Noninvasive Electrical Stimulation Improves Photoreceptor Survival and Retinal Function in Mice with Inherited Photoreceptor Degeneration

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Purpose. Neurons carry electrical signals and communicate via electrical activities. The therapeutic potential of electrical stimulation (ES) for the nervous system, including the retina, through improvement of cell survival and function has been noted. Here we investigated the neuroprotective and regenerative potential of ES in a mouse model of inherited retinal degeneration.

Methods. Rhodopsin-deficient (*Rho*$^{-/-}$) mice received one or two sessions of transpalpebral ES or sham treatments for 7 consecutive days. Intraperitoneal injection of 5-ethynyl-2′-deoxyuridine was used to label proliferating cells. Weekly electroretinograms were performed to monitor retinal function. Retinal morphology, photoreceptor survival, and regeneration were evaluated in vivo using immunohistochemistry and genetic fate-mapping techniques. Müller cell (MC) cultures were employed to further define the optimal conditions of ES application.

Results. Noninvasive transpalpebral ES in *Rho*$^{-/-}$ mice improved photoreceptor survival and electroretinography function in vivo. ES also triggered residential retinal progenitor-like cells such as MCs to reenter the cell cycle, possibly producing new photoreceptors, as shown by immunohistochemistry and genetic fate-mapping techniques. ES directly stimulated cell proliferation and the expression of progenitor cell markers in MC cultures, at least partially through bFGF signaling.

Conclusions. Our study showed that transpalpebral ES improved photoreceptor survival and retinal function and induced the proliferation, probably photoreceptor regeneration, of MCs; this occurs via stimulation of the bFGF pathways. These results suggest the exciting possibility of applying noninvasive ES as a versatile tool for preventing photoreceptor loss and mobilizing endogenous progenitors for reversing vision loss in patients with photoreceptor degeneration.

Keywords: electrical stimulation, retina, retinitis pigmentosa, photoreceptor degeneration, Müller cells

Retinitis pigmentosa (RP) and age-related macular degeneration (AMD) have the common end result of photoreceptor death and are leading causes of irreversible blindness. Their treatment remains a critical unmet medical need. Although exciting progress has been made in gene and stem cell therapies for RP or AMD,[1,2] treatments have been limited to specific patient populations, and the procedures are invasive, with potential tumor growth or retinal damage.[3] As neurons communicate via electrical signals, electrical stimulation (ES) of varying current forms, strength, frequency,

duration, or intervals has been shown to induce neural plasticity and protection of the damaged nervous system, including that of the retina.[4,5] Noninvasive ES is emerging as a promising and versatile tool for modulating neuronal activities and functional rehabilitation.

There is increasing evidence that ES improves vision in the diseased retina. The therapeutic potential of ES for photoreceptor degeneration was initially recognized when implantation of a subretinal microchip in the peripheral retina elicited visual improvement from areas far from the implant site.[6] Subsequently, noninvasive ES was shown to prevent photoreceptor degeneration in Royal College of Surgeons (RCS) rats and preserve electroretinograms in rabbits carrying a rhodopsin mutation.[7,8] The protective effects of minimal or noninvasive ES have since been reported in patients with AMD, RP, and retinal artery occlusion.[6,9–13,14] Importantly, no significant safety-related adverse effects were observed in any of these studies; however, due to the lack of standardization of ES parameters in clinical trials and animal studies, the effects of ES have often been inconsistent or even controversial.[15]

To date, it is not yet known why or how ES improves vision. There is evidence that ES stimulates Müller cell (MC) release of neurotropic factors. These include brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF-1), which exert neuroprotective effects on photoreceptors and retinal neurons.[16–20] The fact that ES reversed vision loss in some patients[6] raises the question of whether or not ES can also promote retinal neuron self-repair under optimal conditions. MCs are a source of retinal progenitors with the potential of replenishing damaged retinal neurons.[21] This prompted us to ask if ES, under optimal conditions, promotes MC release of neurotrophic factors and also triggers the progenitor-like or neurogenic potential of MCs. Here we investigated the effects of ES in vitro and in vivo with mice carrying rhodopsin deficiency (*Rho*$^{-/-}$), which display progressive photoreceptor degeneration that mimics RP in humans.[22] We showed that noninvasive ES improves photoreceptor survival and retinal functions in *Rho*$^{-/-}$ mice and promotes MC reprogramming and proliferation. Together, our results suggest a practical non-pharmacological approach for preventing photoreceptor degeneration in RP or other photoreceptor degenerative diseases.

Materials and Methods

Animals

Rhodopsin knockout (*Rho*$^{-/-}$) mice (n = 12) were originally generated by Peter Humphries, Trinity College, Dublin.[23] Mice carrying a conditional tdTomato (tdT) reporter gene (*ROSA26*)[24] driven under an inducible CreERT promoter (*Sox2*^{ERT}*-ROSA26*) were generated by crossing *ROSA26* mice with mice carrying CreERT knocked-into the Sox2 gene (*Sox2-Cre*^{ERT}).[25,26] Both *ROSA26* and *Sox2-Cre*^{ERT} mice were bought from Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Schepens Eye Research Institute and followed the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were kept in a 12-hour light/dark cycle with free access to food and water.

Noninvasive ES In Vivo

Mice were anesthetized with isoflurane. Conducting electrode gel (Spectral 360; Parker Laboratories, Fairfield, NJ, USA) was applied to the upper and lower eyelids of the mice to allow good electrical contact with the skin. Mice were stimulated by a TheraMac ES device (Acuity Medical, Annapolis, MD, USA), which was selected for the study because of an earlier report implicating potential benefit of the device in human patients with AMD.[10,13,14] To further explore the protective effect of noninvasive ES on mouse photoreceptors, the portable electrode probe of this ES device was placed on four spots of the skin around the mouse orbit—two on the upper eyelid and two on the lower eyelid, for 40 seconds per spot. ES was generated as a series of positive monophasic rectangular pulse trains, followed by a series of negative monophasic rectangular pulse trains, both at increasing frequencies from 2 pulls per second (PPS) to 200 PPS (Fig. 1B); the current was set to 100 µA throughout the stimulation. A ground electrode was placed at the mouse abdomen. To minimize variations between the left and right eyes, mice were randomly assigned to groups that received ES in either the left or right eye. The contralateral eye received no treatment or a sham procedure, in which the probe was placed on the four spots of the eyelid for 40 seconds each without evoking ES. Data recording and analysis were carried out in a masked fashion.

Electroretinography

As the rods of *Rho*$^{-/-}$ mice are dysfunctional, only photopic electroretinograms (ERGs) were recorded once a week, starting 1 day before the first ES. To this end, mice were dark-adapted overnight and anesthetized with an intraperitoneal (IP) injection of ketamine (120 mg/kg)/xylazine (20 mg/kg). Pupils were dilated with 0.5% tropicamide. Mice were then placed on a 37°C warming pad in a Ganzfeld bowl (Diagnosys LLC, Lowell, MA, USA) throughout the recording. Electroretinographs of both eyes were recorded with two contact electrodes being placed centrally on the corneas. The electrodes were lubricated with GenTeal gel (Novartis, Basel, Switzerland). A ground electrode and a reference electrode were inserted subcutaneously at the base of the tail and forehead, respectively.

Retinal Histology and Immunohistochemistry

As previously described,[27] mouse eyeballs were fixed in 4% paraformaldehyde (PFA) at 4°C overnight and cryoprotected in 20% sucrose solution, followed by embedding in optimal cutting temperature compound (Sakura, Torrance, CA, USA). Frozen retinal sections (10 µm) were collected and incubated in a blocking buffer (1% BSA, 0.3% Triton X-100 [Sigma-Aldrich, St. Louis, MO, USA] in PBS) for 1 hour at room temperature, followed by incubation with primary antibodies at 4°C overnight. After three rinses with PBS, the sections were incubated with a secondary antibody at room temperature for 1 to 2 hours. Primary antibodies used included rabbit anti-Ki67 (1:100; Abcam, Cambridge, UK), mouse anti-bFGF receptor (1:200; Millipore, Burlington, MA, USA), rabbit anti-recoverin (1:1000; Millipore), mouse anti-cellular retinaldehyde-binding protein (anti-CRALBP; Abcam), rabbit anti-B-opsin (1:250; Millipore), rabbit anti-R/G-opsin (1:250; Millipore), rabbit anti-glutamine synthetase (GS) (1:500; BD Biosciences, San Jose, CA, USA), and mouse anti-vimentin

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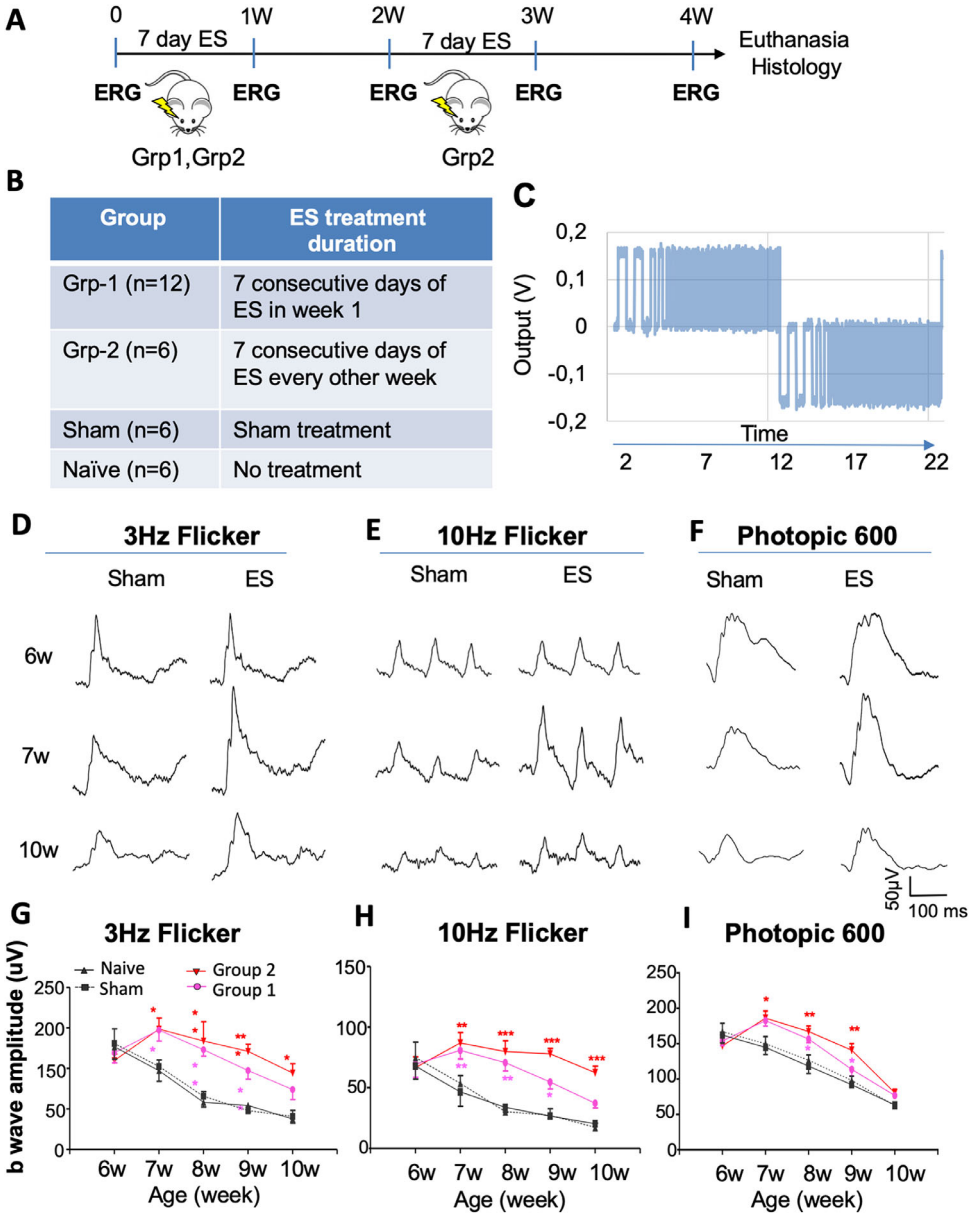

Figure 1. Electrical stimulation (ES) improves ERG responses in *Rbo*$^{-/-}$ mice. (**A**) Illustration representing the experimental paradigm of ES profile. Grp 1 is the group of mice that received 7-day ES treatment in week 1; Grp 2, the group of mice that received 7-day ES treatment every other week (in weeks 1 and 3). (**B**) Table indicates numbers of animals tested per group. (**C**) Electric waveforms generated by the ES device. (**D–F**) Representative 3-Hz flicker (**D**), 10-Hz flicker (**E**), and photopic 600 (**F**) ERG waveforms taken from 6-, 7-, and 10-week-old sham- or ES-treated *Rbo*$^{-/-}$ mice. (**G–I**) The b-wave amplitudes of photopic 3-Hz (**G**) and 10-Hz (**H**) flickers and photopic 600 (**I**) ERG recordings taken from naïve, sham-, or ES-treated *Rbo*$^{-/-}$ mice. NS, non-significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by 2-way ANOVA.

(1:1000; Sigma-Aldrich). All secondary antibodies (Alexa Fluor 488 anti-rabbit, Alexa Fluor 488 anti-mouse, Cy2 and Cy3 anti-rabbit/mouse) were from Jackson ImmunoResearch Laboratories and were used at 1:400 dilution. Sections were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Abcam).

Primary MC Cultures

Retinas from mouse pups aged postnatal days 5 to 6 (P5–6) were dissected and dissociated with papain (Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 10 minutes. A papain inhibitor terminated the reaction; the retina was further dissociated by gently triturating up and down with a pipette. Dissociated cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 containing 10% fetal bovine serum (FBS) and 1× penicillin/streptomycin (P/S; Gibco; Thermo Fisher Scientific, Waltham, MA, USA). A highly enriched MC culture was obtained after 14 days of incubation as previously demonstrated.[28] All MCs were passaged one time as an important step in the purification process.

ES Treatment in MC Cultures

Primary MCs were plated on a circular cover glass, which was then placed inside a custom-made chamber (~1.0 ml volume) that was continuously perfused at 4 ml/min with

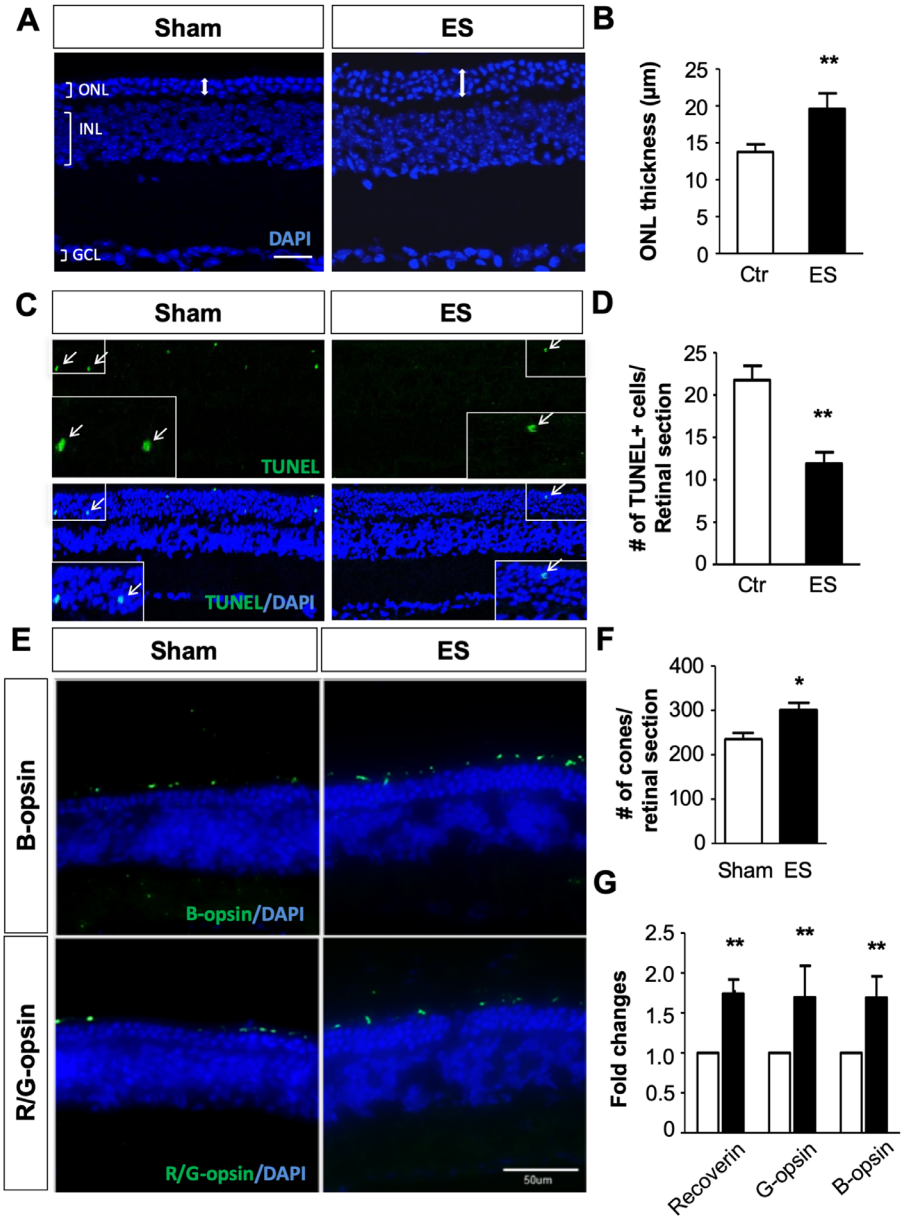

Figure 2. Electrical stimulation (ES) prevents photoreceptor degeneration in *Rho*$^{-/-}$ mice. (**A**, **B**) Epifluorescence photomicrographs of retinal sections of 10-week-old *Rho*$^{-/-}$ mice that were stained with the nuclear marker DAPI (blue; **A**) and quantification of ONL thickness (**B**). *Rho*$^{-/-}$ mice received two sessions of 7 consecutive day of ES or sham treatment at 6 and 8 weeks of age and were killed at 10 weeks of age. Note the thicker ONL (white line) in ES-treated (black bar) retinal sections compared to sham-treated contralateral eyes (white bar). (**C**, **D**) Representative epifluorescence micrographs of TUNEL-stained retinal sections (green; arrowheads), which were counterstained with DAPI (blue) (**C**), and quantification of TUNEL+ cells per retinal section (**D**). Arrows indicate TUNEL+ cells. Note the increasing number of TUNEL+ cells in the sham-treated retinal sections (white bar) compared to ES-treated retina (black bar). (**E**, **F**) Representative epifluorescence photomicrographs of retinal sections immunolabeled for cone-specific markers B-opsin and G-opsin and counterstained with nuclear marker DAPI (blue; **E**), and quantification of cone photoreceptors (B-opsin+ and G-opsin+ cells; **F**) in retinal sections of sham-treated (white bar) and ES-treated (black bar) mice. (**G**) Results of qPCR detecting the levels of expression of rod and cone photoreceptor-specific genes, including recoverin, G-opsin, and B-opsin. Sham treatment, white bar; ES group, black bar. $^{*}P < 0.05$, $^{**}P < 0.01$ by 2-tailed *t*-test. GCL, ganglion cell layer. INL, inner nuclear layer. ONL, outer nuclear layer. *Scale bars*: 20 μm and 50 μm.

Ames medium (pH 7.4) at 36°C and equilibrated with 95% O_2 and 5% CO_2. The ES consisted of 1-ms cathodal (depolarizing) pulses delivered through a 10-kΩ platinum/iridium stimulating electrode (Microprobes, Gaithersburg, MD, USA) centered 25 μm above the glass slide. Identical charge-balancing anodic pulses were delivered without inter-pulse delay after the cathodal pulse to prevent charge build-up in the cells or electrode. Two silver chloride–coated silver wires served as the return; each was positioned ~8 mm from the center of the slide and ~6 mm from the other. In one set of experiments, pulse amplitudes ranged from 50 to 200 μA, and the pulse delivery frequency was fixed at 10 PPS. In a separate experiment, the pulse delivery frequency was 10 to 50 PPS, and the pulse amplitude was fixed at 100 μA. Pulse stimuli were presented for 30 minutes for each parameter set tested. The pulse stimuli were controlled by Multi

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Channel Systems STG2004 hardware and software (Reutlingen, Germany).

Evaluation of MC Differentiation In Vitro

As previously described,[28] MCs were incubated at 37°C in neurobasal medium (Invitrogen; Thermo Fisher Scientific) containing 1% FBS (Sigma-Adrich), 1% P/S (Gibco), 1% B27 supplement (Invitrogen), 0.1% ascorbic acid (200-mM; Sigma-Aldrich), and 1% GlutaMAX 100X (Invitrogen). The differentiation medium was changed every 2 days. After 7 to 21 days of incubation, cells were fixed in 4% PFA for 10 minutes on ice. After washing with PBS, cells were incubated overnight at 4°C in blocking medium containing primary antibodies. The primary antibodies used were mouse anti-βIII-tubulin (1:500; Millipore) and rabbit anti-recoverin antibodies (1:1000; Millipore). The secondary antibodies used were Cy3 anti-mouse (1:500) or DyLight 488 anti-rabbit antibodies (1:500) (both from Jackson ImmunoResearch Laboratories) for 1 hour at room temperature.

Evaluation of MC Proliferation In Vitro

MC proliferation was tracked as previously described.[28] Briefly, MCs were incubated with DMEM/F-12 medium (Gibco) containing 10% FBS (Sigma-Aldrich) and 1× P/S (Gibco). Two microliters of 5-bromo-2′-deoxyuridine (BrdU; 50 µM) were added to 598 µl culture medium following ES or sham treatment. After 24-hour incubation, cells were fixed in 4% PFA for 10 minutes on ice. After washing with PBS, cells were permeablized by incubation with PBS containing 2-N HCl and 0.3% Triton X-100 at room temperature for 30 minutes. Cells were rinsed with PBS again and incubated at 4°C overnight in blocking buffer containing rat anti-BrdU antibody (1:500; Abcam). Following a PBS rinse, cells were incubated for 1 hour at room temperature with the secondary anti-rat Cy3 antibody (1:1000 diluted in blocking buffer). The cells were mounted with mounting medium containing DAPI (Abcam).

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed to identify apoptotic cells in the retinal sections and MC cultures using an In Situ Cell Death Detection Kit according to the manufacturer's protocol (POD; Roche Diagnostics GmbH, Basel, Switzerland).

Detection of MC Proliferation In Vivo

5-Ethynyl-2′-deoxyuridine (EdU) was injected IP daily (10 µg/g) for up to 4 weeks or until mice were killed. Their eyeballs were collected and prepared for retinal histology and immunohistochemistry for in vivo quantification. Frozen retinal sections were prepared as described above and stained for EdU using Click-iT EdU and EdU Imaging Kits (Molecular Probes, Eugene, OR, USA) following the manufacturer's instructions. Three or more non-consecutive retina sections containing optic nerves were quantified randomly. Entire retina sections were quantified under microscope, in a blinded fashion. As the number of EdU/recoverin double-positive cells were few, whole eyeball with 47 or 48 retina sections in a total of three or more mice per group were quantified. Only bright EdU+ signals colocalized with the nuclei within the outer nuclear layer which expressed recoverin were quantified as positive. The result was shown as per 10 retinal sections within the chart presented.

Induction of Retinal Detachments in Mice

Adult *Sox2^{ERT}-ROSA26* mice were given daily IP injections of 2 mg of tamoxifen per 20 g mice for 4 days to induce tdT expression in MCs. Retinal detachment was then induced in *Sox2-Cre^{ERT}ROSA26* mice under anesthesia with ketamine (120 mg/kg)/xylazine (20 mg/kg), IP, as previously described.[27,29] Pupils were dilated with 0.5% tropicamide. A sclerotomy was generated 1.5 mm lateral from the limbus with a 20-gauge needle, and 1 to 2 µl of 14-mg/ml sodium hyaluronate (Healon GC; Johnson & Johnson Vision, Jacksonville, FL, USA) was injected subretinally using a 25-gauge needle syringe to generate retinal detachment in one quadrant of the eye. ES was given to the retinal detached eye daily for 7 consecutive days as described above, and mice were killed 14 days after the first ES.

Results

Transpalpebral ES Improved Retinal Function in *Rho$^{-/-}$* Mice

To investigate if noninvasive transpalpebral ES provides functional benefits with regard to inherited photoreceptor degeneration, we studied *Rho$^{-/-}$* mice, a mouse model of human RP, which display progressive degeneration of photoreceptors over a 3-month period. ES was applied to one eye of *Rho$^{-/-}$* mice, beginning when the mice reached 6 weeks of age, as their retina has developed a stable ERG but the cone degeneration still has not been detected.[22] ES was given daily for 7 consecutive days for 1 week or every other week to evaluate for extended benefit (Figs. 1A–1C). The contralateral eye received sham stimulation was served as a control. ERG was performed weekly to evaluate the retinal function. No significant differences were noted in baseline ERG a-wave and b-wave amplitudes between the sham- and ES-treated eyes before the ES treatment. All *Rho$^{-/-}$* mice showed an absence of scotopic ERG response because of the rhodopsin deficiency. Remarkably, when compared to the sham-treated contralateral controls at 1 week after the first ES treatment, treated eyes showed significantly improved retinal function as demonstrated by 3-Hz and 10-Hz flickers and photopic 600 ERG (Figs. 1D–1F). Marked increases in b-wave amplitudes of photopic Pho600, 3-Hz, and 10-Hz flicker (a typical indicator of cone function) were detected in ES-treated eyes of 7-, 8-, and 9-week-old *Rho$^{-/-}$* mice (or 1, 2, and 3 weeks after the first ES), respectively, as compared to the sham-treated contralateral eyes. The b-wave amplitudes of mice that received sham or no treatment were not significantly different. The effect of the 7-day ES treatment was temporary, however. By 4 weeks after the first ES, no significant improvement was detected between the ES and sham groups (Figs. 1G–1I). We next administered ES for 7 consecutive days every other week (when mice reached 6 and 8 weeks of age). Adding an additional session of ES prolonged the benefit; 3-Hz and 10-Hz flicker b-wave amplitudes were significantly higher in the eyes of 10-week-old (4 weeks after the first ES treatment) *Rho$^{-/-}$* mice that received

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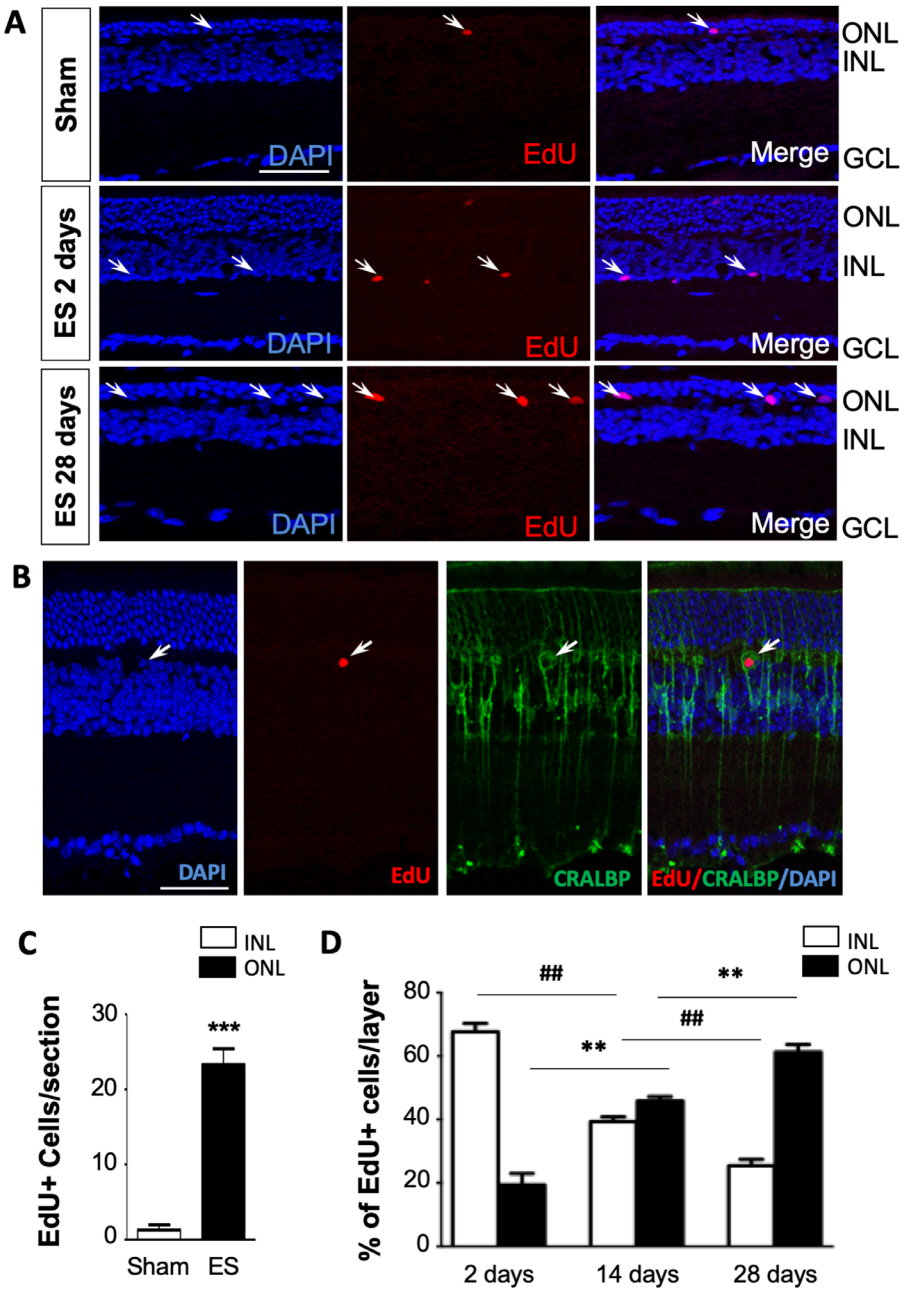

Figure 3. Electrical stimulation (ES) supports MC proliferation and migration toward the ONL. (**A**) Epifluorescence photomicrographs of retinal sections immunolabeled for EdU (red; arrowheads) and counterstained with the nuclear marker DAPI (blue). Note the increased number of EdU+ cells in the ES-treated retina compared to the sham group, especially in the ONL from 2 to 28 days after the first ES treatment in the $Rho^{-/-}$ mice (n = 6/group). (**B**) Retinal sections taken from $Rho^{-/-}$ mice 2 days after the first ES showing the colocalization of EdU immunolabeling (red) and the MC marker CRALBP (green). Arrowheads indicate EdU+/CRALBP+ cells. The section was counterstained with the nuclear marker DAPI (blue). (**C**) Quantification of EdU+/CRALBP+ cells per retinal section at 2 days after the first ES (n = 6/group). $^{***}P < 0.001$ by *t*-test. (**D**) Quantification of EdU+ cells in the INL and ONL. $^{\#\#}P < 0.005$ compared with INL cell counts on day 2 (n = 5 group); $^{**}P < 0.005$ compared to ONL cell counts on day 2 by *t*-test. *Scale bars*: 50 µm.

two sessions of ES than in the sham-treated contralateral eye (Figs. 1G–1I). Thus, transpalpebral ES temporarily but effectively improves retinal function in $Rho^{-/-}$ mice, and additional sessions of ES can prolong this effect.

ES Prevented Photoreceptor Loss and Preserved the Retinal Outer Nuclear Layer

To determine if ES improved retinal function by preserving photoreceptors, we examined retinal histology after mice were killed at 4 weeks after the first ES. Quantification of outer nuclear layer (ONL) thickness showed significantly increased ONL thickness in the ES-treated retinas compared to the sham-treated contralateral eyes, indicating improved photoreceptor cell survival (Figs. 2A, 2B). In agreement with the observation, TUNEL assay detected significantly fewer TUNEL+ apoptotic photoreceptors in ES-treated retinas than in the sham group (Figs. 2C, 2D). Moreover, immunolabeling of cone-specific markers—blue cone opsin (B-opsin) and green cone opsin (G-opsin)—in retinal sections revealed

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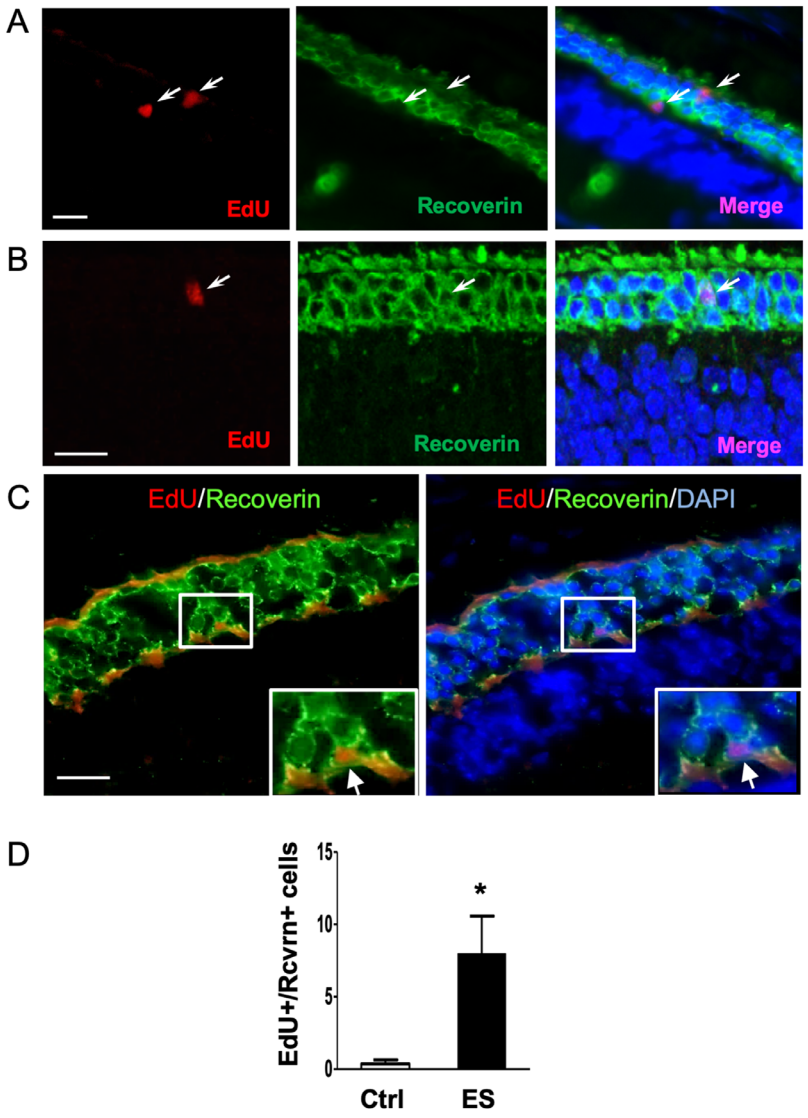

Figure 4. Differentiation of EdU+ cells into photoreceptor cells. Epifluorescence photomicrographs showing retinal sections of *Rho*$^{-/-}$ mice at 10 days (**A-C**) and 4 weeks (**B, C**) after the first ES. Retinal sections were double immunolabeled for EdU (red) and the photoreceptor marker recoverin (green) and counterstained with the nuclear marker DAPI (blue). Note that the EdU+ cells found in the ONL were positive for recoverin taken from retinal sections at 4 weeks but not 10 days after the first ES (arrowhead). Scale bar: 10 µm. (D) Counts of EdU+/Recoverin+ cells and data are presented as number of EdU+/Recoverin+ cells per 10 retinal sections counted; value = means ± S.E.M. $^{*}P < 0.05$.

significantly increased numbers of cones in ES-treated retina compared to sham-treated controls (Figs. 2E, 2F). Supporting the above finding, assessment by quantitative PCR (qPCR) for photoreceptor gene expression also demonstrated significantly higher levels of expression of photoreceptor genes, including recoverin, G-opsin, and B-opsin, in ES-treated retinas compared to sham-treated eyes (Fig. 2G). These data suggest that transpalpebral ES promotes photoreceptor survival in *Rho*$^{-/-}$ mice.

ES Stimulated the Repair Potential of MCs in *Rho*$^{-/-}$ Mice

Evidence suggests that ES reverses vision loss in some human patients with terminal-stage RP, which prompted us to ask whether noninvasive ES can also activate the endogenous regenerative potential of MCs, a source of residential progenitor cells of the retina.[28] To label proliferating retinal progenitors, 6-week-old *Rho*$^{-/-}$ mice received daily IP injections of the cell proliferation marker EdU while they were given ES or sham treatment. Two days after the first ES, the mice were killed and their retinal sections were double immunolabeled for EdU and various retinal cell markers to determine the cell origin of EdU+ cells (Fig. 3A). EdU+ cells were seen to colocalize with MC marker CRALBP (Fig. 3B), but not with other retinal cell markers, such as recoverin (rod photoreceptor), protein kinase C alpha (bipolar cells), Iba1 (microglia), and RECA1 (endothelial cells) (data not shown). In consistent with their colabeling with a MC marker, EdU+ cells were first noted in the inner nuclear layer (INL), where MC nuclei normally reside. An over 20-fold increase in the number of EdU+/CRALBP+ cells was observed in the ES-treated retinas over that of sham-treated retinas (Fig. 3C). In the next series of experiments, we examined EdU+ cell

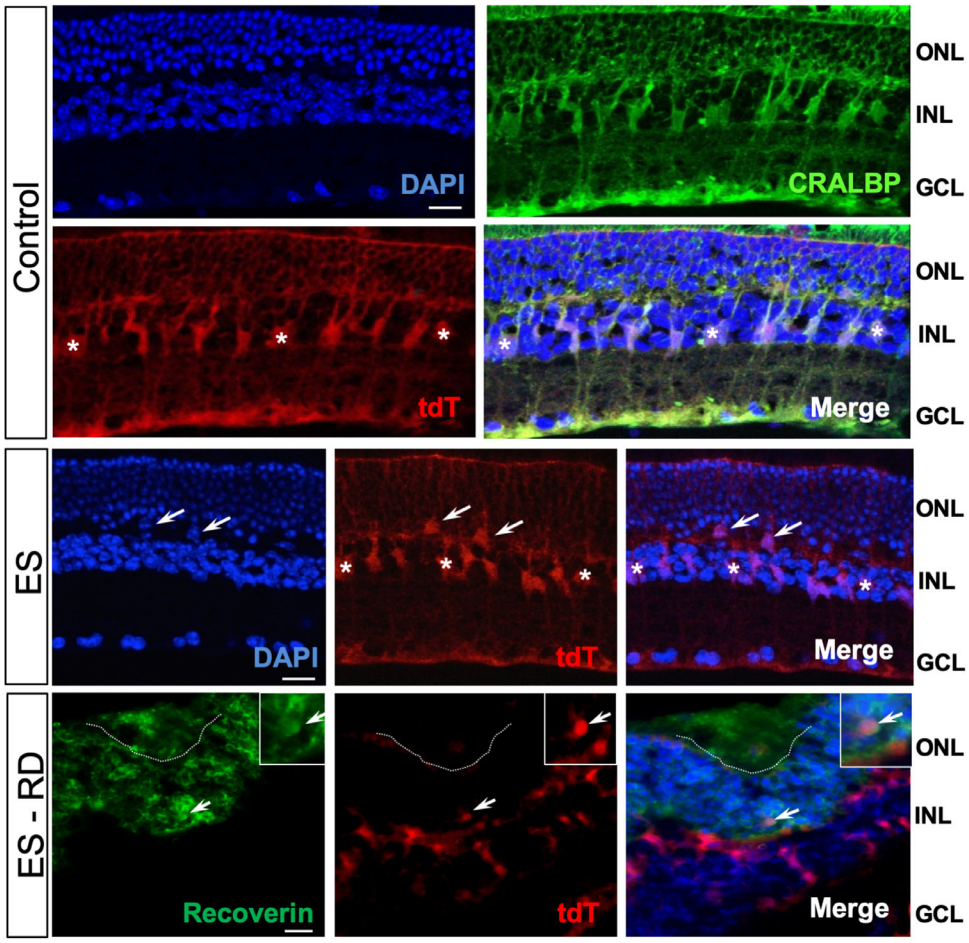

Figure 5. Electrical stimulation (ES)-induced MC migration and transdifferentiation in *Sox2^{ERT}-ROSA26* mice. (**A**) Epifluorescence photomicrographs of retinal sections from naïve *Sox2^{ERT}-ROSA26* mice immunolabeled for the MC marker CRALBP (green) and counterstained with the nuclear marker DAPI (blue). Note that tdT colocalized with CRALBP immunolabeling, and MC nuclei are visible as a well-aligned single band in the INL. *Scale bar*: 10 µm. (**B**) Epifluorescence photomicrographs of retinal sections from *Sox2^{ERT}-ROSA26* mice 3 days after the first ES showing tdT+ cells and MCs migrated out of the INL to the ONL (arrowheads). *Scale bar*: 10 µm. (**C**) Epifluorescence photomicrographs of retinal sections taken from detached retina (DR) of *Sox2^{ERT}-ROSA26* mice 2 weeks after ES, immunolabeled for photoreceptor marker recoverin (green) and counterstained with nuclear marker DAPI (blue). Note the tdT+ cells in the ONL colocalized with recoverin immunolabeling (arrows), indicating MC transdifferentiation into a photoreceptor. Quantification was not performed. *Scale bar*: 20 µm.

migration in the retina by giving *Rbo$^{-/-}$* mice 7 consecutive days of ES and daily IP injections of EdU beginning from the first ES or sham treatment until the mice were killed. On day 2 after the first ES, nearly 70% of EdU+ cell nuclei were localized to the INL, where MC bodies reside, and less than 20% had migrated to the ONL. In contrast, by day 28, over 60% of EdU+ cells had migrated to the ONL and less than 30% of EdU+ cell nuclei were seen in the INL (Fig. 3D). On day 10 after the first ES, EdU+ cells that had migrated to the ONL did not express the photoreceptor cell marker recoverin (Fig. 4A). By 4 weeks after the first ES, EdU+ cells became recoverin+ (Figs. 4B, 4C), suggesting the transdifferentiation of proliferating MCs into photoreceptors. Cell quantification at 3 weeks after stimulation revealed an over 10-fold increase, albeit a small number in total, of EdU+/recoverin+ cells per eye in ES-treated retinas compared to the contralateral control eyes (Fig. 4D).

ES-induced MC migration toward the ONL and transdifferentiation into photoreceptor cells were further corroborated using a ROSA26 fate mapping system in mice. To this end, *ROSA26* reporter mice were crossed with *Sox2-Cre^{ERT}* mice, which drive the expression of Cre recombinase specifically in MCs of the retina. This generated *Sox2^{ERT}-ROSA26* mice, in which *ROSA26* reporter gene expression can be induced permanently in all MCs and their progeny cells following IP tamoxifen injection. As expected, 4 consecutive days of tamoxifen (IP) induced tdT expression in all MCs of *Rox2^{ERT}-ROSA26* mice, in which the cell bodies localized to the INL, appearing as a single band (Fig. 5A). Among them, 92.1 ± 4.2% tdT+ cells were colocalized with MC marker CRALBP immunolabeling. Three days after ES, many tdT+ cells were seen shifting or migrating toward the ONL (Fig. 5B). In a normal retinal environment, we did not detect any tdT+ cells that expressed photoreceptor markers, suggesting that injury signal is required for MC transdifferentiation into photoreceptors. Thus, we induced photoreceptor injury in *Sox2^{ERT}-ROSA26* mice (n = 3) using a retinal detachment model; mice were then treated with 7 consecutive days of ES. By day 14 after the first ES, tdT+/recoverin+ cells could be detected in retinal sections of two out of three mice that received ES, but none from the three sham-treated eyes. Even in ES-treated eyes, tdT+/recoverin+

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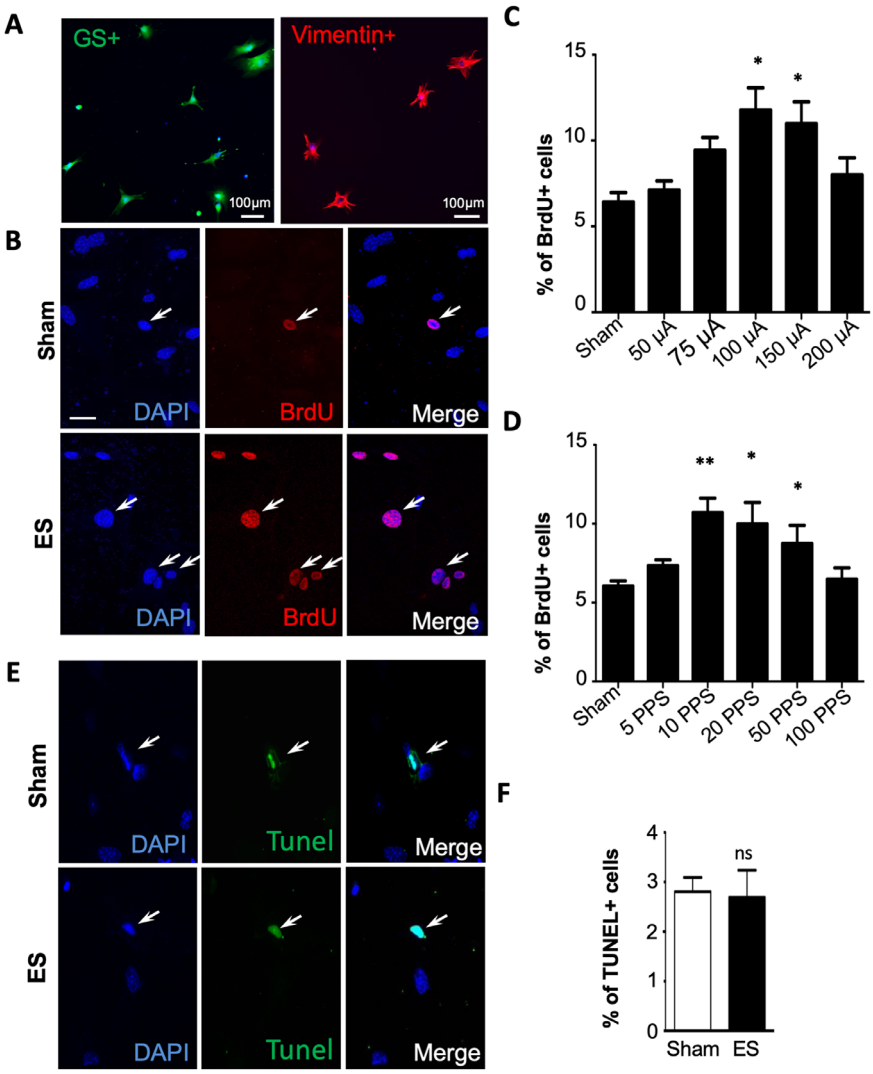

Figure 6. Electrical stimulation (ES) induces cell proliferation in isolated MC cultures. (**A**) Epifluorescence photomicrographs showing cultured MCs immunolabeled for MC specific markers, GS (green) and vimentin (red). (**B–D**) Representative photomicrographs (**B**) and quantification (**C**, **D**) of BrdU+ cells in BrdU pulse assays in ES- or sham-treated MC cultures. Isolated MCs were stimulated with sham and ES of various current amplitudes (**C**) or frequencies (**D**) and cultured for 48 hours before being immunolabeled for BrdU (red; arrowheads) and counterstained with DAPI (blue). (**E, F**) Photomicrographs (**E**) and quantification (**F**) of TUNEL+ cells in ES (100 µA, 20 Hz) or sham-treated MC cultures (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$. ns, non-significant difference compared to the sham group by paired *t*-test. *Scale bar*:10 µm.

cells were so scarce that only four tdT+/recoverin+ cells were detected at the injury site (Fig. 5C). These results suggest that upon noninvasive ES, MCs can be inducted to proliferate and migrate toward the ONL, whereas in rare cases they are found to colocalize with the photoreceptor marker recoverin.

ES Promoted MC Proliferation and Neuron Transdifferentiation in Culture

To test if ES acts directly on MCs to promote cell proliferation and stimulate the generation of new neurons, we isolated MCs in culture (Fig. 6A) as previously described[28] and subjected the cells to ES of various current intensities (50–200 µA) and frequencies (5–100 PPS). As previously demonstrated, our procedure yielded 95.5 ± 1.7% MCs as determined by immunolabeling for the MC markers GS and vimentin; no neuron- or photoreceptor-like cells were observed. BrdU incorporation assay (Sigma-Aldrich) was used to quantify for cell proliferation, and TUNEL was applied for detecting apoptotic cells. We found that 100- to 150-µA and 10- to 50-PPS ES induced significant increases in BrdU incorporation compared to sham treatment (Figs. 6B–6D). TUNEL was carried out in MC cultures treated by various ES parameters, and no apparent differences in TUNEL+ cells were noted at all conditions. Counts of TUNEL+ under the sham treatment or optimal ES conditions were provided (Figs. 6E, 6F), suggesting that ES does not induce MC apoptosis. To further determine if ES directly activates the progenitor cell properties of MCs and stimulates the transdifferentiation into photoreceptor cells, qPCR was applied to check for expression of progenitor cell and mature neuron markers in MC cultures at various time

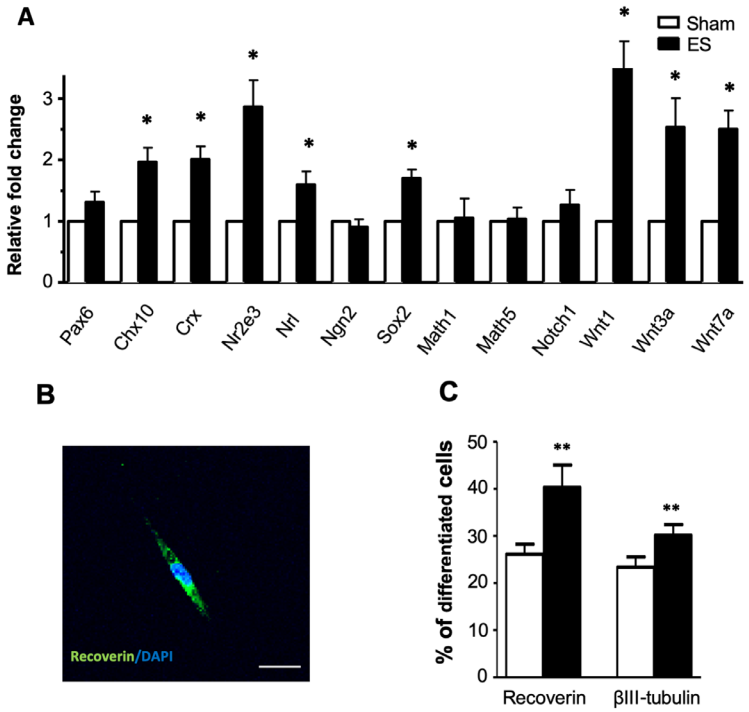

Figure 7. Electrical stimulation (ES) promotes MC expression of progenitor cell markers and photoreceptor cell fate. (**A**) qPCR results showing increased expression of neurogenic factors (*Chx10, Sox2, Wnt*) and photoreceptor progenitor cell markers (*Crx*, *Nr2e3*, *Nrl*) in ES-treated MCs as compared to the sham group (n = 4/group). (**B**) Representative photomicrograph of a cultured MC expressing the photoreceptor marker recoverin at 2 weeks after ES and incubation. (C) Percentage of ES-treated MCs expressing recoverin or the neuron marker βIII-tubulin after 2 weeks of incubation as compared to sham-treated MC cultures (n = 5/group). $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the sham group by paired *t*-test. *Scale bar*: 10 µm.

points after ES. Two days following ES, MCs exhibited significantly increased expression of the neurogenic signals *Sox2, Wnt1, Wnt3a*, and *Wnt7a* and photoreceptor progenitor cell markers *Chx10, Crx, Nr2e3*, and *Nrl* compared to sham-treated MC cultures (Fig. 7A). Two weeks after ES, some MCs could be seen to develop typical photoreceptor morphology and expressed mature photoreceptor-specific marker recoverin as shown by immunohistochemistry (Fig. 7B). Results of immunohistochemistry showed significantly increased numbers of cells expressing the photoreceptor and retinal neuron markers recoverin and βIII-tubulin in ES-treated cultures compared to the sham-treated group (Fig. 7C). Thus, ES directly stimulates MCs to promote their progenitor cell potential and photoreceptor progeny.

ES Induces bFGF Signaling

We proposed that ES induced retinal production of neurogenic stimulating agents and trophic factors to promote MC proliferation and neurogenic potential. To test this, we quantified the retinal expression of candidate neurogenic growth factors after ES using qPCR. These included seven mitogens that have been reported to promote MC or neural stem cell proliferation.[30–34] We found that the mRNA level of bFGF was significantly upregulated in ES-treated retinas compared to the sham-treated group (Fig. 8A). Moreover, MC cultures treated with bFGF showed significantly enhanced BrdU incorporation compared to the sham group. In contrast, administration of bFGF antibody abolished ES-induced enhancement of BrdU incorporation (Fig. 8B). Together, these data support that ES induced retinal production of bFGF to contribute to the enhanced proliferative and neruogenic potential of MCs.

Discussion

Here we reported that transpalpebral ES promoted photoreceptor survival and function. The study provides evidence that might demonstrate a regenerative potential of noninvasive ES in the neural retina by inducing bFGF release and triggering MC proliferation and progenitor cell properties. Moreover, adding multiple sessions could extend these benefits of ES. In the present study, the results of our EdU/recoverin double immunolabeling and *Sox2^{ERT}-ROSA26* lineage tracing experiments suggest that ES may be able to induce residential retinal progenitors to reenter the cell cycle and differentiate into photoreceptors. Although the cell counts revealed a 10-fold increase of EdU+/recoverin+ cells in ES-treated retinas compared to non-stimulated contralateral controls, the incidence of MC transdifferentiation remains very small, unlikely to contribute to the functional benefit. Colocalization studies of MC marker CRALBP with tdT in the retinas of *Sox2ERT-ROSA26* mice showed over 90% tdT+ cells expressing the MC marker, supporting the possibility of a MC origin of new photoreceptors. However, we cannot rule out that some of the tdT+/recoverin+ cells may be derived from other retinal cell types. Together, these data point to the potential of ES to mobilize endogenous retinal progenitors, likely from a MC origin. The number of photoreceptors generated from MCs was small; thus, the increase of recoverin+ cells observed

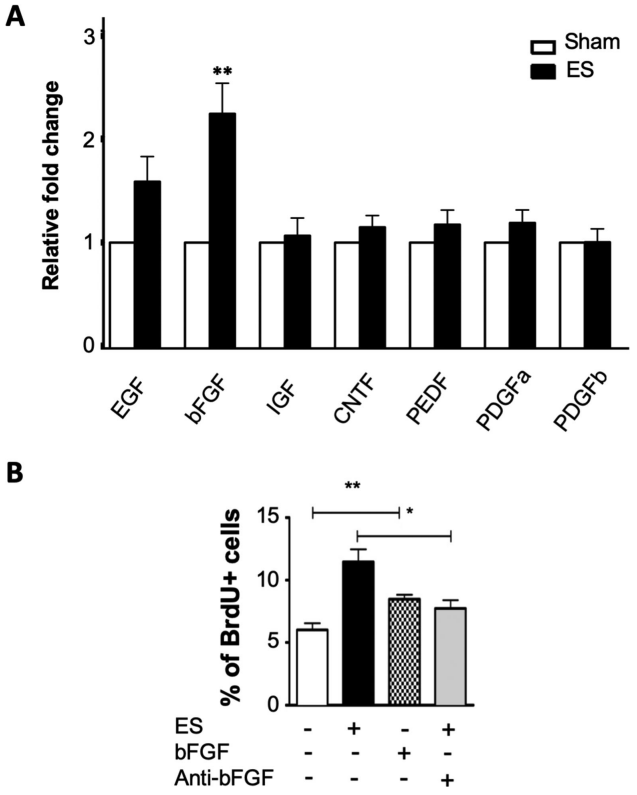

Figure 8. Electrical stimulation (ES) functions partly through the bFGF pathway to promote MC proliferation. (**A**) qPCR results showing increased mRNA levels of bFGF in ES-treated retina as compared to the sham-treated group (n = 4/group; $^{*}P < 0.01$ by unpaired *t*-test). (**B**) Quantification of MC proliferation in isolated ES- or sham-treated cultures followed by incubation with or without bFGF or its neutralizing antibody (n = 5/group). Note that ES or bFGF alone enhanced MC proliferation in culture whereas anti-bFGF reduced the proliferative effect of ES. $^{*}P < 0.01$, $^{**}P < 0.005$ by *t*-test.

in ES-treated mice is primarily a consequence of improved photoreceptor cell survival after ES. These results collectively suggest that noninvasive transpalpebral ES may be a versatile tool for prolonging the preservation of retinal function and possibly reversing vision loss in patients with RP. It must also be mentioned that we cannot completely rule out the possibility that ES may also increase the percentage of recoverin+ cells through improvement of the survival of rod photoreceptors, as we observed in differentiated MC cultures.

The present findings offer the foundation for clinical observations that ES treatment may have the potential to improve sight in patients with photoreceptor degeneration.[6,12,35] Our data agree with earlier findings that ES enhanced ERG b-wave and ONL thickness or photoreceptor survival.[7,36] Moreover, our results suggest that ES drove molecular changes in MCs toward a progenitor-like status. Zebrafish MCs dedifferentiate and evolve into the desired cell types after retinal injury.[37] Significant progress has been made in understanding the molecular basis for the lack of spontaneous retinal regeneration in mammals.[38] Mammalian MCs have been shown to possess the ability of re-entering the cell cycle, dedifferentiating, and acting like retinal progenitor cells to generate new neurons in response to the activation of certain signaling pathways and transcription factors.[39–41] A recent report indicated that, if provided with the appropriate stimuli, mammalian MCs can be mobilized in vivo to repeat the type of regenerative responses found in non-mammalian vertebrates.[21,42] In line with the emerging evidence that electrical activity may be sufficient to induce neuron survival and regeneration in the CNS,[43] we showed here that ES directly induced MC proliferation and expression of progenitor cell and photoreceptor markers in culture. In *Rho*$^{-/-}$ mice, proliferating MCs migrated to the ONL and transdifferentiated into photoreceptor cells following ES. Although it remains unknown if newly generated photoreceptors contributed to the improved retinal function in the *Rho*$^{-/-}$ mice, this study demonstrates that photoreceptor regeneration could also be possible in mammals if correctly stimulated with noninvasive ES.

ES might improve photoreceptor survival and function by triggering neurotrophic factor production and reducing microglia activity leading to a homeostatic microenvironment in *Rho*$^{-/-}$ mice.[22,44] Earlier studies have demonstrated increased CNTF and BDNF levels after transcorneal ES in RCS rats. The same effect was observed after ES to femoral motoneurons in RCS rats.[30,45] Our qPCR results indicate significantly increased mRNA expression of bFGF in the ES-treated MC cultures. Moreover, addition of bFGF alone to purified MC cultures significantly increased cell proliferation, and administering anti-bFGF antibody to the ES-treated MC cultures attenuated the proliferative effect of ES. Thus, ES may upregulate not only neurotrophic agents but also neurogenic signaling to promote MC proliferation and retinal neuron repair.

Neurons and glial cells in the retina are known to have different sensitivity to ES.[46–48] For example, if retinal ganglion cells (RGCs) are directly activated (i.e., not secondary to activation by one or more presynaptic neurons), they can follow pulse trains at rates up to several hundred pulses per second.[49] The ability to follow high-rate trains varies by cell type, however;[46] for ES that activates neurons presynaptic to RGCs, such as bipolar cells and photoreceptors, the sensitivity is greatly reduced.[50] In *Rho*$^{-/-}$ mice, we used the ES with a mixture of stimulation frequencies covering from 2 to 200 PPS because it was shown to improve vision in human patients.[9] When varying ES currents and frequencies were evaluated in MC cultures, we noted that ES at 10 to 20 PPS resulted in the optimal cell proliferation and neurogenic responses of MCs. This ES condition coincides with that reported in vivo,[51] which showed optimal morphological photoreceptor and ONL preservation; in contrast, this ES frequency is far slower than that at which RGCs are directly activated but faster than the peak sensitivity to indirectly activate RGCs. These data suggest that activation of MC proliferation and release of neurotrophic agents may be central to ES-mediated photoreceptor rescue. Further elucidation of the underlying mechanisms and the cell types involved may allow precise selection of ES parameters for optimal outcomes of visual preservation and restoration in the future.

Rho$^{-/-}$ mice are an excellent model system of human RP, as rhodopsin mutation is found in 25% of the autosomal dominant inherited RP in patients.[52] Invasive ES technologies (such as subretinal, epiretinal, or suprachoroidal prostheses or episcleral implants) have been tested clinically in patients with RP or choroideremia.[53–55] In the present study, we showed that even noninvasive ES is sufficient to confer a neuroprotective effect on photoreceptors and at least temporarily rescue photoreceptor cell function in eyes with inherited retinal degeneration. To date, no adverse

effects of ES have been reported. Although the benefits of ES are reportedly transient,[7] we showed that the benefits may be prolonged by repeated ES sessions. Still, little is known about the optimal waveforms, frequencies, and duration of ES for preventing vision loss in photoreceptor degeneration. Moreover, additional studies are needed to determine whether the improvement in retinal function as shown by ERG is translated into improved visual perception and function in humans and whether the protective effect of ES is applicable to other forms of RPs or retinal degeneration.

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