrode (catalogue #45-0115, BTX), and a custom-made electrode made using a 2.5 mm square box filament, 4.5 mm wide (Catalogue #FB245B, Sutter Instrument Company, Novato, CA, USA). Tissue sections were obtained using a HM505E Microm cryostat (Microm, Walldorf, Germany). Microscopic examination was performed using a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Thornwood, NY, USA). Automated cell imaging and counting was done in a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific). For Western blot transfer we used the iBlot Dry Blotting System (Invitrogen), and after development membranes were imaged in an Image Station 4000MM (Kodak, Rochester, NY, USA). Real-time PCR was performed using MyIQ Single Color Real-Time Detection System (Bio-Rad Laboratories).

3. Detailed methods

3.1. Ex vivo electroporation

Chick embryos were euthanized by decapitation on embryonic days (ED) 4–6 (H&H Stages 24–29). After initial testing of parameters, the ideal stage to perform this procedure was determined to be stage 27 (ED5; See Results section 4.2). The eyes were enucleated and placed in a Petri dish containing warm (37°C) CMF-HBSS. The retinal pigmented epithelium (RPE) was carefully dissected using fine forceps, and the RPE-devoid eyes were transferred to a custom-made electroporation chamber as follows (Figure 1): The lid of a 1.5 ml Eppendorf tube filled with 120 μ l of plasmid solution was used as a well in order to perform the procedure with the least amount of plasmid solution possible. Unless otherwise noted, plasmid concentration was 1.5 μ g/ μ l in sterile PBS. A thick gold tipped electrode was used as an anode, whereas an electrode made out of a 2.5 mm square box filament, 4.5 mm wide, was bent with forceps into a U shape and used as a cathode. The U-shaped cathode was placed in the plasmid-filled well, and the eyes were deposited in this chamber with the lens facing up. The anode was then placed on the anterior part of the eye, next to the lens, and 5 square pulses of 15 Volts, 50 milliseconds duration, and 950 milliseconds interval were delivered using an ECM 830 electroporation apparatus.

3.2. Dissociated retinal cell culture

RPE-devoid electroporated eyes were immediately placed in warm CMF-HBSS, where the lens, cornea and vitreous were removed. The retina was then transferred to a clean dish with CMF-HBSS and dissected into smaller pieces using tungsten needles. Then they were dissociated by mild trypsinization for 20min in a 37°C water bath, followed by gentle pipetting up and down 20 times with a plugged silicone coated pasteur pipette that was flamed to reduce bore size. The newly separated cells were seeded at low density (6×10^5 cells/well) in medium 199 with penicillin/glutamine, 10% certified fetal bovine serum, and linoleic acid-bovine serum albumin at 100 μ g/ml, on polyornithine-coated 6-well plates or 35-mm dishes. The cultures were then maintained in a humidified 37°C incubator with 5% CO₂. For a detailed protocol see (Vergara and Canto-Soler, 2012).

3.3. Eye cup culture, retinal flat mounts and histological sectioning

Following electroporation, RPE-devoid eye cups were placed in medium 199 supplemented with 5% certified fetal bovine serum, 100 μ g/ml linoleic acid BSA complex, 2 mM glutamine, and 100 U/mL penicillin, and incubated for 24 hours at 37°C and 5% CO₂. At that point, the retinas were flat-mounted and examined using a Zeiss Axioplan 2 fluorescence microscope. For histological sectioning retinas were fixed for 10 minutes in 4% paraformaldehyde, washed in PBS, and incubated in 25% sucrose in phosphate buffer, to be embedded in OCT Tissue-Tek. Ten micrometer thick sections were obtained using an HM505E Microm cryostat, and analyzed under fluorescence microscopy.

3.4. Immunocytochemistry and cytological staining

After 4 days in culture cells were stained with Hoechst 33342 to label nuclei, and with ethidium homodimer for cell death determination, following manufacturer's directions. After a 30 minute incubation at 37°C, plates were imaged for cell counting using a Cellomics ArrayScan VTI HCS Reader. The number of live cells was obtained by subtracting the dead cell number from the total cell count. For immunocytochemical analysis, the dishes were collected at 4 days after seeding and fixed with 4% PFA for 10min, and washed in PBS (3×5min). The cells were then blocked with 10% goat serum and 0.25% Triton-X in PBS for 1hr at room temperature (RT), incubated overnight with a primary antibody (GFP 1:10000, PAX6 1:50 and 7G4 Visinin 1:2000) in 2% goat serum and 0.04% Triton-X in PBS at 4°C. The next day, the dishes were washed in PBS (3×5min) and incubated with an Alexa Fluor-conjugated secondary antibody (goat anti-mouse 546 or goat anti-rabbit 488; 1:1000) in PBS for 1hr in the dark at RT. The dishes were then washed in PBS (3×5min), incubated in DAPI (1:1000 in PBS) for 10min, rinsed in PBS and imaged using a Cellomics ArrayScan VTI HCS Reader.

3.5. Western blot

Culture medium was replaced with lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100) containing "Complete MiniTM" protease inhibitor, and cell lysates were collected in 1.5ml Eppendorf tubes with the aid of a cell scraper, followed by centrifugation at 14,000 g for 15 min at 4°C. Ten micrograms of each protein sample were electrophoresed on Nu-PAGE Novex 4–12% bis-tris gels, and transferred to a nitrocellulose membrane using an iBlot Dry Blotting System according to the manufacturer's instructions. After blocking with casein, membranes were incubated with primary antibodies for 1 hour at room temperature, washed, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution) for 30 minutes. Signal development was done using the SuperSignal West Femto chemoluminescence reagent, and imaged in an Image Station 4000MM. Primary antibodies used were: mouse monoclonal anti-PAX6 (1:200) and E7 anti- β tubulin (1:500).

3.6. Real time PCR

RNA was isolated with RNeasy mini kit according to the animal cells spin protocol. To remove any remaining DNA, samples were digested with DNase I following manufacturer's instructions. First strand synthesis including reverse transcriptase negative control was done using oligo-dT primers and SuperScript III First Strand Synthesis System. Quantitative PCR was performed with IQ SYBR Green Supermix and 0.3 μ M primers in a MyIQ Single Color Real-Time Detection System. PCR cycles were: 3 minutes at 95°C, 40 cycles of 20 seconds at 95°C, 20 seconds at 57°C, and 20 seconds at 72°C, followed by a melt curve analysis. Analysis was done using an R script utilizing the qpcR package (Ritz and Spiess, 2008).

3.7. Data analysis and statistics

Retinal cell cultures were performed pooling together the retinas from 5 different eyes per experimental condition. All experiments were performed 3 independent times (3 biological replicates), and each time 3 to 6 wells of a 6-well plate were analyzed per experimental condition. Eighty fields per well were imaged at 20X magnification using a Cellomics ArrayScan VTI HCS Reader, resulting in the counting of over 3,000 cells per condition, per experiment. Cells were identified by nuclear fluorescent staining, and their fluorescent intensities in the red and green channels were quantified using the Cellomics ArrayScan target activation software (Thermo Fisher Scientific). Data analysis was performed using the R software for statistical computing, and statistical significance was calculated using Student's t-test or one-way ANOVA for independent samples followed by Turkey HSD test. The sample number "n" was considered 3, reflecting the number of independent biological replicates per condition. A p-value <0.05 was considered significant. Results are expressed as "mean \pm standard error of the mean".

4. Results and troubleshooting

4.1. Ex vivo electroporation setup

Primary retinal cultures involve enucleating the embryonic eyes and carefully removing the sclera, RPE, cornea, lens, and vitreous body using microsurgical tools. The neural retina is then cut into small pieces that are dissociated into single cells by trypsinization, and plated at low density in poly-ornithine coated dishes (a detailed protocol has been recently published in Vergara and Canto-Soler, 2012). We reasoned that the cells are most fragile after dissociation, and thus opted for an ex vivo approach - that is, performing the transfection on the enucleated eyes before dissociation. This approach has the additional advantage that it can also be applied to studies on retinal explant cultures.

We performed these experiments on embryos ranging from H&H stage 24 to 29, corresponding to embryonic days 4 to 6. At these stages the retina is mostly composed of proliferating uncommitted progenitor cells. However, as differentiation progresses from ED4 to ED6, a gradually increasing number of progenitors in the posterior region of the retina exit the cell cycle to become the different retinal cell-type precursors (Belecky-Adams et al., 1996; Gutierrez et al., 2011). From these, approximately 80% correspond to ganglion, amacrine, horizontal and bipolar precursors, while only around 20% correspond to photoreceptor precursors (Belecky-Adams et al., 1996). Interestingly, these cell-type precursors seem to maintain a significant level of plasticity, as evidenced by their behavior when dissociated and cultured in vitro. In this scenario, approximately 80% of the differentiated cells are photoreceptors, while the remaining 20% differentiate into all other retinal cells types (Belecky-Adams et al., 1996).

We started by testing various electroporation parameters including electrode type, voltage and number of electric pulses delivered, plasmid concentration and location in relation to the retina and embryonic stage at the time of electroporation. For this purpose we developed a rapid screening assay using a GFP-encoding plasmid, and a semi-quantitative scoring system based on assessment of the proportion of the retinal surface displaying GFP in the electroporated retinas flat-mounted after 24 hours in culture. With the goal of developing a method that would be practical to other researchers, we initially tested cuvette-type electrodes (for their ease of use and commercial availability), injecting the plasmid solution in the vitreal cavity of stage 29 eyes (ED6, one of the most commonly used and best characterized stages for retinal cultures). However, despite varying the electroporation parameters, we were unable to detect appreciable GFP expression under these conditions. Slightly higher GFP expression was obtained when younger eyes (stages 27 and 24) were treated using the same system, but the transfection efficiency was still very low (Table 1).

Due to these unsatisfactory results, we set out to improve the technique by devising our own custom-made electrode system. The best results were obtained by using a combination of a thick gold tipped electrode (the same type used for in vivo electroporation on ED3–4; Vergara and Canto-Soler, 2012), and an electrode made out of a 2.5 mm square box filament, 4.5 mm wide, normally used as a heating filament for pulling capillaries, bent with forceps into a U shape (Figure 1B). In this system the eye is gently deposited inside the well made by the custom-made electrode, with the photoreceptor side of the retina facing the electrode's "walls", and the gold tipped electrode is placed on top of the eye, next to the lens (Figure 1C). We began testing this system in stage 24 eyes (since they had produced the best results in the previous experiments), by injecting the plasmid solution in the vitreous and connecting the custom-made electrode to the positive pole to direct the plasmid towards the retina. Under such conditions we were able to achieve improved levels of transfection (Table 1). However, when the same system was applied to stage 29 eyes the efficiency was much lower. We then decided to modify the system by removing the RPE and bathing the outer surface of the retina in the plasmid solution, reversing the polarity of the electrodes to direct the plasmid towards the inside of the eye. As shown in Table 1, this approach resulted in much higher electroporation efficiencies for stage 29 eyes, especially when using a plasmid concentration no lower than 1.5 $\mu\text{g}/\mu\text{l}$. A surface area equivalent to 3–4 quadrants of the retinal flat-mount displayed GFP expression under these conditions (Table 1, Figure 2A). Transversal sections of these electroporated retinas showed cells expressing high GFP levels spanning the thickness of the retina (Figure 2B). Therefore, this electroporation setting was chosen as the starting point for further optimization of the system. It should be noted that this system requires a large volume of plasmid solution. Thus in order to minimize the amount of plasmid required, it is convenient to place the eye in the smallest size well that will accommodate it. We found that the cap of a 1.5 ml Eppendorf tube served that purpose satisfactorily (it required a volume of 120 μl) and could be easily sterilized (Figure 1A, D). Moreover, several eyes could be electroporated using the same solution (we did not observe loss of efficiency when sequentially electroporating up to 6 eyes with the same plasmid solution).

4.2. Improving transfection efficiency

Since our previous experiments indicated that the developmental stage of the animal seemed to have an important effect on electroporation efficiency, we reasoned that it might prove useful to fine-tune the technique by testing intermediate stages in an attempt to further optimize efficiency. Therefore we proceeded to electroporate eyes corresponding to stage 27, 28 and 29 embryos (ED5, 5.5 and 6 respectively), since primary retinal cultures derived from these stages have been well characterized and there is only a small variation in eye size among them.

Immediately after electroporation the retinas were dissociated into single cells, plated, and maintained in culture for 4 days. This is a common time point for analysis of this type of cultures since it allows enough time for the cells to attach and differentiate but still maintain good survival. Under these conditions we observed many GFP positive cells with both neuronal and photoreceptor characteristics in all stages tested (Figure 2C). We then set out to quantify the transfection efficiency achieved by our protocol, defining efficiency as the percentage of GFP expressing cells over the total cell count. The best results were obtained using stage 27 eyes (ED5), which displayed an efficiency of $22\pm 0.5\%$ over the total cell population (Figure 2D), a five-fold increase over previously available methods. Consistent with our previous assessment, when the same experiment was performed on stage 28 retinas (ED 5.5) the efficiency decreased to $16\pm 1.5\%$, and experiments on stage 29 eyes (ED6) yielded efficiencies of $12\pm 0.4\%$ (Figure 2D). Even though at these stages the protocol still outperforms other transfection methods, these results emphasize the impact of the

developmental stage on the transfection efficiency that can be achieved. In addition, it is noteworthy that when GFP expression was assessed within the photoreceptor population alone, the transfection efficiency increased to $25\pm 0.8\%$ for stage 27 eyes, whereas it reached $20\pm 0.8\%$ and $14\pm 0.5\%$ for stages 28 and 29 respectively, making this model particularly suitable for studies on photoreceptor development and physiology (Figure 2E).

4.3. Characterization of the electroporated retinal cultures

In order to further validate this system we compared the overall characteristics of cultures from retinas electroporated at stage 27 to those of stage 27 unelectroporated controls, at 4 days in culture. The electroporated cultures appeared morphologically normal, with their cells displaying the same differentiation characteristics as the controls, including shape and neurite outgrowth distinctive of the different photoreceptor and neuronal cell types (Figure 3A–C).

Cell death was assessed by ethidium homodimer staining followed by automated fluorescence imaging and cell counting. The results from 3 independent experiments showed no significant differences in cell survival between GFP plasmid electroporated and control unelectroporated cells (Figure 3D). In addition, we performed immunostaining for visinin, a calcium binding protein expressed in photoreceptors (Hatakenaka et al., 1985)(Figure 3C). Quantification of visinin(+) cells revealed similar ratios of photoreceptors to non-photoreceptor neurons in electroporated and unelectroporated cultures, suggesting that the electroporation technique does not bias the differentiation potential of retinal precursors (Figure 3E).

4.4. Application of ex vivo electroporation to functional studies

Next we decided to test the applicability of the method to study the role of developmentally important genes in the retina by performing gain and loss-of-function experiments using *PAX6* as a candidate target. We chose this gene because it is one of the best studied regulators of eye development, and because it has been hypothesized that it can play a role in the cell fate determination of retinal progenitors, biasing their differentiation against a photoreceptor phenotype (Adler and Canto-Soler, 2007; Canto-Soler and Adler, 2006; Marquardt et al., 2001; Oron-Karni et al., 2008; Philips et al., 2005; Toy et al., 2002; and others). Moreover, since *Pax6* expression is restricted to a small subpopulation of cells in the retinal cultures, it provides a particularly suitable scenario to test the power of the system in loss-of-function experiments.

4.4.1. Gain of function of PAX6—In order to overexpress *PAX6* in chick retinal cells we electroporated a *PAX6*-IRES-GFP plasmid on stage 27 eyes, followed by dissociation and culture, and evaluated the results after 4 days in vitro using GFP plasmid electroporated samples as controls. A marked upregulation in *PAX6* protein levels was observed by Western blot in lysates from *PAX6* plasmid electroporated samples compared to controls (Figure 4A). In addition, immunocytochemistry followed by automated high-throughput fluorescence imaging showed a 2.7-fold increase in the percentage of *PAX6*(+) cells in the *PAX6* treated samples compared to GFP controls (Figure 4B). Analysis of the intracellular fluorescence intensity in *PAX6* positive transfected cells revealed that *PAX6* expression levels in individual cells was significantly higher in *PAX6* plasmid treated cultures than in controls (Figure 4C). Moreover, and in agreement with the published literature, co-immunostaining for visinin and GFP revealed a highly significant decrease in the percentage of cells differentiating as photoreceptors in *PAX6* transfected cells: from $56\pm 1.4\%$ of transfected cells co-expressing GFP and visinin in controls to only $16\pm 1.2\%$ in *PAX6* transfected cells, corresponding to a 3.5 fold decrease (Figure 4D). Importantly, the

percentage of photoreceptors among untransfected cells between cultures electroporated with the control and *PAX6* plasmids remained unchanged (Figure 4D).

4.4.2. Loss of function of PAX6—For the purpose of downregulating PAX6 in retinal cell cultures, we employed a plasmid-based RNAi strategy. The plasmid constructs used included a β -actin promoter driven RFP reporter, followed by a chick U6 promoter driving the expression of a hairpin miRNA-like operon designed to specifically downregulate either *PAX6* or *LacZ* as a control (Das et al., 2006). The efficacy of the *PAX6* RNAi construct for decreasing PAX6 levels was not readily observable to significant levels by Western blot (not shown). However, this was not unexpected considering that only a small proportion of the cultured cells normally express this protein at appreciable levels. Nonetheless, a decrease in *PAX6* mRNA was demonstrated by the more sensitive technique of real time PCR (Figure 5A). Moreover, we performed immunocytochemistry followed by automated high-throughput imaging and analysis, in which protein expression levels are measured cell-intrinsically, therefore enabling the researcher to study changes in the global cell population as well as in the sub-population of interest. Using this technique to analyze the transfected cell population, we detected a significant decrease (-1.26 fold) in the percentage of PAX6 positive cells in the retinas electroporated with the *PAX6* RNAi construct compared to control cultures electroporated with *LacZ* RNAi, whereas the percentage of PAX6(+) cells in the untransfected population remained constant in both conditions (Figure 5B). In addition, the levels of PAX6 expression among PAX6(+) cells transfected with the *PAX6* RNAi construct displayed significantly lower values than their *LacZ* RNAi transfected counterparts (49.1% decrease in average fluorescence intensity; Figure 5C).

Importantly, this level of PAX6 downregulation in the retinal cultures had an appreciable and statistically significant biological effect, resulting in an increase in the number of photoreceptors in the transfected population: from $57 \pm 0.4\%$ in control conditions to $66 \pm 1.4\%$ in *PAX6* RNAi treated cultures (a 1.16 fold increase; figure 5D). Meanwhile, the percentage of photoreceptors in the untransfected population did not change between these treatment groups (Figure 5D). It is worth mentioning that the treatment did not seem to have an effect on cell survival, as assessed by quantification of calcein incorporation (data not shown), and therefore the observed effect is likely to be due to a re-specification of cell fate in retinal progenitors, in agreement with one of the proposed roles of PAX6 in these cells (Oron-Karni et al., 2008; Toy et al., 2002). Finally, similar results were obtained using a second RNAi construct for *PAX6* and a *GFP*-RNAi construct for control (data not shown), confirming the reproducibility and specificity of the treatment.

Taken together, these results not only support previous findings on the role of PAX6 in retinal fate specification, but also validate the potential of the ex vivo electroporation method to study specific aspects of retinal cell biology through gain and loss-of-function approaches.

5. Discussion

5.1. Comparison of ex vivo electroporation to other transfection methods

The continued applicability of primary retinal cell cultures for basic science or industrial purposes necessitates the ability to transfect these cells with an acceptable level of efficiency. Until now, the techniques most commonly used to manipulate gene expression in these cultures included calcium phosphate-mediated and lipid-mediated transfection, which are performed on the dissociated cells just before plating or on cells that are already attached to the dish. Both techniques, however, present several important disadvantages including variability in efficiency between experiments, considerable toxicity and low transfection efficiencies, most commonly in the order of 3–5% (Boatright et al., 1997b; Dudek et al.,

2001; Kumar et al., 1996; Toy et al., 2000; Werner et al., 1990; Yuan et al., 2005). On the other hand, electroporation lacks the toxic effects common to chemical transfection methods and has been used to manipulate gene expression in a variety of cell types (Martinez and Hollenbeck, 2003). However primary neuronal cells, including retinal cells, consistently show low transfection efficiencies (Martinez and Hollenbeck, 2003). One way in which this problem has been circumvented, in the case for example of promoter analysis, has been by the use of plasmid constructs with an enzymatic reporter system, based on colorimetric or chemoluminescence detection, performed on culture supernatants or cell lysates (Boatright et al., 1997a; Boatright et al., 1997b; Kumar et al., 1996; Werner et al., 1990; and others). This is a useful approach in specific situations, since such detection methods amplify the signal so that small differences between treatments can be detected to an extent that allows for statistical analysis. However the results are based on an effect exhibited by only a small number of cells, the method does not discriminate the cell types that are responsible for the effect, and it is not applicable to every circumstance.

Likewise, techniques that are employed with great success in the chick embryo *in vivo* can be inadequate or inefficient when applied to dissociated retinal culture systems. For example, the study of retinal development has significantly benefited from the use of RCAS retroviral vectors. These versatile vectors are very efficient at infecting proliferating cells and thus have been successfully used for gene overexpression and downregulation in the developing retina *in vivo* and in cultured explants, where retinal progenitors actively proliferate (Gutierrez et al., 2011; Harpavat and Cepko, 2006; Ishii et al., 2004; Sato et al., 2002; Vergara and Canto-Soler, 2012; Yang, 2002; and others). However these viruses are not able to infect non-proliferating cells efficiently, and since dissociated retinal cells in culture lose their proliferating capacity, this characteristic makes them poor vehicles for genetic misexpression in such culture systems (Hughes, 2004). A different approach is to transfect the retinas *in vivo* before proceeding with the culture. The most common methods to do so include viral vector infection (such as RCAS) and plasmid electroporation. However for technical reasons both of these procedures are typically performed at early developmental stages (Vergara and Canto-Soler, 2012). This implies a considerable time lag (usually a few days) between genetic misexpression and culture, and therefore, though valuable, these techniques are not suitable for dissecting cell-autonomous from non-cell-autonomous effects of the treatment.

The unsatisfactory performance or technical hurdles associated with these methods prompted us to develop and optimize a technique that would increase the efficiency of transgene expression in chick retinal cell cultures over that of other currently available protocols, allowing us to take full advantage of the potential of this system. In the current protocol, enucleated eyes were devoid of RPE, an plasmid electroporation was carried out using custom-made electrodes, followed by retinal tissue dissociation and culture. This procedure resulted in transfection efficiencies in the order of 22–25 % of cultured cells, encompassing both photoreceptors and non-photoreceptor neurons, and without affecting normal cell survival and differentiation. We found that certain parameters such as the developmental stage of the animal had a significant influence in the transfection efficiencies that could be achieved, with younger embryos resulting in higher efficiency. Therefore we concluded that H&H stage 27 embryos offer a good balance of size and performance, with the advantage of being a stage that is commonly used and has been well characterized for this type of studies. In addition, for best performance we advise using plasmid concentrations no lower than 1.5 $\mu\text{g}/\mu\text{l}$.

5.2. Potential of the *ex vivo* electroporation technique for the study of gene function

Our laboratory is particularly interested in transcription factors important for cell fate determination during early development, and especially in the specification of photoreceptor

versus non-photoreceptor fates. Thus, a method like the one described here is extremely valuable to us and others studying related topics, since at the time the culture is initiated the retina consists of a large proportion of uncommitted progenitors that differentiate in vitro according to their intrinsic program and external factors, allowing the investigator great control of experimental conditions and a flexibility for imaging and analysis that is difficult to obtain in vivo (Adler, 2000, and references therein).

We tested the feasibility of this ex vivo electroporation technique for cell-autonomous studies of gene function in a biologically relevant context by performing up- and down-regulation studies for *PAX6*, since this is one of the best characterized regulators of eye development in many species including the chick (Adler and Canto-Soler, 2007; Canto-Soler and Adler, 2006; Marquardt et al., 2001; Philips et al., 2005; and others).

By electroporating a *PAX6* expressing plasmid we demonstrated that we are able to obtain a marked upregulation in the expression levels of this protein that could be measured in the whole culture as well as cell-intrinsically, providing proof of principle of the effectiveness of the technique. Moreover, this upregulation was accompanied by a decrease in the number of transfected cells adopting a photoreceptor fate as assessed by visinin expression, providing some support to the hypothesis that *PAX6* plays a role in non-photoreceptor cell fate specification.

However, even though overexpression studies provide important information on the potential role or application of the molecule under investigation, loss-of-function approaches can offer better insight into the physiological functions of an endogenous gene product. On the other hand, when compared to overexpression experiments, loss-of-function studies present an additional challenge: only the sub-population of cells that normally expresses the target gene will be capable of responding to the treatment. This may prove an important limitation when methods sensitive enough to detect small differences in the parameters under study are not available. Therefore in order to test the power of our system for loss-of-function studies we decided to apply ex vivo electroporation to the downregulation of endogenous gene expression under the most adverse experimental conditions.

To this end, and after considering various possibilities, we concluded that *PAX6* would again provide a good candidate gene for these analyses, since it is expressed in only 30–35% of the total cell population in the retinal cultures. In addition, our preliminary studies showed that we are able to transfect a control plasmid in approximately 25% of *PAX6* positive cells using our protocol. This means that the population of cells under scrutiny (those in which endogenous gene expression is downregulated by the treatment) constitutes about 8% of the total cell count in the cultures. It is easy to imagine numerous situations in which researchers are faced with this kind of scenario when designing experiments to test gene function in retinal cultures. In such cases, the use of traditional transfection protocols that deliver efficiencies in the order of 5% implies attempting to measure an effect that is manifested in only 1.5 % of the total cell population. In these situations, a potentially important outcome of the downregulation of the endogenous gene could be masked or deemed as non significant due to a dilution effect. This is particularly likely to occur when using techniques that measure parameters extracted from the culture as a whole, and in some cases the number of affected cells may not even be high enough to deliver meaningful results even when changes are assessed cell-intrinsically. However, by using the ex vivo electroporation protocol to transfect an RNAi plasmid construct targeting *PAX6* we have been able to successfully downregulate endogenous *PAX6* levels in cultured retinal cells. Furthermore, despite the modest downregulation of *PAX6* levels achieved under these conditions, this treatment was able to induce a biologically and statistically significant effect in progenitor cell fate specification, increasing the proportion of cells that acquire a

photoreceptor phenotype. These results demonstrate the power of the system for discriminating the biological effect of genetic downregulation with highly satisfactory levels of significance.

6. Conclusions

Primary cell cultures provide a unique system to study retinal cell development and physiology for basic research or industrial applications. In this context, the availability of efficient and reliable transfection techniques is critical for the analysis of the genetic mechanisms underlying those processes. Previously available transfection protocols have provided useful information in numerous studies; however, their low efficiency diminishes their value for many experimental paradigms. The new *ex vivo* electroporation procedure presented in this article yields transfection efficiencies five-fold higher than currently available methods, and when used in conjunction with automated high-throughput cell analysis it results in a powerful system for functional studies in a cell-specific manner. The combination of this approach with *in vivo* studies offers great potential for advancing the field of retinal research.

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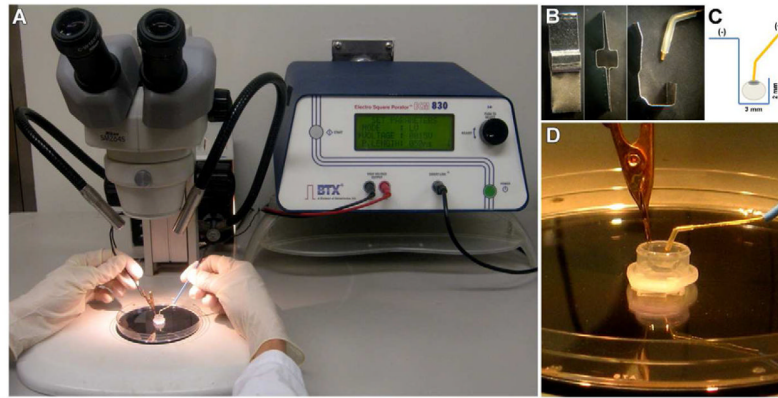
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Highlights

- We describe a high-efficiency electroporation method for chick retinal cell cultures.
- It yields 5-fold higher transfection efficiencies than currently available protocols.
- It can be coupled to high-throughput, single cell analysis.
- Its features provide high-power detection for cell-autonomous gene function studies.

**Figure 1. Electroporation setup**

(A) Electroporation equipment. (B) From left to right: square box filament used to make electrode, front and side view, and magnified image of the two electrodes used. (C) Diagram of electrodes setup. (D) Close-up view of electroporation chamber.

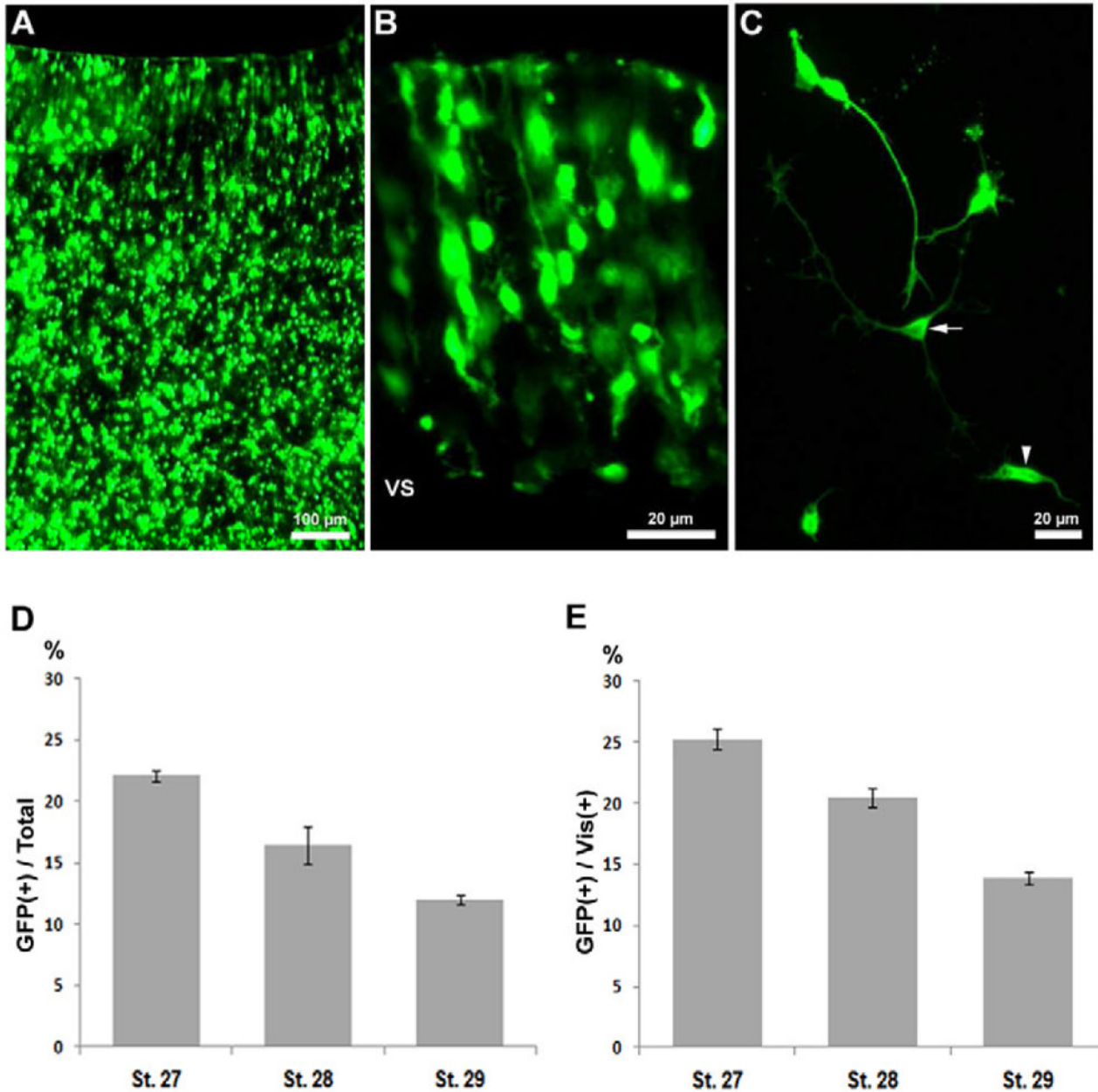


Figure 2. Electroporation efficiency

(A) Fluorescent micrograph of flat mounted retina of a H&H st. 29 embryo electroporated with GFP expressing plasmid and cultured for 24 hours. (B) Transverse section of electroporated retina shown in (A). (C) Fluorescence image of electroporated retinal cells after 4 days in dissociated culture. GFP expression is observed in cells displaying both neuronal (arrow) and photoreceptor (arrowhead) morphology. (D) Efficiency of electroporation at H&H stages 27, 28 and 29, assessed after 4 days in dissociated culture and expressed as percentage of GFP expressing cells over the total cell population. (E) Efficiency of electroporation of photoreceptor cells, expressed as percentage of GFP expressing cells within the visinin (Vis) positive population, at H&H stages 27, 28 and 29. In (D) and (E), more than 3,000 cells per condition were counted within each experiment,

and experiments were repeated 3 independent times (n=3). Error bars in (D) and (E) represent S.E.M. Abbreviation: VS: vitreal surface.

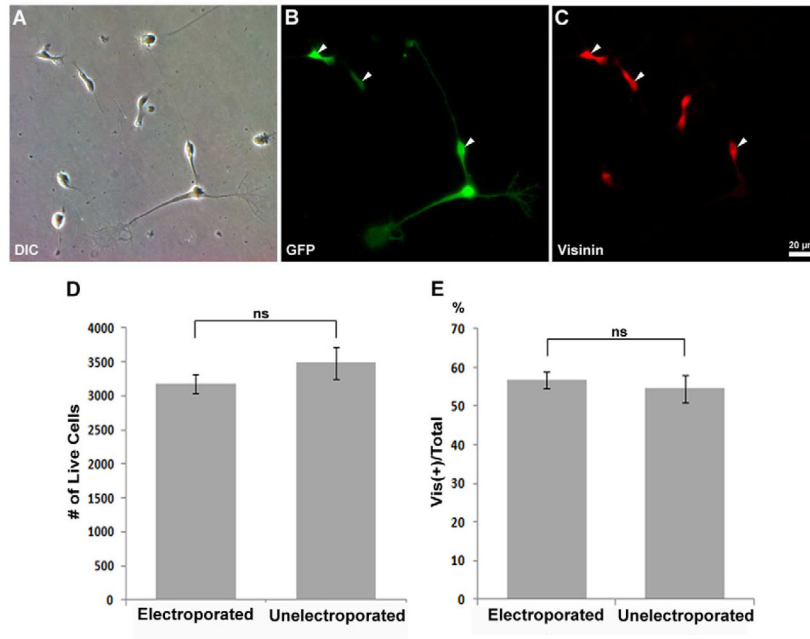


Figure 3. Electroporation does not affect the characteristics of the retinal cultures

(A–C) Representative image of cultured retinal cells from H&H st. 27 embryos, incubated for 4 days after electroporation with GFP expressing plasmid. (A) DIC image of the field; (B) GFP expressing cells; (C) Cells immunolabeled with anti-visinin antibody are shown in red. Arrowheads point at cells in which GFP and visinin expression are co-localized. Scale bar in (C) applies to (A–C). (D) No statistically significant difference in the number of live cells was observed between GFP plasmid electroporated and unelectroporated cells in culture ($p > 0.05$). (E) The percentage of photoreceptors expressed as visinin (Vis) positive cells within the total cell population displayed similar results for both GFP-plasmid electroporated cells and unelectroporated controls. No statistically significant difference was observed between these two groups ($p > 0.05$). In (D) and (E), more than 3,000 cells per condition were counted within each experiment, and experiments were repeated 3 independent times ($n=3$). Error bars in (D) and (E) represent S.E.M.

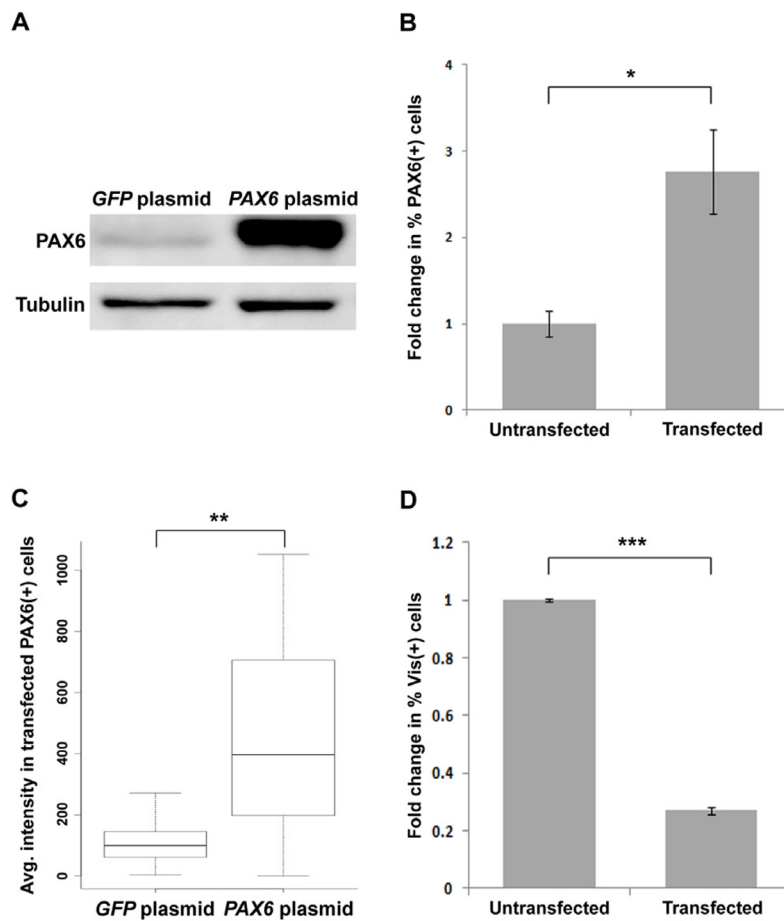


Figure 4. Application of the electroporation technique to the upregulation of PAX6 in retinal cells

(A–C) Ex vivo electroporation of *PAX6*-expressing plasmid efficiently upregulates PAX6 protein levels in cultured retinal cells, as assessed after 4 days in vitro. (A) Western blot showing the effect of *PAX6* or *GFP* (control) plasmid electroporation on PAX6 protein levels in cell culture lysates. (B) PAX6 upregulation was also observed cell-intrinsically by immunofluorescence with anti-PAX6 antibody followed by automated measurement of nuclear fluorescence intensity. Results are displayed as fold change in the percentage of PAX6(+) cells between *PAX6* and control *GFP* electroporated cultures. No change was observed between these two treatments in the percentage of PAX6(+) cells in the population that remained untransfected, whereas in transfected cells this percentage increased 2.7 fold under *PAX6* plasmid transfection conditions compared to control (*, $p < 0.05$). (C) PAX6(+) transfected cells also exhibited significantly higher intracellular expression levels of this protein when transfected with *PAX6* plasmid versus control, as evidenced by an increase in nuclear fluorescence intensity after immunolabeling with anti-PAX6 antibody (**, $p < 0.01$). (D) Effect of *PAX6* plasmid electroporation on photoreceptor differentiation, as determined by the fold change in visinin (Vis) positive cells between *PAX6* and control electroporated cultures. No change was observed between these two treatments in the cell population that remained untransfected, whereas in transfected cells the percentage of Vis(+) cells decreased 3.5 fold in *PAX6* plasmid transfection conditions compared to control (***, $p < 0.001$). The total number of electroporated cells was similar among experimental conditions. In (B–D), more than 3,000 cells per condition were counted within each experiment, and experiments

were repeated 3 independent times (n=3). All experiments were performed in H&H stage 27 embryos. Error bars represent S.E.M.

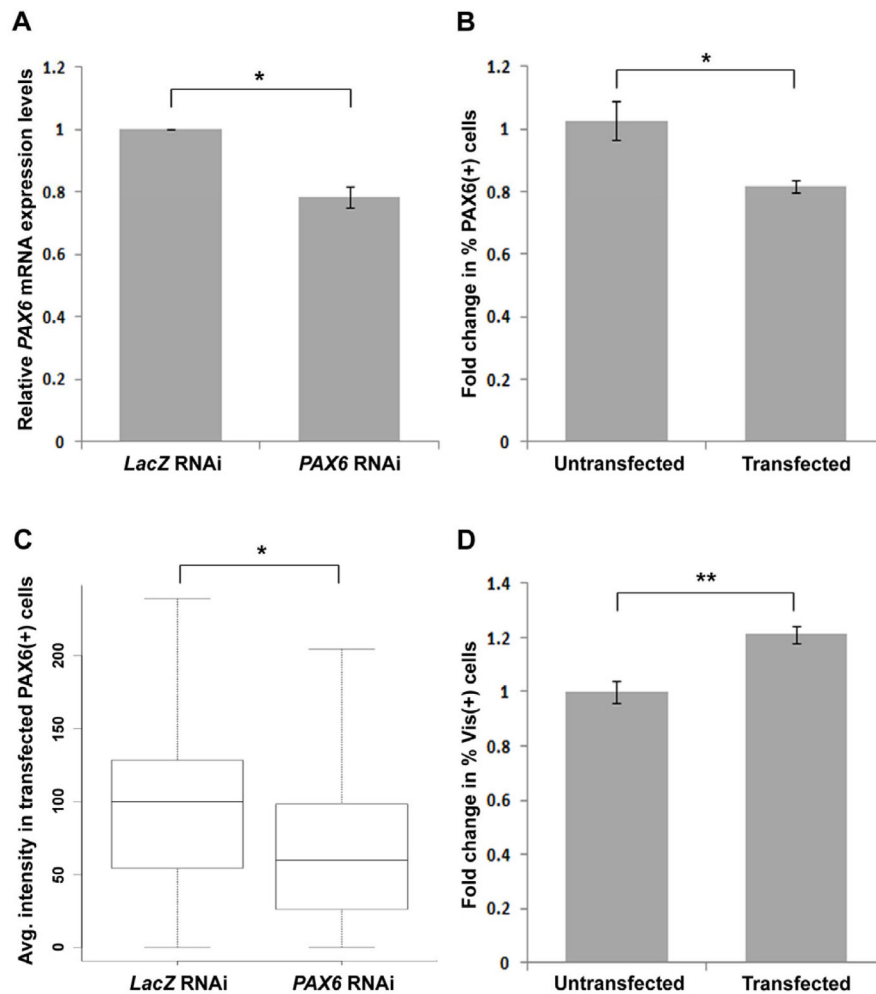


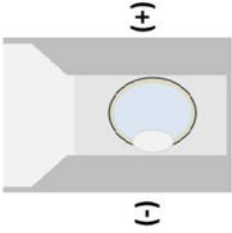
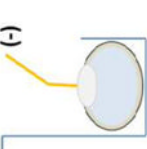
Figure 5. Ex vivo electroporation can be successfully applied to the downregulation of endogenous PAX6 levels in retinal cells

(A) Real time PCR analysis of cell culture lysates after 4 days in vitro showed a significant decrease in *PAX6* mRNA levels in retinal cells electroporated with *PAX6* RNAi plasmid compared to *LacZ* RNAi electroporated controls (*, $p < 0.05$). (B) Immunofluorescent analysis with anti-PAX6 antibody displayed as fold change in the percentage of PAX6(+) cells between *PAX6* RNAi and control *LacZ* RNAi electroporated cultures. No change was observed between these two treatments in the untransfected population, whereas in transfected cells the percentage of PAX6(+) cells decreased 1.26 fold with *PAX6* RNAi plasmid compared to *LacZ* control plasmid (*, $p < 0.05$). (C) PAX6(+) transfected cells displayed significantly lower expression levels of this protein when transfected with *PAX6* RNAi plasmid versus *LacZ* RNAi control, as indicated by the decrease in nuclear fluorescence intensity after immunolabeling with anti-PAX6 antibody (*, $p < 0.05$). (D) Effect of *PAX6* RNAi treatment on photoreceptor differentiation, as determined by the fold change in visinin (Vis) positive cells between *PAX6* RNAi and control *LacZ* RNAi electroporated cultures. The percentage of Vis(+) cells in the untransfected population remained the same between these two treatments, whereas in transfected cells this percentage increased 1.16 fold in *PAX6* RNAi plasmid transfection conditions compared to control (**, $p < 0.01$). The total number of electroporated cells was similar among experimental conditions. In (A–D), more than 3,000 cells per condition were counted within

each experiment, and experiments were repeated 3 independent times (n=3). All experiments were performed in H&H stage 27 embryos. Error bars represent S.E.M.

Table 1

Screening of experimental parameters for retina electroporation.

Electrode type	Plasmid Delivery §	Embryonic stage (H&H)	Parameters		Results †
			Plasmid conc.	Volts and pulses	
Cuvette *	Injected in vitreous (eye intact) 	St. 29	1.5 µg/µl	15V, 3 pulses	(-)
				20V, 3 pulses	(-)
				20V, 5 pulses	(-)
Custom electrodes	Injected in vitreous (eye intact) 	St. 29	1.5 µg/µl	20V, 5 pulses	(+/-)
		St. 24	1.5 µg/µl	15V, 3 pulses	(++)
				18V, 3 pulses	(++)
				20V, 5 pulses	(++)
		St. 29	0.5 µg/µl	15V, 3 pulses	(+)
				15V, 5 pulses	(+)
15V, 5 pulses	(+++)				
	1.5 µg/µl		20V, 5 pulses	(+++)	

* Due to the difference in eye size, 4mm-gap cuvettes were used for stage 27–29 eyes, whereas 2mm-gap cuvettes were used for stage 24 eyes.

§ A GFP expressing plasmid was used for electroporation.

† Results were scored on flat-mounted retinas observed under fluorescence microscopy after one day of incubation. Scoring scale: (-) non or very few fluorescent cells observed; (+/-) fluorescent cells cover an area smaller than one retinal quadrant; (+) fluorescent cells cover an area > 1 but < 2 quadrants; (++) fluorescent cells cover an area > 2 but < 3 quadrants; (+++) fluorescent cells cover an area > 3 quadrants.