

Retinal Pigment Epithelium Responses to Selective Retina Therapy in Mouse Eyes

Hoon Dong Kim,¹ Sun Young Jang,² Si Hyung Lee,³ Young Shin Kim,² Young-Hoon Ohn,² Ralf Brinkmann,⁴ and Tae Kwann Park^{2,5}

¹Department of Ophthalmology, College of Medicine, Soonchunhyang University, Cheonan, Republic of Korea

²Department of Ophthalmology, College of Medicine, Soonchunhyang University, Bucheon, Republic of Korea

³Department of Ophthalmology, Eulji University Hospital, Daejeon, Republic of Korea

⁴Medical Laser Center Lübeck GmbH, Lübeck, Germany

⁵Laboratory for Translational Research on Retinal Macular Degeneration, College of Medicine, Soonchunhyang University, Bucheon, Republic of Korea

Correspondence: Tae Kwann Park, Department of Ophthalmology, College of Medicine, Soonchunhyang University, Bucheon Hospital, #1174, Jung-dong, Wonmi-gu, Bucheon, 420-767, Korea; tkpark@schmc.ac.kr.

HDK and SYJ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: March 6, 2016

Accepted: June 2, 2016

Citation: Kim HD, Jang SY, Lee SH, et al. Retinal pigment epithelium responses to selective retina therapy in mouse eyes. *Invest Ophthalmol Vis Sci*. 2016;57:3486–3495. DOI:10.1167/iovs.16-19508

PURPOSE. To investigate the characteristics of retinal pigment epithelium (RPE) and retinal damage induced by selective retina therapy (SRT) in mice, and to elucidate longitudinal changes in RPE cells.

METHODS. C57BL/6J mice received SRT and continuous-wave laser photocoagulation (cwPC). The cell death pattern was evaluated using TUNEL assay, and proliferative potential of the RPE cells was evaluated using 5-ethynyl-2'-dexoyuridine (EdU) assay. To investigate the cell-cell integrity of RPE cells, β -catenin staining was performed. The number and hexagonality of RPE cells in the SRT-treated area were estimated using a Voronoi diagram with time periods of 3 hours to 14 days. Antibodies to microphthalmia-associated transcription factor (Mitf) and orthodenticle homeobox 2 (Otx2) were used to confirm the specific characteristics of RPE cells in the SRT-treated area.

RESULTS. The number of TUNEL-positive cells located in the neural retina was significantly lower in lesions treated with SRT compared to those treated with cwPC. EdU-positive RPE cells were first detected 3 to 12 hours after SRT, and increased until 3 to 7 days after SRT. β -catenin staining showed that hexagonality was compromised and subsequently, RPE cells expanded in size within the targeted location. The number of RPE cells in SRT lesions decreased gradually until 12 hours after SRT and recovered by 14 days. Upregulated expression of Mitf and Otx2 was observed for 2 weeks in the SRT lesions.

CONCLUSIONS. Selective retina therapy seems to induce selective RPE damage without collateral thermal injury in the neural retina. Furthermore, SRT-treated lesions recovered by proliferation of RPE cells that were present in the treated lesions and by expansion of adjacent RPE cells.

Keywords: microphthalmia-associated transcription factor (Mitf), orthodenticle homeobox 2 (Otx2), retinal pigment epithelium, selective retina therapy

Laser photocoagulation is a mainstay for the treatment of several retinal conditions associated with retinal pigment epithelium (RPE) dysfunction.^{1–4} Continuous-wave laser photocoagulation (cwPC) has been used in the treatment of various macular diseases, such as diabetic macular edema (DME), age-related macular degeneration, and central serous chorioretinopathy (CSC), over several decades.^{2–4} Light energy from cwPC is converted into heat in the melanosomes of RPE cells. By heat diffusion, this thermal energy not only damages the RPE but also results in collateral damage of surrounding components, particularly overlying photoreceptors, Bruch's membrane, and choriocapillaries.^{5–8} This is a desired effect because it induces scar formation, which prevents further retinal detachment in retinal holes, and damages photoreceptors during panretinal photocoagulation, which is thought to preserve the central macula in diabetic retinopathy. However, for pathologies like DME or CSC, the selective damage and subsequent restoration of the RPE might be sufficient to increase the RPE pump

function and overall metabolic activity, and collateral damage is not needed.

Selective retina therapy (SRT) is a new, minimally invasive laser technology that has been designed to limit the laser-induced damage solely to the RPE.⁹ By applying laser pulses that are shorter than the thermal confinement time of the absorbing structure, the risk of thermal damage to the surrounding healthy retina and choroid is reduced.^{10–12} The origin of the selectivity for RPE cells relies on the formation of very short-lived (μ s, microseconds) vapor bubbles generated around the strongly absorbing melanosomes within the cells, which disrupt the cellular membrane due to the temporarily strongly increased cell volume.¹³ Several experimental studies have confirmed the safety of SRT and have established the therapeutic range of laser irradiation for retinal tissue.^{12–16} These studies demonstrated that the useful pulse duration for the photodisruption of RPE is in the range of microseconds.^{12,14,15} Indeed, SRT has been applied clinically in the



treatment of several macular diseases through several pilot studies.^{2-4,10} Recently, a functional evaluation using a multifocal electroretinogram after SRT was recently investigated by our research team, concluding that SRT preserved retinal function in rabbit eyes.¹¹ However, until now, research on a systematic approach to RPE cell proliferation and cytokine expression at the cellular level after SRT application has not been reported in an animal model.

In the present study, we evaluated the characteristics of retinal damage induced by SRT and elucidated the longitudinal changes in RPE cells following SRT in the eyes of mice. Moreover, we investigated whether the proliferation of adjacent RPE cells occurred in SRT-treated lesions, and tried to confirm whether these proliferating cells in the lesions showed RPE characteristics using RPE-specific transcription factors.

METHODS

Animals

The experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research to minimize suffering.

In total, 50 healthy male C57BL/6J mice were used (OrientBio, Inc., Seongnam, Korea) at 8 weeks of age. The mice were kept in a temperature- and humidity-controlled room with a 12/12-hour light/dark cycle environment under standardized conditions. Food and water were provided to the mice *ad libitum*. Only the right eyes of the mice were used for laser treatment with SRT and cwPC. Prior to the laser treatments and examinations, all mice were anesthetized with an intraperitoneal (IP) injection of a mixture of Zoletil 50 (125 mg zolazepam and 125 mg tiletamine hydrochloride; Vibrac, Carros, France; 40 mg/kg body weight) and Rompun (2% xylazine hydrochloride; Bayer Animal Health, Leverkusen, Germany; 5 mg/kg body weight). Pupil dilation was performed with 0.5% tropicamide and 2.5% phenylephrine (Mydrin-P; Santen, Osaka, Japan) for laser irradiation and ophthalmologic examinations. The laser treatments and examinations were conducted at the same time of day.

Laser Irradiation

Two laser modalities, SRT and cwPC, were used for the treatment of the mice. The SRT laser (R-Gen; Lutronic, Goyang, Republic of Korea) was an intracavity frequency-doubled Q-switched Nd:YLF laser with a wavelength of 527 nm that applied 1.7- μ s pulses at a repetition rate of 100 Hz with increasing pulse energy. The focus of light was directed to the lesion and controlled by aiming the beam from the slit-lamp microscope-mounted SRT machine. Because SRT induced invisible burns on retina, laser burns could not be detected ophthalmoscopically. Therefore, a dosimetry tool such as reflectometry was used to control the laser power. Methods regarding SRT application with the automatic feedback system using reflectometry were described in detail in our previous report.¹¹ Briefly, the maximal pulse energy that could be delivered in a single burst was determined. This value was set as the highest energy to induce ophthalmoscopically invisible burns after the test shots. After the determination of maximal pulse energy, the first delivered pulse was 10% of the maximum pulse energy, with a stepwise increase of 3.1% for all subsequent pulses up to a maximum of 30 pulses. During the laser irradiation with stepwise increase of energy, a computer analyzed the backscattered light from the retina and calculated a reflectometry value. If the reflectometry

detected microbubbles and the value exceeded a certain threshold, the laser pulse train was ended automatically by a feedback system.^{17,18} The laser energy at a certain threshold could be considered the energy that induces selective RPE damage.^{11,12,18}

Continuous-wave laser PC was performed with the slit-lamp delivery system of a PASCAL Streamline system (Topcon Medical Laser Systems, Inc., Santa Clara, CA, USA), a frequency-doubled Nd:YAG laser with a wavelength of 532 nm.

The right eyes of 40 mice were treated with SRT (maximal pulse energy: 30 μ J, 95.5 mJ/cm², spot size 200 μ m). The right eyes of another five mice were treated with cwPC (power 100 mW; duration 40 ms; spot size 200 μ m; total energy 12.6 J/cm²). The right eyes of the remaining five mice, which received neither SRT nor cwPC, served as controls. When the laser treatments were performed, a thin transparent handheld flat glass coverslip was used anterior to the mouse eyes as a contact lens with application of 0.5% methylcellulose (Gentel; Novartis, Basel, Switzerland) for visualization of the fundus. Thus, in total, 10 or fewer laser spots were made on the retina, distributed in a concentric pattern around the optic nerve head.

Near-Infrared Imaging and Fundus Fluorescence Angiography

Near-infrared (NIR) images and fundus fluorescence angiography (FFA) findings of the retina were obtained for each laser-treated mouse using a confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph 2; Heidelberg Engineering, Heidelberg, Germany). The animals were anesthetized and the pupils dilated. The NIR images were acquired before fluorescein dye injection at each examination period. The angiographic images were captured 3 to 5 minutes after IP injection of 0.1 mL 2% fluorescein sodium (Fluorescite; Alcon Laboratories, Inc., Fort Worth, TX, USA) to identify any angiographic visibility of the laser-treated lesions over time. Imaging studies using NIR and FFA were performed 1 day after cwPC and SRT treatment, and they were repeated 3 and 7 days later.

Intraperitoneal 5-Ethynyl-2'-Deoxyuridine Injection

To investigate the time-dependent proliferative potentials of RPE cell populations following SRT, the mice underwent periodic IP injections of 5-ethynyl-2'-deoxyuridine (EdU, 100 mg/kg; Sigma-Aldrich Corp., St. Louis, MO, USA) in phosphate-buffered saline (PBS) (Table). The periodic IP EdU injection methods described in our previous paper were modified for this study.¹⁹ The IP EdU injection schedule from the time of SRT is presented in Figure 1. The mice received periodic IP injections at the following intervals: 0 to 3 hours ($n = 5$), 3 to 12 hours ($n = 5$), 12 to 24 hours ($n = 5$), 1 to 3 days ($n = 5$), 3 to 7 days ($n = 5$), and 7 to 14 days ($n = 5$) after SRT. The animals were killed at the end of each time interval. EdU assays on whole-mount tissue preparations were performed at each period after SRT.

Tissue Preparation

The mice were deeply anesthetized using IP injection of a 4:1 mixture of Zoletil 50 (80 mg/kg) and Rompun (10 mg/kg) at the end of each examination period. They were then euthanized by intracardial perfusion with 0.1 M phosphate buffer (PB) containing 1000 IU/mL heparin, followed by 4% paraformaldehyde (PFA) in 0.1 M PB. After euthanasia, the right eyeballs were enucleated and redundant eye tissues were trimmed with scissors. After removal of the anterior segment

TABLE. Antibodies Used for Immunohistochemistry and Western Blotting

Target	Host	Manufacturer	Catalog No.	Dilution
β-catenin	Rabbit	Sigma-Aldrich Corp., St. Louis, MO, USA	C2206	1:2000
EdU	Rabbit	Invitrogen, Waltham, MA, USA	C10340	1:200
MitF	Mouse	Exalpha Biologicals, Shirley, MA, USA	X1405M	1:1000
Otx2	Rabbit	Merck Millipore, Billerica, MA, USA	AB9566	1:1000

and the lens by cutting through the limbal cornea, the eye cups were immersed in fixative solution consisting of 4% PFA in 0.1 M PB at 4°C and pH 7.4 for 1 hour. The nictitating membrane remained attached to the nasal side of the limbus to establish the specimen orientation. The samples were then transferred to 30% sucrose in PBS, incubated overnight, and embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA). Serial sagittal sections (in the 12 to 6 o'clock plane) with a thickness of 8 μm were taken from the embedded samples and mounted on adhesive microscope slides (Histobond; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). By visually scanning the serial sections, the transverse chorioretinal sections with the furthest disruption at the RPE-photoreceptor junction were designated the center of the laser lesion. Chorioretinal sections were used for cell death assays for SRT- and cwPC-treated eyes 24 hours after both laser treatments. For immunohistochemistry and the EdU assay after SRT, whole-mount tissue preparations were applied at each time period. Selective retina therapy-treated eyes were dissected into posterior eye cups. After peeling of the neural retina, RPE-choroid complexes were fixed with 4% PFA in 0.1 M PB at pH 7.4 and prepared as flattened whole mounts with four-quadrant cuts.

Cell Death Assay

The in situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Basel, Switzerland) was used for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay to detect cell death after SRT and cwPC. The TUNEL assay was carried out 24 hours after laser treatments according to the

protocols recommended by the kit manufacturer. Briefly, the procedures were as follows. The chorioretinal preparation representing the center of laser-treated lesions was washed with PBS for 10 minutes. After three repeated washings, the sample was incubated in a blocking solution of 3% H₂O₂ in methanol for 10 minutes at 15°C to 25°C. Following the three washings with PBS, the sample was incubated in permeabilization solution consisting of 0.1% Triton X-100 and 0.1% sodium citrate (Sigma-Aldrich Corp.) for 2 minutes on ice (2°C–8°C). The labeling reaction was performed using a TUNEL reaction mixture after repeated tissue washings. In addition, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 0.1 μg/mL; Sigma-Aldrich Corp.) staining was used to visualize the cell nuclei with a 3-minute incubation time. The chorioretinal sections were evaluated under a fluorescence microscope (AxioPlan Microscope; Carl Zeiss, Inc., Oberkochen, Germany) with ×200 magnification and 1.5-second exposure after incubation at 37°C for 60 minutes. Because the RPE layer could not be observed after DAPI staining due to melanin pigments, a Nomarski image was taken with a light microscope equipped with differential interference contrast (DIC) microscope (AxioPlan Microscope) to identify the location of the TUNEL-positive cells in the chorioretinal section.

EdU Assay

The procedure for the EdU assay has been described in detail previously.¹⁹ Briefly, commercially available Click-iT Imaging Kits (Invitrogen, Carlsbad, CA, USA) were used for EdU assays after periodic IP EdU injections following SRT, and the manufacturer's protocols were followed. The tissue prepara-

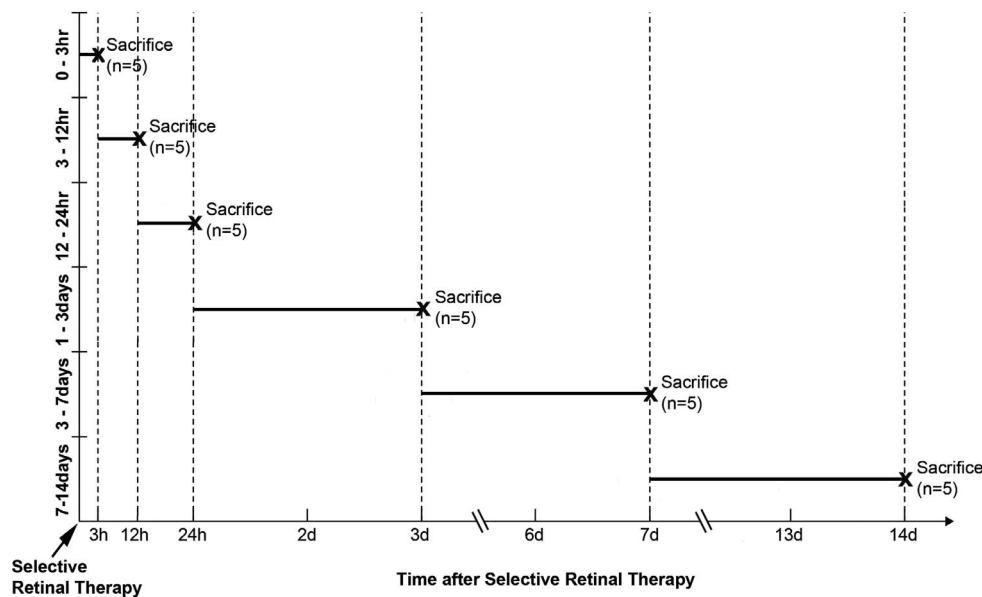


FIGURE 1. Schematics of the protocols for periodic intraperitoneal (IP) EdU injections from the time of selective retina therapy (SRT, arrow). The mice received periodic IP injections at the following intervals; 0 to 3 hours, 3 to 12 hours, 12 to 24 hours, 1 to 3 days, 3 to 7 days, and 7 to 14 days after SRT. The mice were killed at the end of the each injection period.

tion was blocked using 3% bovine serum albumin in PBS for 1 hour. Then, washing for 10 minutes with PBS was repeated three times. After incubation at room temperature for 20 minutes, 0.5% Triton X-100 in PBS was added to each tissue preparation. Click-iT reaction cocktail was used according to protocol. After incubation for 1 hour, washing was performed for 10 minutes. Following application of the primary antibodies to EdU, the tissue preparations were incubated at room temperature for 1 hour. Subsequently, three washes for 10 minutes were carried out. The tissue preparations were then incubated at room temperature for secondary antibody labeling with Alexa Fluor 568 (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hour. The tissues were washed for 10 minutes, and this washing was repeated three times. EdU labeling was then performed using an imaging kit for 1 hour in the dark, and the preparation was mounted with fluorescent mounting medium (Dako, Glostrup, Denmark). Images from the whole-mount preparation were obtained at each period after SRT using confocal fluorescence microscopy (LSM510 META; Carl Zeiss, Inc.) at $\times 200$ magnification and 1.5-second exposure. Finally, consecutive images were taken at 3, 12, and 24 hours and 3, 7, and 14 days after SRT according to the periodic IP EdU injections after SRT application.

Immunohistochemistry

Immunohistochemistry on the chorioretinal sections of the mice retinas was performed to identify the cellular characteristics of the cell populations that were observed in SRT-treated lesions. The antibodies used for immunohistochemistry are listed in the Table. The integrity of cell-to-cell junctions and the hexagonality of the RPE layers were evaluated by labeling with anti- β -catenin antibody. Antibodies to MiTF (microphthalmia-associated transcription factor) and Otx2 (orthodenticle homeobox 2), essential transcription factors for RPE cell differentiation, were applied to identify RPE cell specification in the SRT-treated lesions, and the expression of these transcription factors was observed using immunohistochemistry. For staining of the cell markers, the slides were incubated with antibodies for each cell marker at 37°C for 2 hours after DNA denaturation and antigen retrieval. Detection of the signal was achieved by incubation with the appropriate combination of Alexa Fluor 488 (Thermo Fisher Scientific)– and Alexa Fluor 568–conjugated secondary antibodies. Images from whole-mount preparations were obtained using the LSM510 META confocal fluorescence microscope. The images were taken using image-capture software (LSM image browser; Carl Zeiss, Inc.).

Cell Counting

The numbers of TUNEL-positive cells were estimated on chorioretinal tissues with vertical sections to quantify apoptotic cells after SRT and cwPC because TUNEL labeling indicates cellular apoptosis in the whole laser-treated lesion. The TUNEL-positive cells were manually counted by three independent experienced colleagues who were unaware of the treatment performed on each specimen. Cell counting was done separately for two different layers: the neural retina and the RPE layer. Five laser-treated lesions showing typical morphology were selected and averaged from each animal for the quantitative analysis. The numbers of EdU-positive cells were counted in the same manner.

Retinal pigment epithelium cells labeled with β -catenin in the whole-mount view, located within circles of 200- μ m diameter on the center of the SRT-treated lesions, were counted at each time period for 14 days to evaluate time-dependent changes in the number of RPE cells after SRT.

Because β -catenin is expressed in cell membranes, the number of RPE cells stained with β -catenin was estimated using a Voronoi diagram algorithm program (RhinoCeros 5.0; Roberts McNeel & Associates, Seattle, WA, USA) to confirm the decreased number of RPE cells after SRT and their restoration at later time periods. A Voronoi diagram is a type of geometric tool that can partition a particular plane using a set of points with boundaries determined by distances. An algorithm of the Voronoi diagram has been used to establish a model of biological structure and to analyze cell density, variation in cell size, and the creation of cell barrier.^{20,21} As above, in five laser-treated lesions, the RPE cells with intact β -catenin staining were averaged for analysis. To evaluate changes in variation in RPE cell size and hexagonality, the areas of RPE cells stained with β -catenin within circles of 200- μ m diameter were also estimated. The mean RPE cell areas on Voronoi diagram images were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All numbers are shown as mean $\pm$ standard errors of the mean (SEMs), and coefficients of variance were calculated to evaluate the variation in cell size and structure over time.

Western Blotting

The level of expression of two essential transcription factors for RPE cell differentiation (MiTF and Otx2) in chorioretinal tissue following SRT was analyzed by Western blotting. After enucleation, the anterior segment and retina were removed from the eyeball. Tissue extracts from the RPE-choroid complex of the mouse eyes were sonicated and then placed on ice for 50 minutes. The extracts were centrifuged (16,773g, 20 minutes, 4°C). The supernatant was collected, and the protein concentration in the homogenate was measured using a commercial protein assay kit (Pierce BCA Protein Assay Reagent Kit; Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol. An equal volume (20 μ g) of total protein from each extract was resolved on SDS polyacrylamide gel electrophoresis, and the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 hour at room temperature. The membranes were incubated with appropriate antisera overnight at 4°C (Table). Subsequently, the membranes were incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 90 minutes at room temperature. The blot was developed with the enhanced chemiluminescence system (Clarity ECL Western Blotting Substrate; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Positive labeling bands were detected, and the densitometry of the bands was performed using ImageJ software. Detection of β -actin was assessed for all samples as a positive gel-loading control.

Statistical Analysis

Statistical analyses were performed using SPSS software (ver. 20.0 for Windows; IBM Corp., Armonk, NY, USA). The 2-tailed Student's *t*-test was used to compare the results of the TUNEL-positive cell counting after the cell death assay following treatment with SRT and cwPC. A *P* value < 0.05 was considered to indicate statistical significance for Student's *t*-test. In addition, the quantified results of Western blotting after SRT over time were averaged from five samples, and the results at each time period were compared with the expression level of the control. Bonferroni correction was used for multiple comparisons among examination periods to reduce type I errors. All quantified values are given as mean $\pm$ SEMs. A *P*

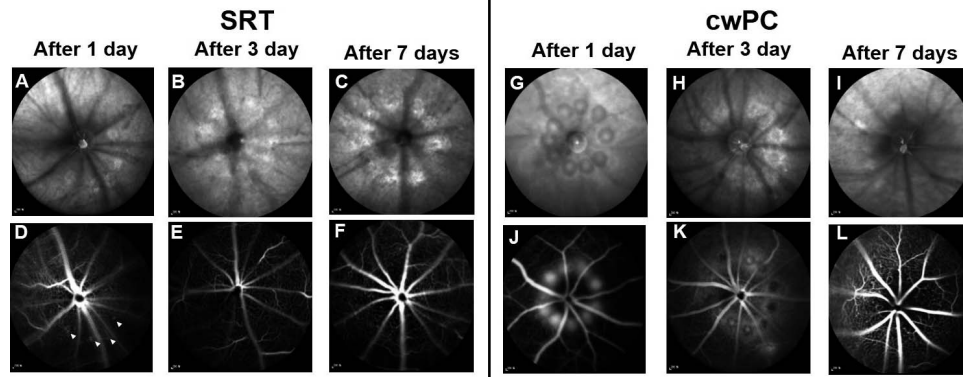


FIGURE 2. Longitudinal findings of near-infrared (NIR) images and fundus fluorescein angiography (FFA) findings following selective retina therapy (SRT) and continuous-wave laser photocoagulation (cwPC) for 7 days. Visible spots were observed continuously in NIR images in SRT (A–C) and cwPC lesions (G–I) for 7 days. Hyperfluorescent spots in FFA images were detected by 1 day after SRT (D, arrowheads); however, hyperfluorescent spots were no longer identified after 3 (E) and 7 days (F). In cwPC-treated lesions, hyperfluorescence was evident for 1 (J) and 3 days (K) after treatment. After 7 days, cwPC-treated lesions did not show hyperfluorescence either (L).

value of 0.0083 was used for statistical significance after the Bonferroni correction.

RESULTS

NIR and FFA Findings in Laser-Treated Lesions

Near-infrared and FFA images were taken 1, 3, and 7 days after treatment with SRT or cwPC. Both SRT- and cwPC-treated lesions were visible around the optic disc on NIR and FFA images taken 1 day after treatment (Figs. 2A, 2D, 2G, 2J). On day 3, FFA detected hyperfluorescence of cwPC-treated lesions, whereas it was difficult to detect in SRT-treated lesions (Figs. 2E, 2K). On day 7 after laser application, hyperfluorescence in cwPC-treated lesions was not visible (Fig. 2L). In the NIR images, hyperreflectance at laser spots was demonstrated until day 7 in both SRT- and cwPC-treated mice.

Quantification of Cell Death Following SRT and cwPC in Chorioretinal Tissue

To determine which cell types were affected by SRT and cwPC treatment, the TUNEL assay was performed 1 day after each treatment. TUNEL-positive cells were detected in the RPE layer of the SRT-treated eyes. However, in the outer nuclear layer (ONL) of the neural retina, TUNEL-positive cells were very rarely detected (Figs. 3A, 3B). In a Nomarski view taken from the same section, waveform discontinuity of the RPE layer was also noted in SRT-treated lesions (Fig. 3C). In contrast, TUNEL-positive cells were abundantly detected not only in the RPE layer but also in the ONL of cwPC-treated eyes (Figs. 3D, 3E). To quantify the amount of apoptotic cell death after treatment with two different laser procedures, the numbers of TUNEL-positive cells detected in the RPE layer and the neural retina in central transverse sections were assessed because those cells

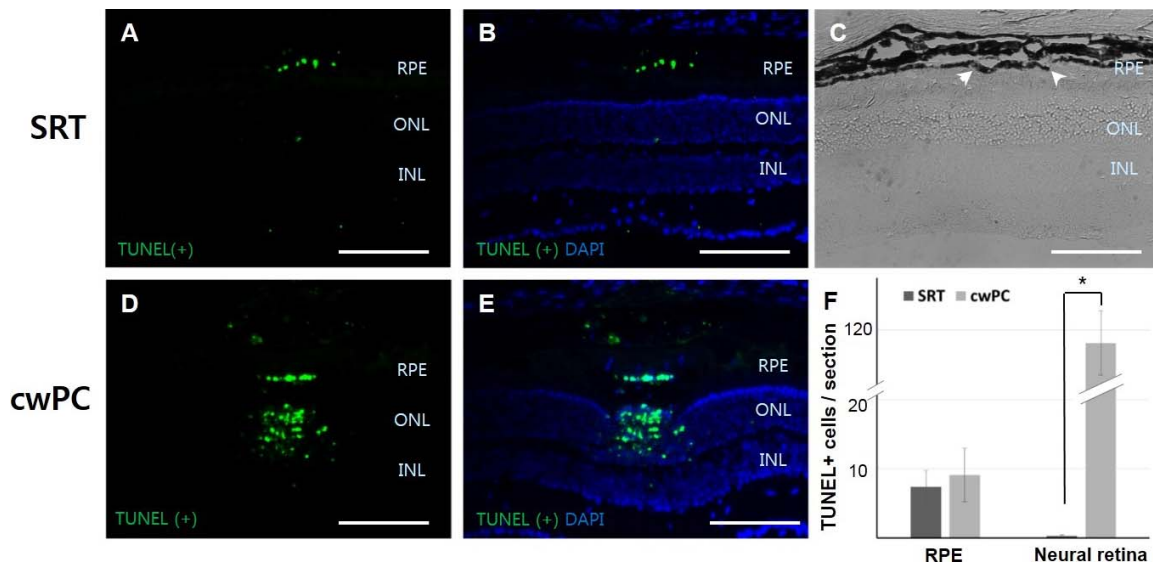


FIGURE 3. Representative images of cell death 1 day after laser treatments with SRT and cwPC. In SRT-treated lesions, TUNEL-positive cells were observed in the RPE layer, while they appeared sporadically in the neural retina (A, B). SRT-treated lesions could be identified by discontinuity of RPE layer on Nomarski image (C). Cell death, labeled with TUNEL, was identified in the RPE and neural retina (D, E). Quantification of TUNEL labeling at 1 day after SRT and cwPC (F). The number of TUNEL-positive cells seen in the RPE layer was not significantly different between SRT- and cwPC-treated lesions. However, the number of TUNEL-positive cells detected in the neural retina was significantly higher in cwPC-treated lesions than in SRT-treated lesions (F). Data are expressed as mean $\pm$ SEM. * $P < 0.05$ by Student's *t*-test. Scale bar: 100 μ m.

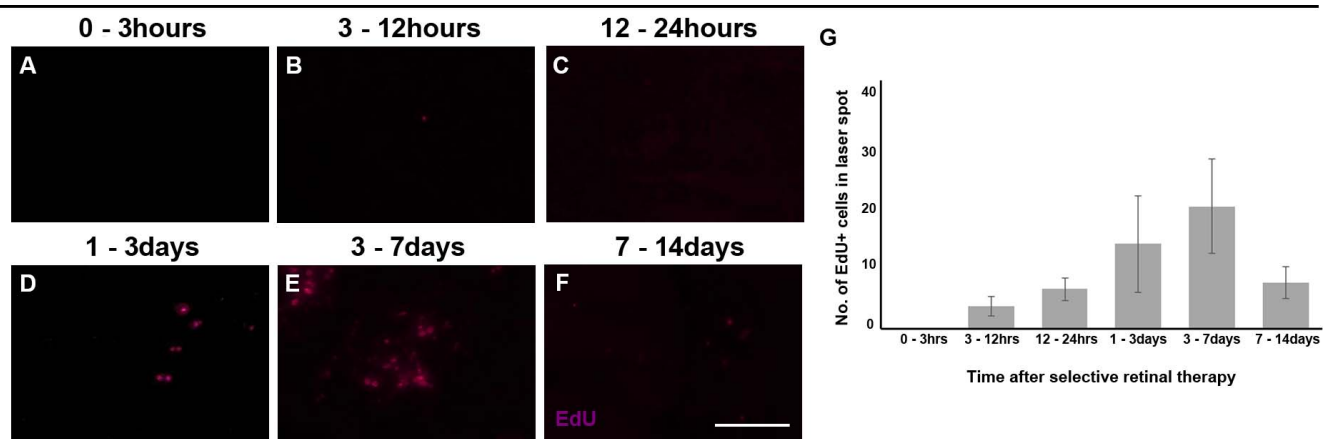


FIGURE 4. EdU expression in SRT-treated lesions at each injection interval. No expression of EdU was found in whole-mount views during 0 to 3 hours (A) after SRT. The EdU-positive cells were first identified during 3 to 12 hours (B), and there was a gradual increase in the number of EdU-positive cells observed until 3 to 7 days (C-E), when peak proliferative activity was reached. The expression level of EdU was markedly reduced during 7 to 14 days after SRT (F). Summarized data of EdU-positive RPE cells are shown (G). Data are expressed as the mean $\pm$ SEM. Scale bar: 100 μ m.

represent the number of apoptotic cells in the whole laser lesion. The number of TUNEL-positive cells in the RPE layer of SRT-treated lesions was 6.82 ± 2.97 , and the number in the RPE layer of cwPC-treated lesions was 8.83 ± 3.60 . There was no significant difference in the number of TUNEL-positive cells in the RPE layer between the two groups ($P = 0.124$). However, the numbers of TUNEL-positive cells from the neural retina, detected mostly in the ONL, were significantly different between the two treated arms. There were significantly fewer numbers of TUNEL-positive cells in the neural retina in lesions treated with SRT (1.20 ± 0.55 cells/section) compared to those treated with cwPC (112.50 ± 18.48 cells/section, $P < 0.001$) (Fig. 3F).

EdU Assay

The EdU assay was performed to estimate the time-dependent changes in the proliferative potentials of RPE cells in SRT-treated lesions. EdU was not seen in the whole-mount views during 0 to 3 hours after SRT (Fig. 4A). However, the RPE cells with EdU expression could be identified at 3 to 12 and 12 to 24 hours (Figs. 4B, 4C) after SRT. Moreover, a considerably increased number of EdU-positive cells were observed during the 1- to 3-day interval (Fig. 4D), and the number of EdU-positive cells was obviously increased during 3 to 7 days after SRT (Figs. 4E, 4G). During 7 to 14 days, fewer EdU-positive cells were observed (Figs. 4F, 4G). The number of cells with EdU expression showed a peak level during 3 to 7 days following SRT (Fig. 4G).

Quantification of RPE Cell Density and Distribution in SRT-Treated Lesions Over Time

To evaluate the longitudinal changes in the RPE cell structure after treatment with SRT, the expression patterns of β -catenin, a component of the cell-to-cell connections, were observed at 3 and 12 hours and 1, 3, 7, and 14 days after the application of SRT. At 3 and 12 hours after laser treatment, β -catenin staining showed compromised hexagonality of the RPE cells, which subsequently expanded in size within the targeted location (Fig. 5). Furthermore, more aberrant β -catenin expression was detected at the membranous portion of the elongated RPE cells. Then, 2 days later, the center of the SRT lesion was gradually covered with RPE cells, and cell-to-cell connections

were also regained. The hexagonality of the RPE cells seemed to be restored 1 week after SRT (Fig. 5).

To evaluate the longitudinal changes in the cell density of RPE cells following SRT, the mean numbers of RPE cells labeled with β -catenin at the center of the SRT-treated lesions in whole-mount views were investigated using a Voronoi diagram. The RPE cell numbers were significantly decreased after 3 hours of SRT ($P < 0.001$), and gradually increased until 3 days (Fig. 4). After 7 days of SRT, the RPE cell numbers were not significantly different from the control level ($P = 0.152$). The mean numbers of RPE cells (46.75 ± 3.04) were restored to 93.8% of the baseline value (49.85 ± 4.62) after 7 days of SRT, and this was maintained until 14 days.

To estimate the variation in the size of RPE cells, areas of individual RPE cells were calculated using Voronoi diagrams, and a coefficient of variance (CV) was identified for each time period (Fig. 5). The mean areas of RPE cells in the laser-treated lesions peaked at 12 hours after SRT, and the CVs of RPE cell areas also significantly increased until 3 days after SRT ($P < 0.001$). Thereafter, the mean areas and CVs of RPE cell areas both markedly decreased after 7 days of SRT (Fig. 5). The average area of individual RPE cells ($251.6 \pm 57.2 \mu\text{m}^2$) within a circle 200 μ m in diameter from the center of SRT-treated lesions reached 103% of the baseline level ($244.25 \pm 65.2 \mu\text{m}^2$) after 7 days. One week after SRT, the numbers of RPE cells were restored to the normal range, and the variation in sizes had also recovered. These immunohistochemistry results imply that the expansion of RPE cells in laser-treated lesions persisted until 3 days after SRT, and the uniformity of RPE cell sizes had recovered by 1 week following SRT.

Expression Patterns of MiTF and Otx2 After SRT Application

Immunohistochemistry was performed to evaluate changes in two essential transcription factors associated with RPE cell differentiation following SRT. Antibodies to MiTF and Otx2 were used to investigate the consecutive responses in RPE-specific characteristics in the cell population observed in SRT-treated lesions. Microphthalmia-associated transcription factor and Otx2 are widely known as key transcription factors for RPE cell differentiation during eye development.^{22,23} Upregulation of the expression of MiTF and Otx2 at 3 and 7 days was identified in SRT-treated lesions; in particular, their colocaliza-

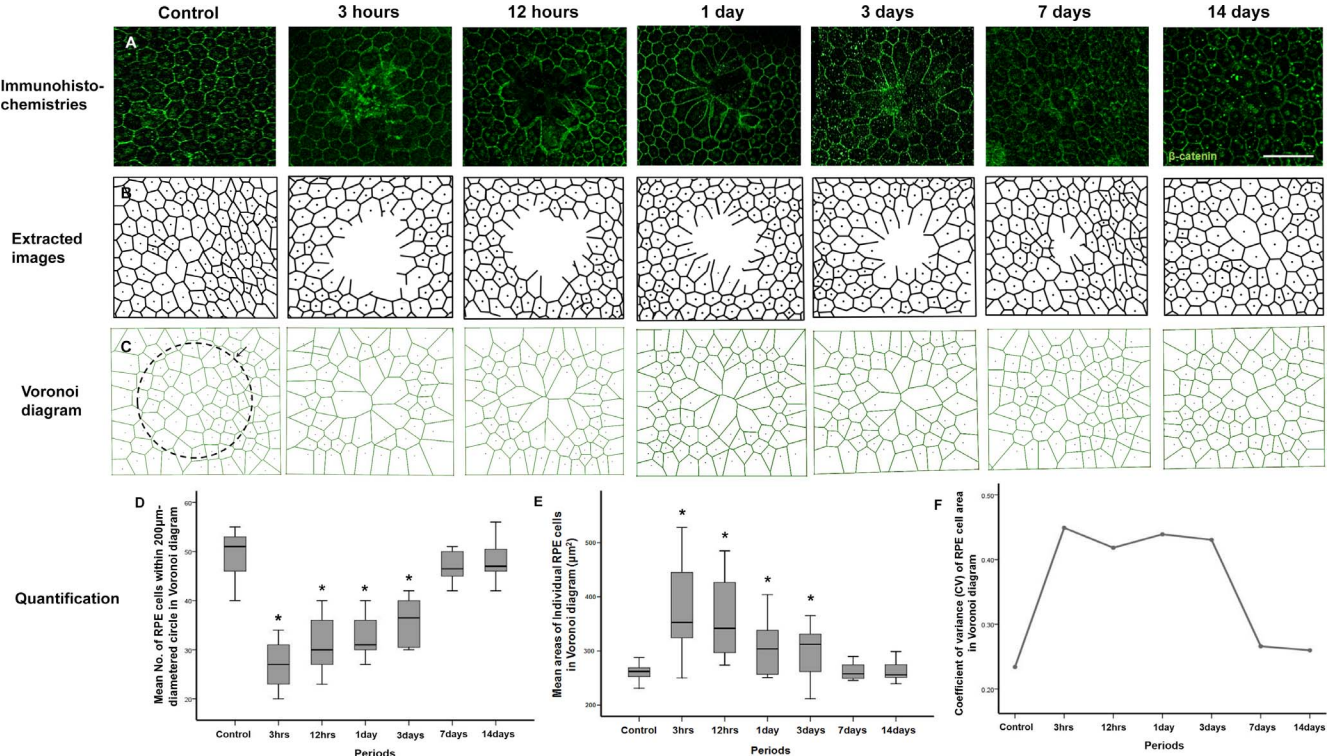


FIGURE 5. Longitudinal changes in β -catenin-labeled RPE cells in the laser-treated lesions in a whole-mount view (A) after SRT. Extracted images (B) clearly show the RPE cell walls. A Voronoi diagram was used to estimate the areas of RPE cells in SRT-treated lesions (C), and the mean numbers and areas of RPE cells with β -catenin labeling located within a circle 200 μm in diameter are shown (arrow). There were significantly fewer RPE cells after SRT, but they recovered after 7 days (D). The average areas of individual RPE cells were significantly increased (E) with high variation (F) after SRT (G), but they normalized after 7 days (E) with uniformity in cell size (F). Data are expressed as the mean $\pm$ SEM. $*P < 0.001$ by Student's *t*-test with Bonferroni correction. Scale bar: 100 μm .

tion was also observed after merging the distribution of both transcription factors (Figs. 6A, 6B).

Western blot analysis was also performed to quantify the time-dependent changes in the expression of MiTF and Otx2 following SRT (Figs. 6C-E). The expression of MiTF was

significantly increased 3 days after treatment compared with the control, and elevated expression was relatively maintained until 14 days after SRT (Fig. 6D). The level of Otx2 expression also showed a significant increase 3 days after the treatment. The expression then decreased gradually; however, it was still

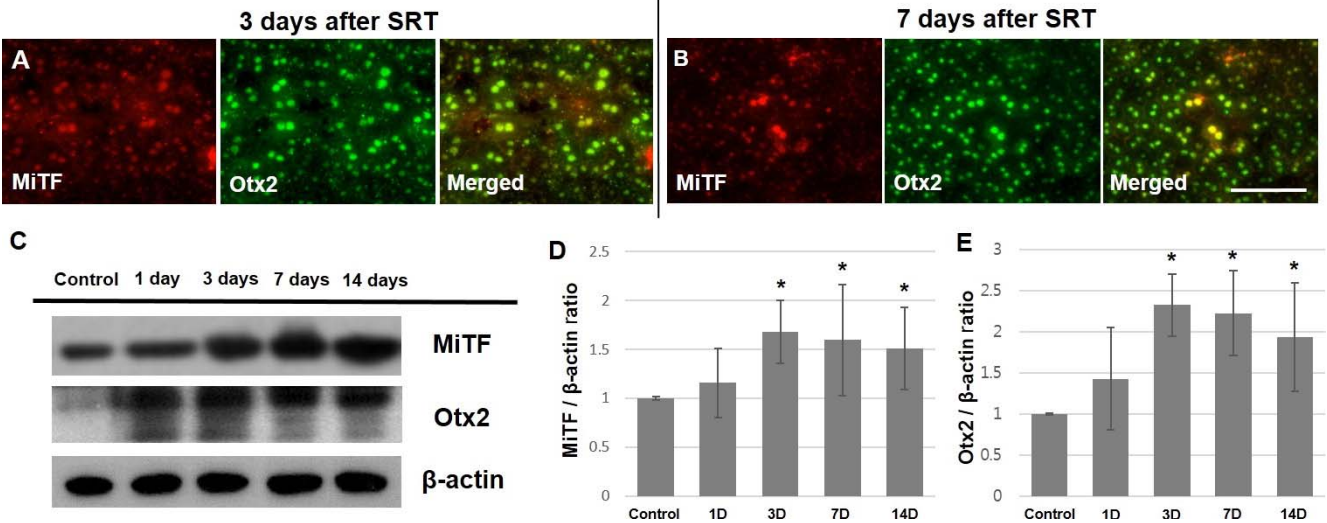


FIGURE 6. Expression patterns of transcription factors associated with RPE cells, MiTF and Otx2, following SRT. Colocalization of MiTF and Otx2 was observed at 3 days (A) and 7 days (B) after SRT. Western blotting (C) and quantified results (D, E) of transcription factors associated with RPE cells, MiTF and Otx2, following SRT. The expression of MiTF (C, D) and Otx2 (C, E) was significantly higher than that in the controls after 3 days. Additionally, the elevated levels were maintained until 14 days after SRT (B, C). Individual data were semiquantified by densitometry and normalized to the β -actin level. Data are also described as mean $\pm$ SEM. $*P < 0.0083$ by Student's *t*-test with Bonferroni correction. Scale bar: 50 μm .

significantly higher than the control level at 14 days after SRT (Fig. 6E). After 14 days, the immunoreactivity levels of both MiTF and Otx2 were significantly higher than those of the control ($P = 0.001$ and $P = 0.03$, respectively). Overall, the levels of MiTF and Otx2 were significantly elevated 3 days after SRT, and their increased levels were sustained for 2 weeks.

DISCUSSION

The RPE cell plays a fundamental role in maintaining retinal structure and visual function.^{24,25} Because RPE cell dysfunction has been considered an important component of the pathophysiology of several macular diseases,^{24–27} the RPE is a primary therapeutic target for laser photocoagulation.^{6,28} Generally, in laser treatment, the expected therapeutic effect is not from the laser lesion itself but from the subsequent biological reaction in the retinal tissue.²⁹ Theoretically, laser photocoagulation can be used to induce scar formation in certain retinal diseases, for example, peripheral retinal holes and diabetic retinopathy. However, for pathologies such as DME and CSC, especially macular problems, selective damage to and subsequent restoration of RPE is necessary to increase the RPE pump function and overall metabolic activity.^{2,4} In contrast to cwPC, which induces collateral thermal damage in the overlying neural retina, SRT with microsecond pulses can confine the laser energy to the RPE layer.^{9,12} It is known that the spatial extent of the temperature increase can be diminished through use of a multiple pulsed laser with a short duration and low repetition rate.^{2,30}

In the present study, we investigated the characteristics of retinal damage induced by SRT and elucidated the longitudinal changes in RPE cells following SRT in the eyes of mice. A number of studies have described specific cellular responses to laser treatment, whereas only a limited number of studies have demonstrated the cellular response following SRT. Moreover, longitudinal changes in RPE cell proliferation and differentiation have not been previously investigated. In our previous experiment, RPE proliferative activity following cwPC was elevated during the first week, and cell apoptosis was prominent during the first 24 hours.¹⁹ Because we also expected to observe obviously TUNEL-positive cells after 24 hours of SRT-like cwPC lesions in the previous study, the TUNEL assay was performed 24 hours after SRT application to evaluate the pattern of cell death. Cell death following SRT was not identified in the neural retina, and the distribution of cell death was limited to the RPE layer. In contrast, TUNEL-positive cells were abundantly detected not only in the RPE layer but also in the ONL of cwPC-treated eyes. These results support the safety of SRT for the neural retina and selectivity for the RPE, and are consistent with the outcomes from previous studies on the safety of SRT.^{12,14,18,31}

Several studies have confirmed the safety of SRT. Park et al.¹⁸ showed that the structures of photoreceptors were preserved and that RPE was disrupted in rabbit eyes after SRT using optical coherence tomography (OCT). Framme et al.³¹ also used OCT to show the selectivity of SRT for RPE in humans. In both studies, the authors reported that thickening of the RPE layer was observed within a period of 3 or 4 weeks, and they assumed that the thickening might be due to RPE proliferation. To verify this hypothesis, the current study investigated RPE cell proliferation as well as differentiation changes over time in mice using advanced surrogate markers. In the EdU assay, cells with proliferative potential in the RPE layer were identified, and the number of EdU-positive cells showed a peak during 4 to 7 days after SRT, suggesting that SRT could induce morphologic changes and stimulate the proliferation of RPE cells to fill the gap caused by the application of

laser. However, these results were slightly different from the results from our previous study using cwPC, in which peak proliferation occurred during the first 3 days of laser application. We speculate that the reason for this difference is that SRT induces less damage to the RPE layer compared to cwPC, and that the cell's nature to cover the damaged site is migrating into the lesion before actual proliferation.

Indeed, β -catenin staining showed that hexagonality was compromised after SRT and that subsequently, RPE cells expanded in size within the targeted location during the early time intervals (3–24 hours). Also, we found that β -catenin expression in the membranous portion of elongated RPE cells was more aberrant 1 day after SRT. We assumed that this may have resulted from the loosening of the cell-cell connections for further cell migration. A Voronoi diagram, which was used to quantify cell hexagonality, revealed that the number and hexagonality of the RPE cells were restored 1 week after SRT, indicating that morphologic changes and cell proliferation mostly occurs during the first week after laser application.

Although mouse models, which have a physiology and anatomy similar to those of humans, have served as a powerful tool to investigate the retinal pathophysiology in people, the melanin content of the mouse retina is still quite different from that of humans. Although the C57BL/6J mice used in this study have a pigmented retina, the distribution of melanin pigments in mouse retina is different from that in humans. This difference in melanin content may lead to different heat absorption and diffusion characteristics, especially in experiments that use laser treatment. Nonetheless, mouse models are useful for rapid histopathologic analysis and a systematic approach at the cellular level, and they can provide the possibility of expanding this study to examine the safety of SRT in mouse models of DME, CSC, and AMD.^{32,33}

We investigated whether the proliferation and migration of RPE cells could be induced by SRT. Retinal pigment epithelial cells form a stable monolayer without migration or proliferation properties in the normal state; however, they can start to migrate and proliferate in disease states.^{34–36} Microphthalmia-associated transcription factor and Otx2 have been considered key regulatory transcription factors for RPE specification and differentiation, and they can be upregulated after damage to the RPE layer.^{35–37} Microphthalmia-associated transcription factor may be involved in promoting melanin pigment synthesis and regulating cell proliferation.^{36,37} Moreover, Otx2 reportedly has a role in maintaining the identity and survival of adult retinal differentiated cells, and elevated expression of Otx2 has been related to reentry of RPE cells into mitosis.³⁸ In the current study, the time-dependent expression of MiTF and Otx2 was assessed after SRT application. Significantly upregulated expression of both the RPE-specific transcription factors was detected at 3 days in SRT-treated lesions.

Interestingly, we noted that transcription factors related to RPE cell differentiation (MiTF and Otx2) were upregulated 3 days after SRT and remained elevated for 14 days after lesioning, while the proliferative potential of the RPE cells (EdU levels) peaked at 7 days and then declined. Also, during the late phase of the experiment, the RPE cells adjacent to laser-treated lesions gradually became EdU positive, indicating that the RPE response to laser treatment might proceed with temporal and spatial divergence. Although it is not possible to elucidate the exact reason for the difference in the time course between RPE differentiation and proliferation through this study, we assume that the EdU injection methods might induce these divergences. EdU injections were performed periodically in this study. Thus, the study showed EdU expression only during each interval, not the total cumulative EdU expression from the onset of SRT application. Retinal pigment epithelium

proliferation did not occur simultaneously in all RPE cells within laser-treated lesions, and different RPE cells showed active proliferative potentials over different periods. Therefore, RPE-specific transcription factors including Mitf and Otx2 could be upregulated in different RPE cells in different time periods to replace laser-injured lesions by RPE cells with regular size and hexagonality. These temporal and spatial divergences might induce the difference in the time course between RPE proliferation and differentiation.

In conclusion, SRT induced selective RPE cell death without collateral thermal damage in photoreceptor cells. The results suggest that SRT-treated RPE areas recovered with expansion and proliferation of the surrounding RPE cells, and the cells that were found in SRT-treated lesions showed RPE cell characteristics. Consequently, SRT can induce RPE cell regeneration to the pretreatment level and may be introduced as a safe and effective treatment option for several diseases in which RPE cell abnormalities are considered the primary cause.

Acknowledgments

Supported by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant HT13C0005), and by the Soonchunhyang University Research Fund.

Disclosure: **H.D. Kim**, None; **S.Y. Jang**, None; **S.H. Lee**, None; **Y.S. Kim**, None; **Y.-H. Ohn**, None; **R. Brinkmann**, None; **T.K. Park**, None

References

- Early Treatment Diabetic Retinopathy Study Research Group. Treatment techniques and clinical guidelines for photocoagulation of diabetic macular edema: Early Treatment Diabetic Retinopathy Study Report Number 2. *Ophthalmology*. 1987; 94:761-774.
- Roider J, Liew SH, Klatt C, et al. Selective retina therapy (SRT) for clinically significant diabetic macular edema. *Graefes Arch Clin Exp Ophthalmol*. 2010;248:1263-1272.
- Prahs P, Walter A, Regler R, et al. Selective retina therapy (SRT) in patients with geographic atrophy due to age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 2010;248: 651-658.
- Elsner H, Pörksen E, Klatt C, et al. Selective retina therapy in patients with central serous chorioretinopathy. *Graefes Arch Clin Exp Ophthalmol*. 2006;244:1638-1645.
- Jain A, Blumenkranz MS, Paulus Y, et al. Effect of pulse duration on size and character of the lesion in retinal photocoagulation. *Arch Ophthalmol*. 2008;126:78-85.
- Marshall J. Thermal and mechanical mechanisms in laser damage to the retina. *Invest Ophthalmol Vis Sci*. 1970;9:97-115.
- Marshall J, Hamilton AM, Bird AC. Histopathology of ruby and argon laser lesions in monkey and human retina. A comparative study. *Br J Ophthalmol*. 1975;59:610-630.
- Schatz H, Madeira D, McDonald HR, Johnson RN. Progressive enlargement of laser scars following grid laser photocoagulation for diffuse diabetic macular edema. *Arch Ophthalmol*. 1991;109:1549-1551.
- Brinkmann R, Roider J, Birngruber R. Selective retina therapy (SRT): a review on methods, techniques, preclinical and first clinical results. *Bull Soc Belge Ophthalmol*. 2006;302:51-69.
- Koinzer S, Elsner H, Klatt C, et al. Selective retina therapy (SRT) of chronic subfoveal fluid after surgery of rhegmatogenous retinal detachment: three case reports. *Graefes Arch Clin Exp Ophthalmol*. 2008;246:1373-1378.
- Kim HD, Han JW, Ohn YH, et al. Functional evaluation using multifocal electroretinogram after selective retina therapy with a microsecond-pulsed laser. *Invest Ophthalmol Vis Sci*. 2014;56:122-131.
- Brinkmann R, Huttmann G, Rogener J, Roider J, Birngruber R, Lin CP. Origin of retinal pigment epithelium cell damage by pulsed laser irradiance in the nanosecond to microsecond time regimen. *Lasers Surg Med*. 2000;27:451-464.
- Neumann J, Brinkmann R. Cell disintegration by laser-induced transient microbubbles and its simultaneous monitoring by interferometry. *J Biomed Opt*. 2006;11:041112.
- Schuele G, Rumohr M, Huttmann G, et al. RPE damage thresholds and mechanisms for laser exposure in the microsecond-to-millisecond time regimen. *Invest Ophthalmol Vis Sci*. 2005;46:714-719.
- Neumann J, Brinkmann R. Self-limited growth of laser-induced vapor bubbles around single micro-absorbers. *Appl Phys Lett*. 2008;93:033901.
- Neumann J, Brinkmann R. Boiling nucleation on melanosomes and microbeads transiently heated by nanosecond and microsecond laser pulses. *J Biomed Opt*. 2005;10:024001.
- Seifert E, Roh YJ, Fritz A, et al. Automatic irradiation control by an optical feedback technique for selective retina treatment (SRT) in a rabbit model. *Proc SPIE*. 2013;8803:880303.
- Park YG, Seifert E, Roh YJ, et al. Tissue response of selective retina therapy by means of a feedback-controlled energy ramping mode. *Clin Experiment Ophthalmol*. 2014;42:846-855.
- Lee SH, Kim HD, Park YJ, et al. Time dependent changes of cell proliferation after laser photocoagulation in mouse chorioretinal tissue. *Invest Ophthalmol Vis Sci*. 2015;56:2696-2708.
- Bucht C, Söderberg P, Manneberg G. Simulation of specular microscopy images of corneal endothelium, a tool for control of measurement errors. *Acta Ophthalmol*. 2011;89:e242-e250.
- Kim Y, Kim B, Park H, et al. Image analysis algorithm for the corneal endothelium. *J Biomed Eng Res*. 2006;27:125-130.
- Adjianto J, Castorino JJ, Wang ZX, et al. Microphthalmia-associated transcription factor (MITF) promotes differentiation of human retinal pigment epithelium (RPE) by regulating microRNAs-204/211 expression. *J Biol Chem*. 2012;287: 20491-20503.
- Azzolini C, Pagani IS, Pirrone C, et al. Expression of VEGF-A, Otx homeobox and p53 family genes in proliferative vitreoretinopathy. *Mediators Inflamm*. 2013;2013:857380.
- Young RW. Pathophysiology of age-related macular degeneration. *Surv Ophthalmol*. 1987;31:291-306.
- Eom Y, Oh J, Kim SW, Huh K. Systemic factors associated with central serous chorioretinopathy in Koreans. *Korean J Ophthalmol*. 2012;26:260-264.
- Marmor MF. New hypotheses on the pathogenesis and treatment of serous retinal detachment. *Graefes Arch Clin Exp Ophthalmol*. 1988;226:548-552.
- Weinberger D, Fink-Cohen S, Gatton DD, et al. Non-retinovascular leakage in diabetic maculopathy. *Br J Ophthalmol*. 1995; 79:728-731.
- Mainster MA, White TJ, Tips JH, et al. Retinal temperature increases produced by intense light sources. *J Opt Soc Am*. 1970;60:264-270.
- Framme C, Kobuch K, Eckert E, et al. RPE in perfusion tissue culture and its response to laser application. Preliminary report. *Ophthalmologica*. 2002;216:320-328.
- Framme C, Schuele G, Kobuch K, et al. Investigation of selective retina treatment (SRT) by means of 8 ns laser pulses in a rabbit model. *Lasers Surg Med*. 2008;40:20-27.
- Framme C, Walter A, Prahs P, et al. Structural changes of the retina after conventional laser photocoagulation and selective

- retina treatment (SRT) in spectral domain OCT. *Curr Eye Res.* 2009;34:568-579.
32. Chang B, Hawes NL, Hurd RE, et al. Mouse models of ocular disease. *Vis Neurosci.* 2005;22:587-593.
 33. Chang B. Mouse models for studies of retinal degeneration and disease. *Methods Mol Biol.* 2013;935:27-39.
 34. Marshall J. Interactions between sensory cells, glial cells and the retinal pigment epithelium and their response to photocoagulation. *Dev Ophthalmol.* 1981;2:308-317.
 35. Liu Y, Ye F, Li Q, et al. Zeb1 represses Mitf and regulates pigment synthesis, cell proliferation, and epithelial morphology. *Invest Ophthalmol Vis Sci.* 2009;50:5080-5088.
 36. Ma X, Pan L, Jin X, et al. Microphthalmia-associated transcription factor acts through PEDF to regulate RPE cell migration. *Exp Cell Res.* 2012;318:251-261.
 37. Westenskow P, Piccolo S, Fuhrmann S. Beta-catenin controls differentiation of the retinal pigment epithelium in the mouse optic cup by regulating Mitf and Otx2 expression. *Development.* 2009;136:2505-2510.
 38. Torero Ibad R, Rhee J, Mrejen S, et al. Otx2 promotes the survival of damaged adult retinal ganglion cells and protects against excitotoxic loss of visual acuity in vivo. *J Neurosci.* 2011;31:5495-5503.