Laser photocoagulation has been performed for many years, and it remains an effective method of therapy for retinal diseases such as age-related macular degeneration and diabetic macular edema. It involves tissue denaturation at the irradiation site as a result of energy absorption, which is converted into heat. Retinal pigment epithelium (RPE) cells are the primary sites affected by the laser because they are responsible for light absorption. Beyond light absorption, they also play a key role in visual function. Given that the well-known major retinal diseases, such as age-related macular degeneration, diabetic macular edema, and retinitis pigmentosa, are related to changes in RPE function, most studies have focused on examining changes in the RPE layer after laser photocoagulation.

After laser photocoagulation, the epithelial sheet is repaired by a morphologically heterogeneous population of regenerated RPE cells. The regeneration occurs via cell proliferation and epithelial-mesenchymal transition (EMT), due to intrinsic transcription factors and extrinsic cell signaling. Wnt signaling is among the cell signaling events that play an important role in regulating cell proliferation and EMT in various tissues. Of the 19 Wnts identified in mammals, several are expressed in damaged retina, and activation of Wnt/β-catenin signaling reportedly regulates EMT with cell proliferation in RPE sheets upon disruption of cell junctions. However, it remains unclear whether Wnt signaling promotes regeneration in laser-photocoagulated RPE.

**Purpose.** Laser photocoagulation of retinal pigment epithelium (RPE) is used to stimulate the regenerative processes of the RPE. However, the molecular mechanisms that control RPE proliferation and the epithelial-mesenchymal transition (EMT) during regeneration remain poorly understood. We investigated the role of Wnt/β-catenin signaling in the regeneration of mouse RPE after laser photocoagulation.

**Methods.** C57BL/6J mice were photocoagulated unilaterally. To determine the β-catenin-dependent Wnt signal transduction in the photocoagulated RPE, the expression levels of Wnts, β-catenin, and their target genes were analyzed using real-time PCR and Western blotting. Retinal pigment epithelium proliferation and EMT were determined by 5-ethyl-2'-deoxyuridine (EdU) incorporation assay and by profiling expression of EMT markers, respectively, in eyes injected intravitreally with a Wnt/β-catenin signaling antagonist, Dkk-1, after laser photocoagulation.

**Results.** Expression of several of the 19 Wnt genes was significantly increased in laser-treated RPE. The expression levels of β-catenin signaling target genes cyclin D1, Otx2, and Mitf were higher in laser-treated RPE than in control RPE. The number of EdU-positive cells in the laser-treated area was significantly lower in the Dkk-1-injected group than in the laser-treated group or laser-treated + vehicle-injected group. There were more Otx2- and Mitf-positive cells after laser photocoagulation and markedly fewer in the Dkk-1-injected group. Otx2- and Mitf-positive cells were colocalized with EdU-positive cells. The EMT markers vimentin and α-smooth muscle actin (α-SMA) were upregulated in the laser-treated area and significantly downregulated in the Dkk-1-injected group.

**Conclusions.** Laser photocoagulation activates a Wnt/β-catenin signal transduction pathway, promoting both RPE proliferation and EMT. Wnt/β-catenin signaling also upregulates the expression of Otx2 and Mitf, key transcription factors in RPE formation. Our results demonstrate an important role for Wnt/β-catenin signaling in RPE proliferation and EMT, and suggest that manipulating Wnt/β-catenin signaling may have therapeutic potential for RPE regeneration.

Keywords: retinal pigment epithelium (RPE), Wnt/β-catenin signaling pathway, laser photocoagulation, epithelial-mesenchymal transition (EMT)
Mif and Otx2 are key transcription factors in RPE formation in the developing eye.\textsuperscript{11-15} Disruption of either gene, similar to genetic ablation of the RPE, results in microphthalmia and coloboma during murine eye development.\textsuperscript{11,14,16,17} Furthermore, Otx2 regulates the human tyrosinase gene, by interacting with Mif, for melanin synthesis in the adult RPE. Mitf and Otx2 in the RPE protects against light toxicity and against cytotoxic effects. A recent study demonstrated that Otx2 and Mitf are direct transcription targets of Wnt/β-catenin signaling. These results suggest the possibility that the Wnt/β-catenin signal pathway, via the transcriptional activation of Otx2 and Mitf, is involved in RPE regeneration.\textsuperscript{19}

**Materials and Methods**

**Animals**

Eight-week-old C57BL/6j mice (Orient Bio, Inc., Sungnam, South Korea) were housed in a temperature- and humidity-controlled room under a 12/12-hour light/dark cycle and were provided with food and water ad libitum. All animals were kept and experiment procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of Soonchunhyang University Bucheon Hospital.

**Laser Photocoagulation**

Animals were anesthetized by intraperitoneal (IP) injection of a mixture of 40 mg/kg zolazepam/tiletamine ( Zoletil; Virbac, Carros Cedex, France) and 5 mg/kg xylazine (Rompun; Bayer Healthcare, Leverkusen, Germany), followed by pupil dilation with 0.5% tropicamide and 2.5% phenylephrine (Mydrin-P; Santen, Emeryville, CA, USA). Only the right eyes of mice were used for laser treatment. Laser photocoagulation (200-μm spot size, 0.02-second duration, 100-mW laser power) was performed with the slit-lamp delivery system of a PASCAL diode laser (Topcon Medical Laser Systems, Inc., Santa Clara, CA, USA), a frequency-doubled neodymium-doped yttrium aluminum garnet (Nd:YAG) laser diode with a wavelength of 532 nm. To view the mouse retina, a handheld coverslip was used as a contact lens with the application of 0.5% methylcellulose (Genteal; Novartis, East Hanover, NJ, USA) in front of the eyes. The mice were divided into four groups: control, laser treated, laser treated + vehicle, and laser treated + Dickkopf-1 (Dkk-1) (R&D Systems, Minneapolis, MN, USA), a Wnt signaling inhibitor. For histological examinations, 8 or 10 laser spots were distributed in a concentric pattern around the optic nerve head of the eye (Fig. 1). For Western blotting and real-time (RT)-PCR analysis, 50 laser shots were applied around the optic nerve head. If a lesion produced a gaseous bubble, indicating rupture or hemorrhage of Bruch’s membrane, the animal was excluded from the experiment.

**Intraperitoneal Injection of 5-Ethynyl-2′-Deoxyuridine**

Intraperitoneal injection of 5-ethyl-2′-deoxyuridine (EdU; 10 mg/kg; Invitrogen, Carlsbad, CA, USA) was performed to detect cell proliferation induced by the laser treatment. Mice were given an IP injection of EdU in phosphate-buffered saline (PBS) twice a day for 7 days after the laser photocoagulation.

**Fundus Fluorescein Angiography and Infrared Reflectance Imaging**

Immediately after and 7 days after the laser photocoagulation, fundus fluorescein angiographic (FFA) and infrared reflectance (IR) images were taken with a confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph 2; Heidelberg Engineering, Heidelberg, Germany; Fig. 1). Specifically, animals were anesthetized and pupils were dilated. Fluorescein and IR images were captured 3 to 5 minutes after IP injection of 0.1 mL 2% fluorescein sodium (Fluoresceite; Akorn, Lake Forest, IL, USA) and used to identify any vascular leakage at the laser lesion, indicating the formation of choroidal neovascularization.

**Intravitreal Administration of Dickkopf-1 (Dkk-1)**

In total, 1 μL Dkk-1 (30 ng/μL) was injected into the vitreous cavity using a 10-μL Hamilton syringe with a 33-gauge beveled-tip needle (Hamilton, Reno, NV, USA). Only 1 μL phosphate-buffered saline (PBS) was injected as a control vehicle. The pupil was dilated by topical application of phenylephrine (2.5%) and tropicamide (1.0%). A sclerotomy was created approximately 0.5 mm posterior to the limbus with a needle tip. All intravitreal injections were performed using a surgical microscope with a condensing lens system and a small plastic ring filled with 0.5% methylcellulose (GenTeal; Novartis, Basel, Switzerland), which allowed for direct visualization of the fundus during the procedure.

Animals in the control group were not treated with the laser or with Dkk-1. All animals excluding the control group were laser treated and killed at 7 days after the laser photocoagulation. The vehicle group received 1 μL PBS immediately after the laser treatment, and another group of laser-treated animals received Dkk-1, an inhibitor of Wnt signaling.

**Tissue Preparation for Whole Mounts**

At the end of the EdU injection period, mice were deeply anesthetized with an intraperitoneal injection of a 4:1 mixture of zolazepam/tiletamine (80 mg/kg) and xylazine (10 mg/kg), and then were intracardially perfused with 0.1 M PBS containing 150 U/mL heparin, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). For RPE whole mounts, eyeballs were enucleated and the anterior segments, including the cornea, lens, and neural retina, were removed by cutting through the limbal cornea. The RPE-choroid complex was then fixed with 4% PFA in 0.1 M PB at pH 7.4 for 2 hours and prepared with four equidistant cuts.

**RNA Isolation and RT-PCR Analysis**

After the mice were deeply anesthetized, the eyeball was enucleated and the cornea, lens, and neural retina were removed. Total RNA was prepared from the RPE-choroid complex using TRizol reagent (Invitrogen, Tokyo, Japan). RNA (2 μg) was reverse transcribed using Superscript III (Invitrogen, Tokyo, Japan). Quantitative real-time PCR was conducted using SYBR Green kits (Invitrogen, Tokyo, Japan), and samples were quantified by amplifying Gapdh as an internal control for each sample. Polymerase chain reaction amplification was performed using specific primer pairs, listed in the Table.

**Western Blot Analysis**

Mice were anesthetized and perfused intracardially with 10 mL cold Dulbecco’s phosphate buffered saline (DPBS; pH 7.0). Following enucleation of the eyeballs, the cornea, lens, and neural retina were removed and 80 μL lysis buffer was added to the RPE-choroid complex of each mouse. Then the extracts were sonicated, placed on ice for 50 minutes, and centrifuged (17,000 g, 20 minutes, 4°C). The supernatant was collected and the protein concentration in the homogenate was measured.
with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s protocol. An equal amount of total protein (20 μg) from each sample was resolved by SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 hour at room temperature and incubated with anti-vimentin (1:1000; Abcam, Cambridge, UK) and α-smooth muscle actin (α-SMA) (1:1000; Sigma-Aldrich Corp., St. Louis, MO, USA) overnight at 4°C. Then the membrane was incubated with goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 90 minutes at room temperature.

### Table. List of Primers Used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Wnt1</td>
<td>5'-CTTCCTGATGAACTTCACA-3'</td>
<td>5'-ACTGTAAGTGCAAGATTTGG-3'</td>
</tr>
<tr>
<td>Wnt2b</td>
<td>5'-GGAGATTCTGAGCTGAGAT-3'</td>
<td>5'-CACACCCATCTCGTCTTTTA-3'</td>
</tr>
<tr>
<td>Wnt3</td>
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<tr>
<td>Wnt3a</td>
<td>5'-CAGGAACTAGCTGAGATACA-3'</td>
<td>5'-GAGACCATTCTCCAAATTCA-3'</td>
</tr>
<tr>
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<td>5'-TAGCATCTGGAGCCAGCT-3'</td>
</tr>
<tr>
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<td>5'-TGCCAGTTGATATCTGACC-3'</td>
</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Wnt7b</td>
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<td>5'-GGCTTCTGTTGATCGCTAGT-3'</td>
</tr>
<tr>
<td>Wnt10b</td>
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<td>5'-ATCGATAAAGATGGCTGTCG-3'</td>
</tr>
<tr>
<td>Gapdh</td>
<td>5'-ACGGCAATTTCAACGCGACTG-3'</td>
<td>5'-GGTCATGAGCCCTCCACAAT-3'</td>
</tr>
</tbody>
</table>
temperature. The blot was developed with the enhanced chemiluminescence system (ECL; Bio-Rad Laboratories, Hercules, CA, USA), and densitometry of the bands was performed by drawing the regions of the bands of interest. Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA [in the public domain]).

**Immunohistochemistry and EdU Assay Analysis**

Using 0.1% Triton X-100 in PBS (PBST; Sigma-Aldrich Corp.), samples were washed three times for 10 minutes each and blocked with 5% goat serum in PBST for 1 hour at room temperature. After blocking, the samples were incubated with primary antibodies overnight at 4°C. On the following day, the samples were incubated with secondary antibodies for 1 hour at room temperature in the dark. Then EdU immunolabeling was carried out using an EdU imaging kit (Click-it EdU imaging kit; Invitrogen, Carlsbad, CA, USA) for 1 hour in the dark, and the samples were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark). The results were examined using a confocal microscope (LSM 510 META; Carl Zeiss, Oberkochen, Germany) at ×200 magnification, and images were captured using image-capture software (LSM Image Browser; Carl Zeiss, Inc.). The following antibodies were used: anti-β-catenin (1:2000; Sigma-Aldrich Corp.), anti-cyclin D1 (1:200; Thermo Scientific, Fremont, CA, USA), anti-vimentin (1:1000; Abcam), α-SMA (1:1000; Sigma-Aldrich Corp.), Otx2 (1:1000; Millipore, Temecula, CA, USA), and Mitf (1:1000; Exalpa, Shirley, MA, USA). Alexa Fluor-488 anti-rabbit, Alexa Fluor-488 anti-mouse, and Alexa Fluor-568 anti-mouse (1:2000; Molecular Probes, Grand Island, NY, USA) were used as the secondary antibodies.

**Image Analysis and Statistical Analysis**

A quantitative comparison of fluorescence intensity was made using the ImageJ software. The Wilcoxon signed-rank test was used to compare fluorescence intensities between experimental groups. The numbers of EdU-positive cells were counted and compared for each experimental group. The Kruskal-Wallis test with a post hoc analysis was conducted to compare the number of EdU-positive cells among the three groups. Statistical analyses were conducted using the SPSS software (ver. 20.0 for Windows; SPSS, Inc., Chicago, IL, USA). A P value < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Wnt/β-Catenin Signaling Is Activated After Laser Photocoagulation in Mouse RPE**

To examine the function of Wnt signaling in RPE after laser photocoagulation, we performed laser photocoagulation concentrically around the optic nerve head. Laser burns were identified using FFA and IR images immediately after laser photocoagulation and 7 days after photocoagulation (Fig. 1). In FFA images, discrete hyperfluorescent laser lesions were located around the optic nerve head without any definite leakage, and IR images showed laser sites with central hyperreflectance surrounded by peripheral hyporeflectance. At 7 days after laser treatment, hyperfluorescence in the previously visible laser spots was not found in FFA images, suggesting that the blood-retinal barrier was recovering in the RPE layer, while IR images still showed hyperreflectance at the laser sites.

We assessed the cytoplasmic levels of β-catenin proteins and the protein levels of cyclin D1, a target of β-catenin signaling, in RPE 7 days after the laser photocoagulation to determine the activation of Wnt/β-catenin signaling. Immunohistochemical staining of laser-treated RPE cells showed the accumulation of β-catenin in the cytoplasm (Fig. 2B1), whereas the expression of β-catenin in control RPE cells was limited to only the cell-to-cell border (Fig. 2A). A quantitative comparison of β-catenin expression is presented in Figure 2E. The calculated fluorescence intensities obtained from the laser-treated group were statistically significantly higher than those in the control group. The laser-treated group also showed a significant increase in β-catenin expression (P < 0.05). In the control, RPE cells were weakly positive for cyclin D1 in the whole-mount preparations. However, cyclin D1-positive cells were brighter and stronger than nonphotocoagulated RPE cells within the laser-treated area (Figs. 2C, 2D1). Fluorescence intensity significantly increased in cyclin D1 in the laser-treated group versus the control group (P < 0.05; Fig. 2F).

Quantitative real-time PCR analysis also showed that mRNA levels of Wnt1, Wnt2b, Wnt3, Wnt5a, Wnt7a, and Wnt10b were significantly increased in laser-treated RPE compared to control RPE (Fig. 2G). Taken together, these results indicate that the Wnt/β-catenin signal transduction pathway was activated in photocoagulated RPE cells.

**Wnt/β-Catenin Signaling Pathway Induces RPE Cell Proliferation After Laser Photocoagulation**

In the laser-treated areas shown in Figures 2B3 and 2D3, we further found EdU-positive cells, indicative of proliferating cells. This suggests that cell proliferation occurred in the RPE layer after laser photocoagulation via a Wnt/β-catenin signaling pathway. To test this, we performed an intravitreal injection with a Wnt antagonist, Dkk-1, following laser photocoagulation, and then assessed EdU staining in RPE whole mounts. The proliferative activity in the RPE layer after laser photocoagulation was determined by counting the number of EdU-labeled cells in the laser-treated area. There were significantly more EdU-positive cells 7 days after photocoagulation (106.2 ± 7.8 cells/field) compared to the control group (0 cells/field; P < 0.001; Figs. 3A, 3B). Furthermore, the Dkk-1 injection significantly reduced the number of EdU-positive cells (52.3 ± 9.1 cells/field) versus the laser treatment + vehicle-injected group (101.1 ± 9.4 cells/field; P < 0.001; Figs. 3C, 3D). No significant difference was observed between the laser-treated group and the laser treatment + vehicle-injected group (P > 0.05).

Taken together, these results show that cell proliferation was decreased as a result of the inhibition of Wnt/β-catenin signaling. Thus, we conclude that Wnt/β-catenin signaling was involved in cell proliferation in the RPE layer after laser photocoagulation.

To determine whether the proliferating cells took on characteristics specific to RPE cells, immunolabeling for Otx2 and Mitf, known RPE cell-specific markers, was performed. As shown in Figures 4A through 4C, EdU-positive cells colocalized with Otx2-positive cells, which were brighter and larger than the surrounding RPE cells. There were also more Mitf-positive cells within the laser-treated area and they colocalized with EdU-positive cells (Figs. 4D-F), indicating that many of proliferated cells after laser photocoagulation expressed characteristics of RPE cells.

**Wnt/β-Catenin Signaling Upregulates the Expression of Otx2 and Mitf After Laser Photocoagulation**

To examine whether the activation of Wnt/β-catenin signaling regulated the expression of Otx2 and Mitf, we measured the
expression of Otx2 and Mitf after laser photocoagulation. Control RPE cells were weakly positive for Otx2 in the whole-mount preparations (Fig. 5A). Mitf was present throughout the RPE in controls (Fig. 5E). Seven days after photocoagulation, there were markedly more Otx2-positive cells, which were brighter and stronger than the surrounding RPE cells, within the laser-treated area (Fig. 5B). There were also more Mitf-positive cells within the laser-treated area (Fig. 5F). Quantification of relative fluorescence intensity showed significantly increased intensity of Otx2 and Mitf in the laser-treated group compared to the control group ($P < 0.05$; Figs. 5I, 5J).

Immunohistochemical examination of the Dkk-1-injected group showed a decrease in the number of Otx2- and Mitf-positive cells in the laser-treated area versus the vehicle-

**Figure 2.** Retinal laser photocoagulation activates Wnt/β-catenin signal transduction in RPE cells. (A) Normal RPE cells retained β-catenin expression at the cell-to-cell border. (B1) Immunohistochemical staining of β-catenin revealed accumulation in the cytoplasm in laser-treated RPE. (B2, B3) Many EdU-positive cells in the laser-treated area colocalized with β-catenin expression. (C) Control RPE cells were weakly positive for cyclin D1. (D1) The cyclin D1-positive cells were brighter and stronger in laser-treated RPE than in control RPE. (D2, D3) Many of the EdU-positive cells in the laser-treated area colocalized with cyclin D1-positive cells. (E, F) Mean fluorescence intensity calculated using ImageJ for β-catenin and cyclin D1. Quantifying relative fluorescence intensity showed significantly increased intensity in the laser-treated group compared to the control group. (G) Wnt gene expression was determined by quantitative real-time PCR analysis of control or laser-treated RPE. Each assay was performed in triplicate. Wnt1, Wnt2b, Wnt3, Wnt5a, Wnt7a, Wnt7b, and Wnt10b were significantly increased in laser-treated RPE (mean ± SD, $n = 3$, *$P < 0.05$). Scale bars: 100 μm.
injected group (Figs. 5C, 5D, 5G, 5H). Fluorescence intensity analysis also revealed that Dkk-1 injection significantly reduced the fluorescence intensity of Otx2 and Mitf ($P < 0.05$; Figs. 5I, 5J). These results suggest that the Wnt/b-Catenin signaling pathway upregulated the expression of Otx2 and Mitf after laser photocoagulation.

**Wnt/b-Catenin Signaling Induces EMT After Laser Photocoagulation**

To examine whether the activation of Wnt/b-catenin signaling after laser photocoagulation induced EMT, we measured molecular markers for EMT, such as vimentin and $\alpha$-SMA. Immunohistochemical examination demonstrated that the expression levels of vimentin and $\alpha$-SMA were higher in the laser-treated RPE than in the control RPE at day 7 after laser treatment (Figs. 6A, 6B1, 6F, 6G1). Many of the EdU-positive cells in the laser-treated area were colocalized with vimentin and $\alpha$-SMA expression (Figs. 6B2, 6B3, 6G2, 6G3), indicating the occurrence of EMT in the proliferating cells after laser photocoagulation. Assessment of fluorescence intensity showed significantly increased fluorescence intensity of vimentin and $\alpha$-SMA in the laser-treated group versus the controls ($P < 0.05$; Figs. 6E, 6J). These results indicate that EMT occurred with cell proliferation after laser photocoagulation.

To further confirm whether the activation of Wnt/b-catenin signaling induced EMT after laser photocoagulation, a Dkk-1-injected group was examined by immunohistochemical staining and Western blot analysis. Immunohistochemical examination showed that the Dkk-1-injected group had markedly reduced expression levels of vimentin and $\alpha$-SMA (Figs. 6C, 6H) versus the vehicle-injected group (Figs. 6D, 6I). Fluorescence intensity analysis also revealed that Dkk-1 injection reduced the fluorescence intensity of vimentin and $\alpha$-SMA significantly ($P < 0.05$; Figs. 6E, 6J). These findings indicate that the inhibition of Wnt/b-catenin signaling decreased EMT after laser photocoagulation. Thus, we conclude that the Wnt/b-catenin signaling pathway induced EMT with cell proliferation after laser photocoagulation.

**Figure 3.** Immunocytochemical staining of EdU cells from whole-mount preparations 7 days after laser photocoagulation. (A) EdU cells were not detected in control RPE. (B) EdU incorporation was significantly elevated following laser photocoagulation of the RPE (106.2 ± 7.8 cells/field) versus control RPE (0 cells/field; $P < 0.001$). (C) Treatment of laser-treated RPE with vehicle significantly increased the number of EdU-positive cells. There was no difference with laser treatment alone (101.1 ± 9.4 cells/field, $P > 0.05$). (D) Treatment of the laser-treated RPE with Dkk-1 resulted in a significantly lower number of EdU-positive cells (52.3 ± 9.1 cells/field) than did laser treatment + vehicle injection ($P < 0.001$). (E) Summary of the number of EdU cells from whole mounts 7 days after photocoagulation (mean ± SD, *$P < 0.05$ by Kruskal-Wallis test with post hoc analysis).

**Figure 4.** Identification of EdU-positive cells from whole-mount preparations 7 days after laser photocoagulation using Otx2 (green) and Mitf (red). (A–C) Many EdU-positive cells colocalized with Otx2-positive cells ([C] arrowheads). (D–F) Many EdU-positive cells colocalized with Mitf-positive cells ([F] arrowheads). Scale bars: 100 μm.
To determine the time course of EMT after laser photocoagulation, we observed the expression levels of vimentin and α-SMA at different time points (before laser photocoagulation and 1, 3, 7, 14, and 28 days after photocoagulation). Western blot analysis showed that the expression levels of vimentin and α-SMA increased to day 3 and declined thereafter until 28 days (Figs. 7A–C).

**DISCUSSION**

Several studies have histologically analyzed lesions resulting from various laser photocoagulation regimes and have described specific cellular responses to laser photocoagulation.20–23 Because the main target of laser photocoagulation is the RPE layer, recent studies have focused on changes in the RPE layer after laser photocoagulation, and many details have been added to the morphological description of the RPE.6,7,9 One in vitro study showed that on the loss of contact inhibition in ARPE-19 cells, RPE proliferation with EMT occurred via a Wnt signaling pathway.10 The Wnt/β-catenin signaling pathway plays a key role in regulating tissue differentiation not only during embryogenesis but also postnatally, where it functions in stem cell renewal, cancer, and aging. In this study, Wnt/β-catenin signaling activation in RPE after laser photocoagulation was assessed using a mouse model, where there was disruption of cell-to-cell contact, and the involvement of the Wnt/β-catenin pathway in RPE regeneration was evaluated.

Activation of Wnt/β-catenin signaling was previously demonstrated in a laser-induced CNV model,24 and the morphologic endpoint of the laser injury was defined as the appearance of a subretinal bubble caused by the disruption of Bruch’s membrane. However, our study indicated that an adequate power of laser photocoagulation in either a clinically used level or a slightly lower power level was sufficient to induce an activation of Wnt/β-catenin signaling in the RPE. In
FIGURE 6. Representative confocal micrographs from whole-mount preparations 7 days after laser photocoagulation, immunostained with molecular markers of EMT (vimentin, green; α-SMA, red). (A, F) Expression of vimentin and α-SMA was not detected in the control RPE. (B1, G1) Expression levels of vimentin and α-SMA were higher in the laser-treated RPE than in the control RPE on day 7 after laser photocoagulation. (B2, B3, G2, G3) Many EdU-positive cells in the laser-treated area colocalized with vimentin and α-SMA expression. (C, H) Treatment of laser-treated RPE with vehicle increased the expression levels of vimentin and α-SMA. (D, I) Dkk-1 decreased the expression levels of vimentin and α-SMA compared to vehicle-injected RPE. (E, J) Mean fluorescence intensity calculated using ImageJ for vimentin and α-SMA. Quantifying relative fluorescence intensity showed significantly increased intensity in the laser-treated group versus the control group. The Dkk-1-injected group showed lower intensity than the vehicle-injected group (mean ± SD, n = 3 mice per group, *P < 0.05). Scale bars: 100 μm.
other words, unlike the previous study showing that Wnt signaling plays a pathological role in laser-induced CNV, our results suggested that the activation of Wnt signaling after adequate power of laser photocoagulation would be involved in RPE regeneration.

First, we demonstrated that laser photocoagulation activated the Wnt/β-catenin signaling pathway. To assess Wnt/β-catenin signal transduction in the photocoagulated RPE, expression levels of Wnt5a, β-catenin, and their target genes were determined. Immunohistochemical examination indicated that β-catenin accumulated in the cytoplasm in RPE cells 7 days after laser photocoagulation, but not in nonphotocoagulated RPE cells. Another line of evidence supporting the enhanced transcriptional activity of β-catenin was the significantly upregulated expression of cyclin D1, a target gene of Wnt/β-catenin signaling. We also found that expression of Wnt1, Wnt2b, Wnt3, Wnt5a, Wnt7a, Wnt7b, and Wnt10b was significantly increased in the laser-treated RPE versus the control RPE. In particular, Wnt2b expression increased most significantly. Wnt2b, which is expressed in the equatorial margin of the developing retina, is a Wnt family protein that has been shown to control the differentiation of progenitor cells in the retina. Steinfeld et al. investigated whether Wnt2b was a candidate gene involved in RPE specification. These results suggest that the activation of Wnt signaling, through the action of Wnt2b, has an important role in RPE specification after laser photocoagulation. Additional studies are needed to determine how Wnt2b targeting is related to Otx2 and Mitf expression. Wnt1, Wnt2b, Wnt3, Wnt7a, Wnt7b, and Wnt10b activate a β-catenin-dependent pathway, which mediates signaling to the nucleus, where β-catenin activates transcription factors, thereby inducing the expression of various genes. Wnt7a leads to β-catenin nuclear translocation, and cytosolic β-catenin levels are elevated in cells expressing Wnt1 and Wnt3. Consistent with this, we found that β-catenin accumulated in the cytoplasm of RPE cells after laser photocoagulation. Given this pattern of Wnt gene expression, the β-catenin-dependent Wnt pathway is activated in mouse RPE cells after laser photocoagulation. We also found that expression of Wnt5a was increased after laser photocoagulation. Wnt5a activates a β-catenin-independent pathway. The β-catenin-independent pathway is mainly thought to regulate the cytoskeleton, thereby coordinating cell migration and polarity. A recent study revealed that Wnt5a regulates the induction of EMT. Further study is required to identify the role of the β-catenin-independent pathway in RPE cells after laser photocoagulation.

The expression of β-catenin and cyclin D1 after laser photocoagulation colocalized with many EdU-positive cells, suggesting that Wnt/β-catenin signaling could contribute to RPE proliferation. The RPE has a proliferative capacity, and it has been demonstrated that local proliferation occurs after injury. However, until now, no in vivo study has shown that RPE cell proliferation is induced by Wnt/β-catenin signaling after laser photocoagulation. To measure RPE cell proliferation after laser photocoagulation, the number of EdU-positive cells in the laser-treated area was counted in whole mounts. The number of EdU-positive cells was significantly higher in the laser-treated area of the RPE. In addition, the number of EdU-positive cells decreased dramatically after intravitreal injection of Dkk-1, an inhibitor of Wnt signaling. We also found that some EdU-positive cells after photocoagulation were colocalized with Otx2- and Mitf-positive cells, suggesting that many of the proliferating cells had natural characteristics of the RPE. We concluded that the activation of Wnt/β-catenin signaling contributes to the intrinsic capacity for regeneration of the RPE after laser photocoagulation. Thus, we propose that modulating the Wnt/β-catenin signaling pathway is a possible therapeutic strategy to enhance the proliferation of RPE for patients with RPE degenerative diseases.

In a wide array of injury models, investigators have demonstrated that Wnt signaling is activated by injury to the adult retina. Using nonmammalian models of retinal injury, investigators have demonstrated that Wnt signaling is essential for regenerative responses. These nonmammalian models have indicated that the program of regeneration may be recapitulated in the mammalian retina. A few studies have shown that Wnt signaling acts as a mitogenic stimulus to Müller cells, and in an in vitro model, a subset of these proliferating cells differentiated into photoreceptors after exposure to retinoids or hedgehog-conditioned media. These data suggest an important role of Wnt signaling in the regeneration of the mammalian retina. We expanded on these studies to demonstrate that laser photocoagulation upregulated Wnt/β-catenin signaling in the RPE as well as in the neural retina. Our previous study showed that cell proliferative activity in the RPE increased significantly until 7 days after the laser photocoagulation and was maintained afterward. Thus, we identified the effect of a Wnt signaling inhibitor when the cell proliferative activity was maximized at 7 days after photocoagulation. We observed that there were significantly more Otx2- and Mitf-positive cells, which were brighter and stronger than the surrounding RPE cells, after photocoagulation. We also found that Otx2 and Mitf expression significantly decreased after intravitreal injection of a Wnt/β-catenin inhibitor, suggesting that Wnt/β-catenin signaling upregulates the expression of Otx2 and Mitf. Otx2 and Mitf are essential for RPE development, particularly in structural integrity and function. They are initially expressed in the entire optic vesicle, but their expression soon becomes restricted to the presumptive RPE, where it is maintained throughout adulthood.
erates with Otx2 and Mitf to activate functional and structural proteins characteristic of the pigmented phenotype.\textsuperscript{12,19} In this study, upregulation of Otx2 and Mitf expression through Wnt/β-catenin signaling after laser photocoagulation suggests the possible reoccurrence of a developmental stage during RPE regeneration. Thus, Wnt/β-catenin signaling has an important role in RPE specification by upregulating the expression of Otx2 and Mitf after laser photocoagulation.

Our data indicated that Wnt/β-catenin signaling resulted in EMT after laser photocoagulation in RPE. The EMT is a process in which epithelial cells lose their differentiated phenotypes and become mesenchymal-like cells.\textsuperscript{46–49} It is associated with wound healing, tissue regeneration, and organ fibrosis. The EMT and the ectopic proliferation of RPE cells have been suggested to contribute to the development of proliferative vitreoretinopathy (PVR). Thus, many recent studies have focused on the inhibition of EMT for the prevention and treatment of PVR.\textsuperscript{47,50,51} In this study, we found that vimentin and α-SMA, both EMT markers, were upregulated after laser photocoagulation. Many ED1-positive cells colocalized with the expression of vimentin and α-SMA, indicating that EMT occurred with cell proliferation after laser photocoagulation. We also investigated whether EMT would significantly decrease after intravitreal injection of a Wnt/β-catenin inhibitor, meaning that the activation of Wnt/β-catenin signaling would have induced EMT. As shown in Figure 7, EMT was most highly activated at day 3 and declined afterward, until day 28, indicating that EMT is a temporary cellular response after laser photocoagulation. This may suggest the possibility that mesenchymal cells undergo a mesenchymal–epithelial transition (MET) to form secondary epithelia over time. That is, RPE regeneration after RPE damage may be promoted not only by RPE proliferation but also through the process of EMT.

In conclusion, the present study provides the first reported evidence that the Wnt/β-catenin signaling pathway is activated after laser photocoagulation in the RPE of mice. As a result of this activation, the pathway induces RPE proliferation and EMT. It also upregulates the expression of Otx2 and Mitf, and many of the proliferated cells have natural characteristics of RPE cells. Taken together, our results point to the possibility of manipulating the Wnt/β-catenin signaling pathway for RPE regeneration. If laser treatment modalities that minimize photoreceptor cell damage, such as the subthreshold laser currently under development, are discovered, RPE regeneration may be induced for the treatment of RPE degenerative diseases.

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