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ORIGINAL ARTICLE

The role of Wnt/β-catenin signaling in the restoration of induced pluripotent stem cell-derived retinal pigment epithelium after laser photocoagulation

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Abstract

To investigate the role of *Wnt*/β-catenin signaling pathway in the restoration of induced pluripotent stem cell-derived retinal pigment epithelium (hiPSC-RPE) after laser photocoagulation. After differentiation of RPE cells from hiPSCs, laser photocoagulation was performed. Activation of *Wnt*/β-catenin signaling at days 1 and 5 after laser photocoagulation was evaluated by expression of β-catenin. Cell proliferation and alteration in cell-to-cell contact at day 5 after laser photocoagulation with or without Dickkopf-1 (Dkk-1) treatment were studied using ethynyl-2′-deoxyuridine (EdU) assay and zonula occludens-1 (ZO-1) expression analysis, respectively. The mRNA levels of *Wnt* genes at day 5 after laser photocoagulation were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Activation of *Wnt*/β-catenin signaling at days 1 and 5 after laser photocoagulation was confirmed by β-catenin accumulation in the cytoplasm and nucleus of hiPSC-RPE. Many EdU-positive cells also expressed β-catenin, and the number of EdU-positive cells was decreased at day 5 after laser photocoagulation after Dkk-1 treatment, indicating that *Wnt*/β-catenin signaling mediated hiPSC-RPE proliferation. ZO-1 expression was not decreased with Dkk-1 treatment at day 5 after laser photocoagulation, indicating that *Wnt*/β-catenin signaling mediated hiPSC-RPE restoration. At day 5, after laser photocoagulation, mRNA levels of *W*nt2b, *W*nt3, *W*nt5a, *W*nt7a, and *W*nt10b were increased. *W*nt/β-catenin signaling has a crucial role in restoration of hiPSC-RPE proliferation after laser photocoagulation. Manipulation of *W*nt/β-catenin signaling while elucidating the underlying mechanisms of RPE restoration might have a therapeutic potential in retinal degenerative diseases.

Keywords Wnt/β-catenin signaling pathway · Induced pluripotent stem cell · Retinal pigment epithelium · Laser photocoagulation

In Hwan Cho and Seong Jun Park contributed equally to the work presented here and should therefore be regarded as equivalent first authors.
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Introduction

The retinal pigment epithelium (RPE) layer, located between the neurosensory retina and the choriocapillaris, plays an essential role in the maintenance of visual function and the viability of photoreceptor cells [1]. These RPE layers are nonproliferative under normal conditions [2]. However, following an injury, the RPE layers are repaired by morphologically heterogeneous populations of RPE cells [3, 4]. In other words, micro-scale injuries such as laser photocoagulation induce proliferation in RPE cells to repair the damage [5]. Since permanent damage of the RPE layers leads to degeneration of photoreceptor cells and impairment of vision [6], it is important to understand the molecular responses after damage. Elucidating the mechanism and regulation of such responses may provide insights for developing novel therapeutic strategies that would allow the restoration of human RPE layers after any damage. With this aim, many researchers have been developing several experimental models, such as RPE cells damaged by oxidative stress [7, 8], hypoxia [9], and laser photocoagulation [10]. However, none of these models was ideal because none of them fully reflected the disease response of RPE cells after damage. Among these, laser photocoagulation has been clinically used for the treatment of diabetic retinopathy [10, 11] and age-related macular degeneration [12]. Therefore, we believe that it might be clinically most relevant to investigate molecular responses of RPE cells after laser photocoagulation.

Another problem of the abovementioned experimental models was that they did not use human RPE cells and failed to reflect molecular responses of human RPE cells after damage. Because using human cells for such experiments has certain limitations, alternative sources might be needed. Recently developed induced pluripotent stem cells (iPSCs) which are generated from human cells have the potential to be an alternative source of RPE cells [13]. These iPSCs can be expanded in vitro and can be induced to differentiate into RPE cells [14]. Furthermore, RPE derived from hiPSCs (hiPSC-RPE) demonstrated similar physical and functional properties as those of human RPE cells [15, 16]. Therefore, hiPSC-RPE would be an appropriate source of RPE cells, which could then be used for investigating molecular responses of human RPE cells after laser photocoagulation.

The *Wnt*/β-catenin signaling pathway plays a crucial role in the differentiation of optic vesicle cells to RPE cells, angiogenesis, and neurogenesis during development of the eye [17, 18]. Recently, we found that *Wnt*/β-catenin signaling mediates the regeneration of RPE layers by promoting RPE cell proliferation after laser photocoagulation in the normal mouse eye [19]. However, the role of this signaling pathway in the restoration of human RPE cells after laser photocoagulation has not been studied yet. Elucidating the role of *Wnt*/β-catenin signaling pathways in mediating the restoration of hiPSC-RPE could reveal their therapeutic potential in human RPE regeneration.

In this study, we observed early morphological changes in hiPSC-RPE after laser photocoagulation and confirmed cell proliferation around laser-treated areas using the 5-ethynyl-2′-deoxyuridine (EdU) assay. Using the *Wnt* antagonist, Dkk-1, we revealed that the *Wnt*/β-catenin signaling pathway mediates the restoration of hiPSC-RPE and decreases laser spot size after laser photocoagulation. Finally, we detected activation of *Wnt* genes in hiPSC-RPE after laser photocoagulation using quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and methods

Differentiation of human hiPSCs to RPE cells

Human iPSCs (ACS-1011) were purchased from ATCC (Manassas, VA) and maintained on vitronectin (#A14700, Gibco, Carlsbad, CA)-coated plates in Essential 8 media (#A1517001, Gibco, Carlsbad, CA) in a feeder-free system. Undifferentiated iPSCs were washed twice with Dulbecco's phosphate-buffered saline (DPBS) (CA008-600, GenDEPOT, Houston, TX), and separated into single cells using 0.5 mM EDTA (#15575020, Invitrogen, Carlsbad, CA). The single cells (1×10^6 cells/ml) were then seeded in Ultra-Low Attachment Surface 6-well plates (#3471, Corning, NY) and cultured in Essential 8 media, containing 10 μM blebbistatin (#B5060, Sigma-Aldrich, Munich, Germany) and 100 μM Rock inhibitor Y-27632 (#1254, Tocris Bioscience, MO). The formation of embryoid bodies (EBs) was identified. One day later, the medium was replaced with Essential 8 medium and Neural Induction Medium [NIM, consisting of DMEM/F12 (#11330-032, Gibco, Carlsbad, CA) supplemented with 1% N2 (#AR009, Gibco, Carlsbad, CA), 1 x NEAA (#11140-050, Gibco, Carlsbad, CA) and 2 μl/ml heparin (#07980, STEMCELL Technologies, Vancouver, BC, Canada)] in the ratio of 3:1. At day 2, the medium was replaced with Essential 8 medium and NIM in the ratio of 1:1. From days 3 to 7, the medium was replaced with NIM and changed every day.

For differentiation into RPE cells, about 20 embryoid bodies per cm^2 of plate areas were seeded on Matrigel (growth factor reduced, BD Biosciences, San Jose, CA)-coated plates and cultured in medium containing α-MEM (#LM 008-02, WELGENE, Daegu, Korea), 1× N1 (#N6530, Sigma-Aldrich, Munich, Germany), 1× GlutaMax (#35050-061, Gibco, Carlsbad, CA), 125 mg taurine (#T8691, Sigma-Aldrich, Munich, Germany), 10 μg hydrocortisone (#H0396-100MG, Sigma-Aldrich, Munich, Germany), 0.0065 μg triiodo-thyronin (#T6397, Sigma-Aldrich, Munich, Germany), 1× penicillin-streptomycin (#CA005-010, GenDEPOT, Huston, Tx), and 15% fetal bovine serum (FBS) (#16000-044, Gibco, Carlsbad, CA). In addition, 100 ng/ml activin A (#120-14E, Peprotech, Rocky Hill, NJ) was added and the medium was changed every 2 to 3 days

until the pigmentation of cells appeared. Then, the medium was replaced with 5% FBS (#16000-044, Gibco, Carlsbad, CA).

Laser photocoagulation and Dickkopf-1 (Dkk-1) treatment

Laser photocoagulation was performed as previously described [3, 20] using slit-lamp delivery system of a PASCAL diode laser (Topcon Medical Laser Systems, Inc., Santa Clara, CA), a frequency-doubled neodymium-doped yttrium aluminum garnet (Nd:YAG) laser diode with a wavelength of 532 nm (200-μm spot size, 0.1-s duration, and 250-mW laser power). Each plate applied 50 evenly spaced laser shots. During the laser photocoagulation procedure, the medium was temporarily removed and a sterile sheet of black paper was placed on top of the cells to facilitate absorption of the laser energy. Immediately after laser photocoagulation, fresh medium was added to the plates and the plates were returned to the CO_2 incubator.

To determine the role of the *Wnt*/β-catenin signaling pathway in the restoration of hiPSC-RPE after laser photocoagulation, the cells were either exposed or not exposed to 300 ng of Dickkopf-1 (Dkk-1, R&D Systems, Minneapolis, MN), an inhibitor of *Wnt* signaling. The hiPSC-RPE not subjected to laser photocoagulation served as control.

Immunolabeling

The cells were fixed with 4% formaldehyde in phosphate buffer solution (PBS) for 15 min at room temperature. Then, the cells were permeabilized with 0.1% Triton X-100 in PBS (PBST; Sigma-Aldrich Corp., St. Louis, MO) for 15 min and blocked with 5% goat serum in PBST for 1 h at room temperature. After blocking, the cells were incubated with primary antibodies including rabbit anti-Otx2 (1:500; ab21990, Abcam UK, Cambridge, UK) antibody, rabbit anti-ZO-1 antibody (1:250; 61–7300, Invitrogen, Carlsbad, CA), mouse anti-RPE65 (1:500; NB100–355, Novus, Littleton, CO), and mouse anti-β-catenin antibody (1:200; C7207, Sigma-Aldrich, St. Louis, MO) overnight at 4 °C. On the next day, the cells were incubated with secondary antibodies such as Cy2-AffiniPure Fab fragment goat anti-mouse Ig M (115-227-020, Jackson ImmunoResearch Lab, West Grove, PA), Cy2-AffiniPure Fab fragment goat anti-mouse Ig M (115-167-020, Jackson ImmunoResearch Lab, West Grove, PA), Alexa Fluor-488 goat anti-mouse (1:2000; Molecular Probes, Grand Island, NY), and Alexa Fluor-568 goat anti-mouse (1:2000; Molecular Probes, Grand Island, NY) antibodies for 2 h at room temperature. The cells were then washed with PBS and treated with Hoechst 33342, trihydrochloride, trihydrate (1:4000; H1399, Thermo Fisher Scientific Inc., Waltham, MA) for 15 min. For the negative control, the primary antibodies including Otx2 and RPE65 were omitted and the secondary antibodies were applied. Images were obtained using confocal fluorescence microscopy (LSM710; Carl Zeiss, Inc. Jena, Germany).

EdU labeling

For double immunolabeling of EdU with β-catenin and ZO-1, commercial EdU imaging kit (Click-iT EdU Alexa Fluor 647 imaging kit; C10340, Invitrogen, Carlsbad, CA) was used according to manufacturer's instructions. Before fixation of cells, they were treated with 10 μM of 5-ethynyl-2′-deoxyuridine (EdU; Invitrogen, Carlsbad, CA). Then, the immunolabeling of β-catenin and ZO-1 was performed as described above. For detection of EdU, the cells were washed with 3% bovine serum albumin (BSA; 8076.2, Roth, Karlsruhe, Germany) in PBS for 10 min. Click-iT cocktail including reaction buffer, CuSO4, azide, and buffer additive was added and incubated for 30 min. The mixture was then washed with PBS and stained with Hoechst 33342, trihydrochloride, and trihydrate (1:4000; H1399, Thermo Fisher Scientific Inc., Waltham, MA) for 1 min to visualize the cell nuclei. Images of double immunostaining were obtained using confocal fluorescence microscopy (LSM710; Carl Zeiss, Inc. Jena, Germany).

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from hiPSCs, hiPSC-RPE cells, and human adult RPE cells using RNeasy mini kit (74104, Qiagen, Hilden, Germany). Total RNA (2 μg) was reverse-transcribed to cDNA using Superscript III enzyme (Invitrogen, Carlsbad, CA). Polymerase chain reaction (PCR) amplification of cDNA was performed using the specific primer pairs (Table 1).

Quantitative real-time PCR (qRT-PCR)

The mRNA levels of *W*nt ligands were measured by qRT-PCR. Total mRNA was prepared from the cells using TRIzol reagent (Invitrogen, Tokyo, Japan). Then, the mRNA (0.5~1 μg) was reverse-transcribed using Superscript III enzyme (Invitrogen, Tokyo, Japan), and qRT-PCR was performed using SYBR Green kits (Philekorea, Daejeon, Korea) with specific primer pairs (Table 2).

Quantification and statistical analysis

Laser spot size, number of EdU-positive cells, and β-catenin fluorescence intensities were measured by ImageJ software. All quantified values were expressed as mean ± SEM. The Wilcoxon signed-rank test was used to compare between different groups. SPSS software (ver. 21.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses, and p value < 0.05 was considered to be statistically significant.

Table 1 List of primers used for reverse transcription PCR (RT-PCR)

Genes	GenBank no.	Sources	Primer sequences (5′–3′)		Size (bp)
			Forward	Reverse	
RPE 65	NM_00032-9.2	Human	ACATATGCGTATGG ACTTGG	GAACAGTCCATGAA AGGTGA	296
Best1	NM_00418-3.3	Human	CCTTATGGGCTCCA CCTTCAACATC	CAGTAGTTTGGTCC TTGAGTTTGCCC	163
Rlbp1	NM_00032-6.4	Human	AACCAAGACTGGGG TTAAT	ATGTTGCCTATGGA AGACAC	160
Tyr	NM_00037-2.4	Human	AGAGACGACTCTTG GTGAGA	AGTGCATCCATTGA CACATA	222
Nanog	NM_02486-5.3	Human	AGAAGGCCTCAGCA CCTAC	GGCCTGATTGTTCC AGGATT	205
GAPDH	NM_00204-6.6	Human	GTCAGTGGTGGACC TGACCT	CACCACCCTGTTGC TGTAGC	255

Results

Characterization of RPE cells derived from hiPSC

RPE cells were differentiated from hiPSCs as described above. About 3–4 weeks after initiation of RPE differentiation, cells in the plates showed pigmentation (Fig. 1a). The pigmented cells mimicked the typical morphology of RPE cells, such as monolayer growth and hexagonal shape (Fig. 1b). Immunolabeling of these cells revealed expressions of RPE specific transcriptional markers such as Otx2 (optic vesicle and cup specification), ZO-1 (tight junction), and RPE65 (retinoid cycle) (Fig. 1c, d). In negative control, Otx2 and RPE65 were not stained positively (Fig.1e, f). The hiPSC-RPE exhibited RNA expression of typical hiPSC-RPE markers including RPE65 (retinoid cycle), Best1 (chloride

Table 2 List of primers used for quantitative real-time PCR (qRT-PCR)

Genes	GenBank no.	Sources	Primer sequences (5′–3′)		Size (bp)
			Forward	Reverse	
Wnt1	NM_021279.4	Mouse	TCTTTGGCCGAGAG TTCGTG	CATTTGCACTCTTG GCGCAT	124
Wnt2b	NM_009520.3	Mouse	TTGCTCCGGTTTCA CATCCA	CGAGGAAGACATTC CCTCCG	122
Wnt3	NM_009521.2	Mouse	AGCTGCCTCTACTC GTGACA	ATCTTGCTCCCACT GTTGGC	194
Wnt3a	NM_009522.2	Mouse	TGGCTCCTCTCGGA TACCTC	GCACAGAGAATGGG CTGAGT	127
Wnt4	NM_009523.2	Mouse	GCTATTGGGTGGTG CAAACG	GAGCAGAGAGCTTG GATGGG	154
Wnt5a	NM_00125622-4.1	Mouse	GTTGCTCCGGCCCA GAAG	GCGAAGGAGAAAAA CGTGGC	119
Wnt5b	NM_00127175-7.1	Mouse	TGTCCCAACTCTGA ATCCTGC	CAATGCCCTGGGAC CAACAT	180
Wnt7a	NM_009527.3	Mouse	TGTGACCTCATGTG CTGTGG	TCTGACCTGTGACC GCATTCT	159
Wnt7b	NM_00116363-4.1	Mouse	CGCACACTCTGGTC AACCTC	TAGTGGCTGTCTGG TCCTCT	174
Wnt10b	NM_011718.2	Mouse	GTGAGCAAGACCGG CTTAGA	TGGTTACAGCCACC CCATTC	148

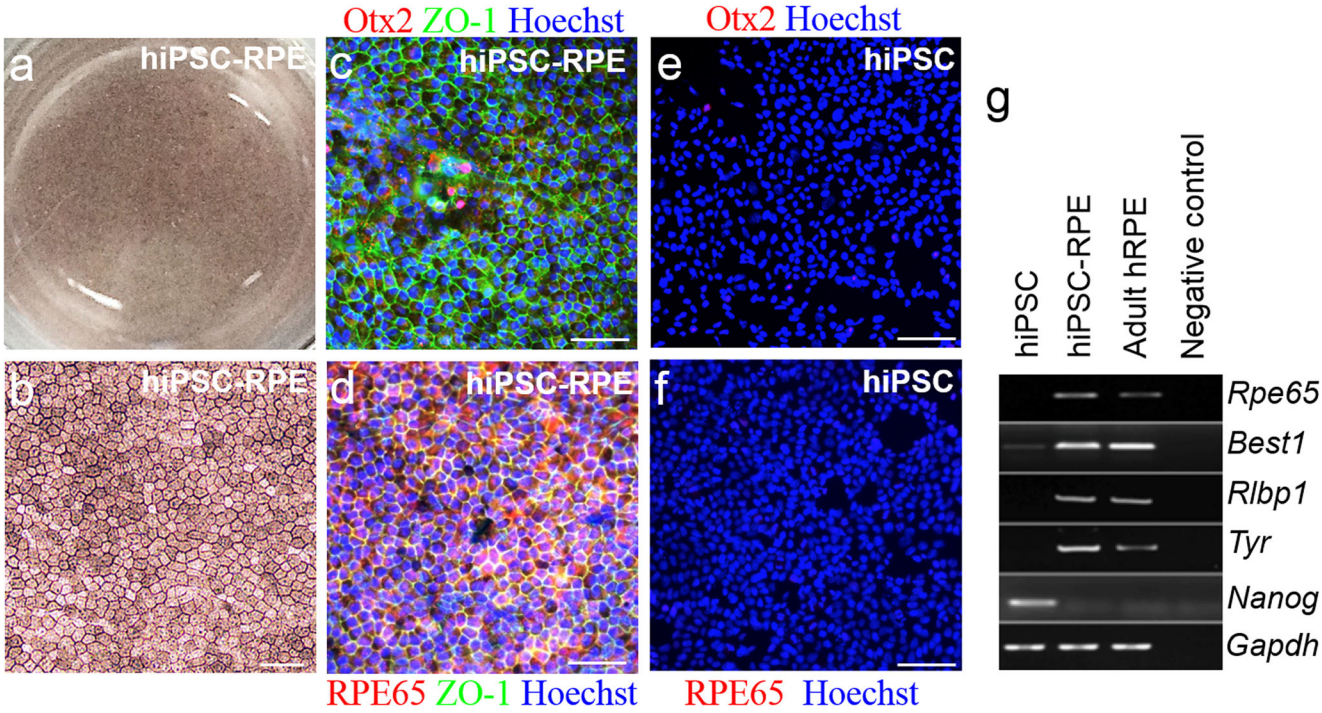

Fig. 1 Characterization of hiPSC-RPE. **a** Low-resolution images of hiPSCs 3–4 weeks after initiation of RPE differentiation showing pigmentation of cells. **b** Phase contrast images of pigmented cells showing a typical morphology of RPE cells such as monolayer growth and hexagonal shape. **c**, **d** Immunolabeling of RPE specific markers (Otx2, ZO-1, and RPE65) in the pigmented cells. **e**, **f** Negative control of RPE specific markers including Otx2 and RPE 65. Scale bars 50 μm. **g** hiPSC-RPE showing RNA expressions of typical hiPSC-RPE markers (RPE65, Best1, Rlbp1, and Tyr) which are not expressed in hiPSCs. The GAPDH gene was used as a loading control. Total RNA without reverse transcriptase served as negative control

channel), Rlbp1 (mature RPE marker), and Tyr (melanogenesis marker) which are not expressed in hiPSC alone. Notably, Nanog (stem cell marker) was endogenously silenced in hiPSC-RPE. Furthermore, these expression patterns were similar to adult human RPE (hRPE) cells (Fig. 1g). Taken together, these results suggest that we successfully differentiated RPE cells from hiPSCs.

Early morphological changes during hiPSC-RPE restoration after laser photocoagulation

To observe early morphological changes in hiPSC-RPE after laser photocoagulation (Fig. 2a, b), we performed time-lapse imaging from hour 1 to hour 30. The laser-treated areas were filled with well-differentiated pigmented cells which have hexagonal shape. The laser spot size was decreased steadily as time passes (Fig. 2c, f). By hours 20 and 30, the cells were increased around the laser-treated area and then were pushed in (Fig. 2g, h).

Wnt/β-catenin signaling pathway is activated after laser photocoagulation in hiPSC-RPE

Previously, we found that the *Wnt*/β-catenin signaling pathway is activated after laser photocoagulation and this pathway induces RPE proliferation in mouse eyes [9]. To determine whether this signaling pathway is also activated after laser photocoagulation in hiPSC-RPE, we performed immunolabeling of β-catenin. At days 1 and 5 after laser photocoagulation, β-catenin was accumulated not only in cell-to-cell border but also in the cytoplasm and nucleus (Fig. 3). Furthermore, we investigated distribution of EdU-positive cells, which represent proliferating cells, in correlation with β-catenin expression. Most EdU-positive cells colocalized with β-catenin expression at both days 1 and 5 after laser photocoagulation (Fig. 3). Beta-catenin/EdU-positive cells were mainly located around the laser-treated area at day 1 after laser photocoagulation (Fig. 3a–c). However, at day 5 after laser photocoagulation, β-catenin/EdU-positive cells were mainly located in the center of the laser-treated area (Fig. 3d–f).

Wnt/β-catenin signaling pathway mediates hiPSC-RPE cell proliferation after laser photocoagulation

The colocalization of EdU-positive cells with β-catenin expression may suggest that the *Wnt*/β-catenin signaling pathway mediates hiPSC-RPE cell proliferation after laser photocoagulation. To evaluate this, we exposed the cells to a *Wnt* antagonist, Dkk-1, after laser photocoagulation, and then assessed β-catenin expression and EdU staining. In control hiPSC-RPE cells, the expression of β-catenin was limited to

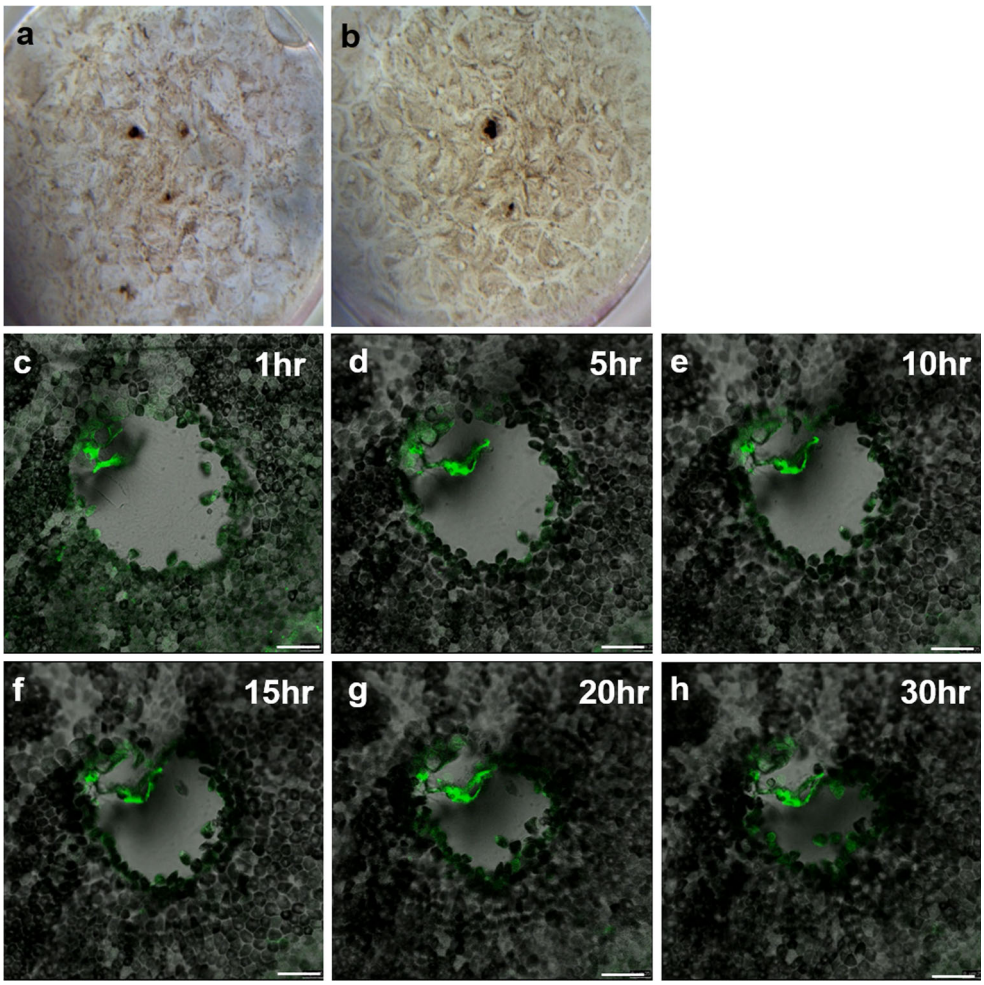

Fig. 2 Time course of morphological changes of hiPSC-RPE after laser photocoagulation. **a**, **b** Gross hiPSC-RPE images before and after laser photocoagulation. **c**–**f** The laser-treated areas were filled with well-differentiated pigmented hexagonal cells, and the laser spot size was decreased steadily as time passes. **g**, **h** By hours 20 and 30, the cells were increased around the laser-treated area and then were pushed in. Scale bars 50 μm

the cell-to-cell border and EdU-positive cells were barely observed (Fig. 4a–c). At day 5 after laser photocoagulation, β-catenin was accumulated in the cytoplasm and nucleus. Most Edu-positive cells colocalized with β-catenin expression and were mainly located in the center of the laser-treated area (Fig. 4d–f). However, at day 5 after laser photocoagulation with Dkk-1 exposure, expressions of β-catenin and Edu-positive cells were reduced compared to those in the cells subjected to laser photocoagulation but without Dkk-1 (Fig. 4g–i). Taken together, these results suggest that cell proliferation was decreased after laser photocoagulation due to inhibition of the *Wnt*/β-catenin signaling pathway in hiPSC-RPE. Therefore, we can conclude that the *Wnt*/β-catenin signaling pathway mediates hiPSC-RPE cell proliferation after laser photocoagulation.

Expression of ZO-1 was not decreased with Dkk-1 after laser photocoagulation in hiPSC-RPE

It has been reported that the loss of cell-to-cell contact is a crucial step for initiating RPE proliferation [21]. To determine whether the *Wnt*/β-catenin signaling pathway altered cell-to-cell contact in hiPSC-RPE after laser photocoagulation, we performed immunostaining of ZO-1 in cells with or without Dkk-1 application. At day 5 after laser photocoagulation, expression of ZO-1 was decreased around the laser-treated area indicating the loss of cell-to-cell contact and many EdU-positive cells were detected (Fig. 5a–c). However, at day 5 after laser photocoagulation and application of Dkk-1, expression of ZO-1 was not decreased and the cells were densely packed around the laser-treated area. Number of EdU-positive cells was also decreased (Fig. 5d–f).

Changes in laser spot size, number of EdU-positive cells, and relative β-catenin fluorescence intensity after laser photocoagulation with or without Dkk-1

We performed quantitative analysis to evaluate the changes in laser spot size, number of EdU-positive cells, and relative β-catenin fluorescence intensity. The laser spot size was significantly larger with the application of Dkk-1 than without the application of Dkk-1 both at days 1 and 5 after laser photocoagulation (Fig. 6a, $p < 0.001$, $p < 0.001$, respectively). The laser spot size was significantly decreased without Dkk-1

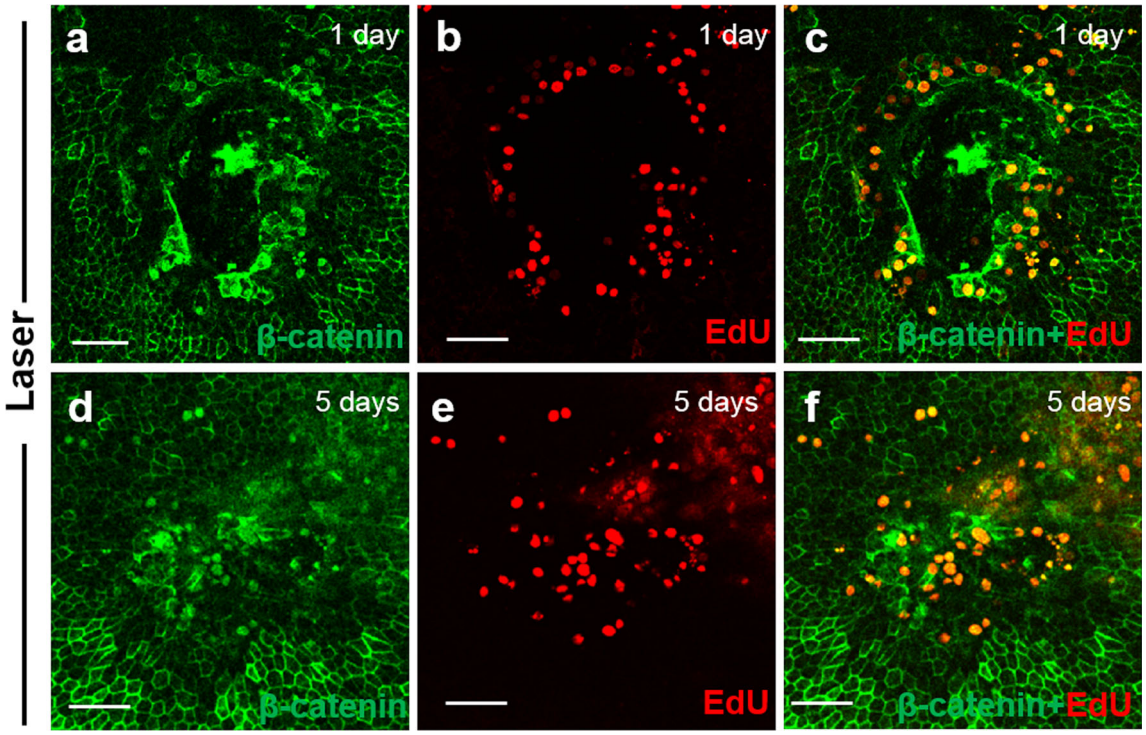

Fig. 3 Immunostaining of β-catenin and EdU at days 1 and 5 after laser photocoagulation. After laser photocoagulation, β-catenin accumulated in cytoplasm and nucleus. **a**–**c** At day 1 after laser photocoagulation, most Edu-positive cells are colocalized with β-catenin expression and distributed around the laser-treated area. **d**–**f** At day 5 after laser photocoagulation, most Edu-positive cells are colocalized with β-catenin expression and distributed in the center of laser-treated area. Scale bars 50 μm

application (Fig. 6a, $p < 0.001$, $p < 0.001$, respectively) but was not significantly decreased with Dkk-1 application compared to control at day 1 and day 5 after laser photocoagulation (Fig. 6a, $p = 0.745$, $p = 0.421$, respectively). The number of EdU-positive cells and relative β-catenin fluorescence intensity was significantly higher without Dkk-1 application than with Dkk-1 application both at day 1 and day 5 after laser photocoagulation (Fig. 6b, c, EdU: day 1; $p < 0.001$, day 5; $p < 0.001$, β-catenin: day 1; $p < 0.001$, day 5; $p < 0.001$). The number of EdU-positive cells and relative β-catenin fluorescence intensity was significantly increased without Dkk-1 application (Fig. 6b, c, EdU; $p < 0.001$, $p < 0.001$, respectively, β-catenin; $p < 0.001$, $p < 0.001$, respectively) but was not significantly increased with Dkk-1 application compared to control at day 1 and day 5 after laser photocoagulation (Fig. 6b, c, EdU: $p = 0.946$, $p = 0.784$, respectively, β-catenin: $p = 0.884$, $p = 0.622$, respectively). There were significantly more EdU-positive cells and higher β-catenin fluorescence intensity at day 5 than day 1 after laser photocoagulation ($p < 0.001$ and $p < 0.001$, respectively).

mRNA levels of *Wnt* genes after laser photocoagulation in hiPSC-RPE

Quantitative real-time PCR analysis showed that mRNA levels of both the canonical pathway including *W*nt2b, *W*nt3, *W*nt7a, and *W*nt10b, and the non-canonical pathway including *W*nt5a were increased at day 5 after laser photocoagulation in hiPSC-RPE compared hiPSC-RPE not subjected to laser photocoagulation ($p < 0.05$, Fig. 7). In particular, the increase in *Wnt*2b was most significant. These results suggest that both canonical and non-canonical *Wnt*/β-catenin signaling pathways were activated after laser photocoagulation in hiPSC-RPE.

Discussion

Damage of RPE layer is a main underlying mechanism of various retinal degenerative diseases including age-related macular degeneration, Stargardt disease, and choroideremia [22]. Therefore, it is important to understand the molecular responses after damage of RPE layer. However, it is impossible to investigate responses of RPE cells after damage separately in vivo condition because there are many adjacent cells such as neural and glial cells. Because of this limitation, many researchers have been developing in vitro experimental models such as RPE cells damaged by oxidative stress [7, 8], hypoxia [9] and laser photocoagulation [10]. But, most of these experimental models were used ARPE-19 cells. Therefore, these models failed to fully reflect the disease response of human RPE cells after damage.

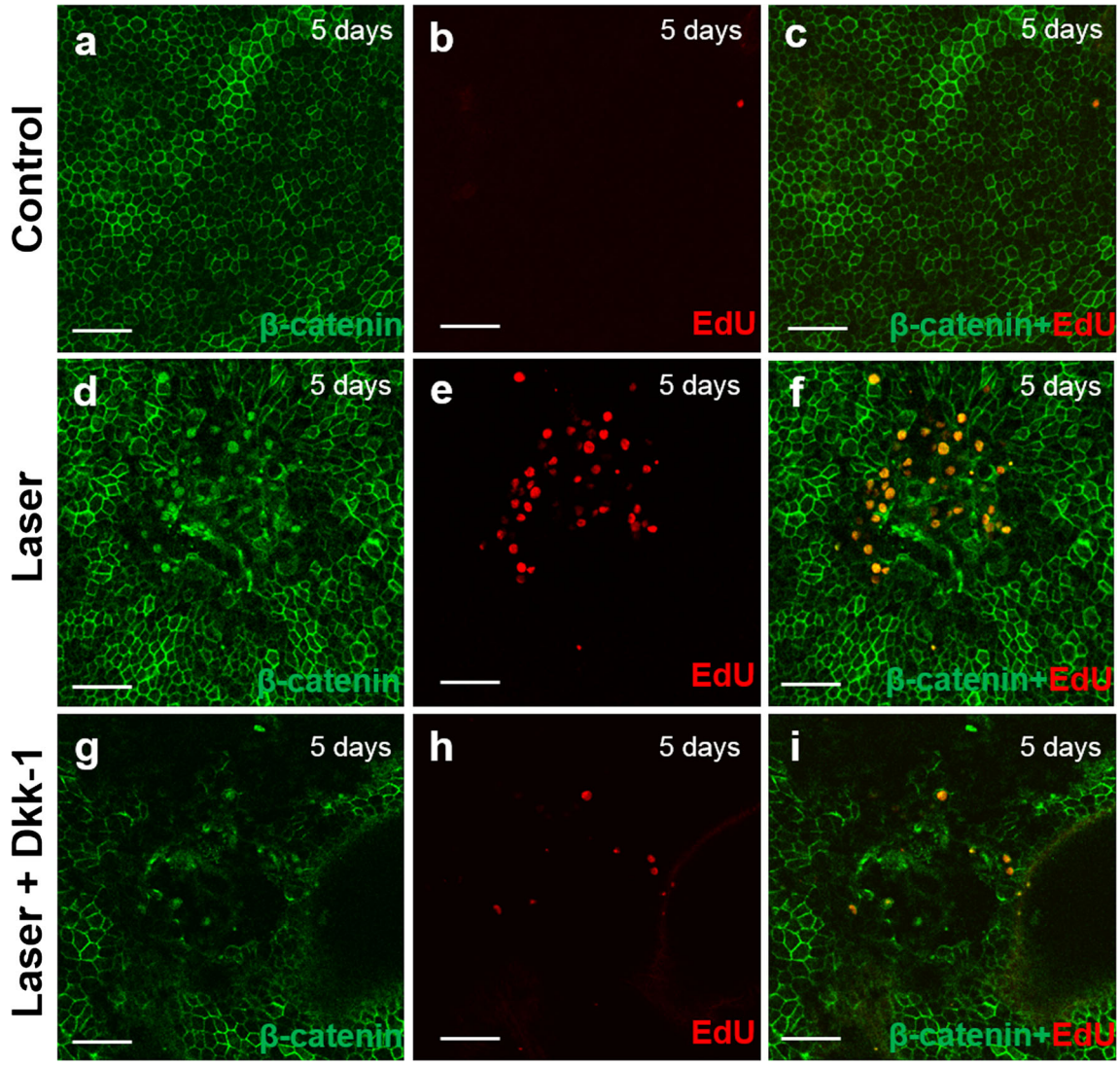

Fig. 4 Immunostaining of β-catenin and EdU in control hiPSC-RPE cells and at day 5 after laser photocoagulation with or without Dkk-1. **a**–**c** In control hiPSC-RPE, expression of β-catenin is limited to the cell-to-cell border and Edu-positive cells were barely observed. **d**–**f** At day 5 after laser photocoagulation, most Edu-positive cells are colocalized with β-catenin and distributed in the center of laser-treated area. **g**–**i** At day 5 after laser photocoagulation with Dkk-1 application, expressions of β-catenin and EdU-positive cells are decreased compared to after laser photocoagulation without Dkk-1 application. Scale bars 50 μm

In the present study, we used hiPSC-RPE to investigate molecular responses of cells after laser photocoagulation. Since hiPSC-RPE is a fully differentiated cell and demonstrated similar physical and functional properties as those of human RPE cells [15, 16], it might be the best cellular source to speculate molecular responses of human RPE cells after damage. In addition, laser photocoagulation has been clinically used for the treatment of diabetic retinopathy and age-related macular degeneration. Therefore, we believe that it might be clinically most relevant to investigate molecular responses of RPE cells after laser photocoagulation. Furthermore, the results of our study might be applicable for future human clinical trials.

After laser photocoagulation, the light energy is primarily absorbed in the melanosomes of RPE cells and is converted into heat. This thermal energy causes destruction of RPE cells in the laser-treated area [3]. Subsequently, RPE cells around the laser-treated area undergo apoptosis [3]. The living or surviving cells start filling the laser-treated area [3]. By time-lapse imaging, we observed this process took from hour 1 to hour 30 after laser photocoagulation. We could only obtain the images only up to hour 30 because after that, cellular transformation occurred.

During early restoration of hiPSC-RPE after laser photocoagulation, laser-treated areas were filled with well-differentiated pigmented cells which have hexagonal shape. The laser spot size was decreased steadily as time passes. By hours 20 and 30, the cells were increased around the laser-treated area and then were pushed in. Although their method of damage to RPE cells was different from our experiment,

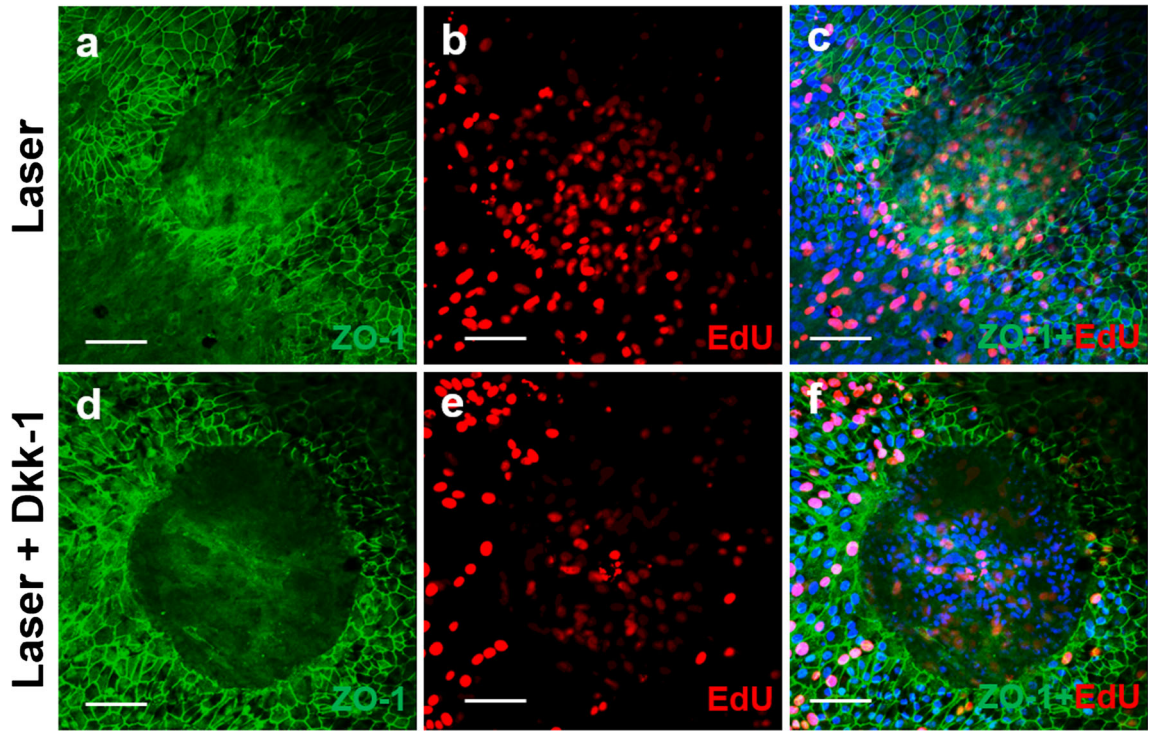

Fig. 5 Immunostaining of ZO-1 and EdU at day 5 after laser photocoagulation with or without Dkk-1 application. **a**–**c** At day 5 after laser photocoagulation, ZO-1 expression was decreased around the laser-treated area. EdU-positive cells mainly distributed in the center of laser-treated area. **d**–**f** At day 5 after laser photocoagulation with Dkk-1 application, ZO-1 expression is not decreased and is densely packed around the laser-treated area. EdU-positive cells are decreased with Dkk-1 application compared to laser without Dkk-1 application. Scale bars 50 μm

Greene et al. also reported that hiPSC-RPE secreted proteins associated with cell proliferation, contraction, and migration during wound healing [23].

To investigate differences between early and late changes during restoration of iPSC-RPE after laser photocoagulation, we chose day 1 and day 5 as the observing time point in the present study. Since there have been no previous study investigating restoration of hiPSC-RPE after laser photocoagulation, we referred previous study using ARPE-19 cells [13]. They reported that cell migration is main mechanism at early period and cell proliferation is main mechanism at late period in the restoration of ARPE-19 after laser photocoagulation [13]. Therefore, we chose day 1 to observe early changes in the restoration of hiPSC-RPE after laser photocoagulation.

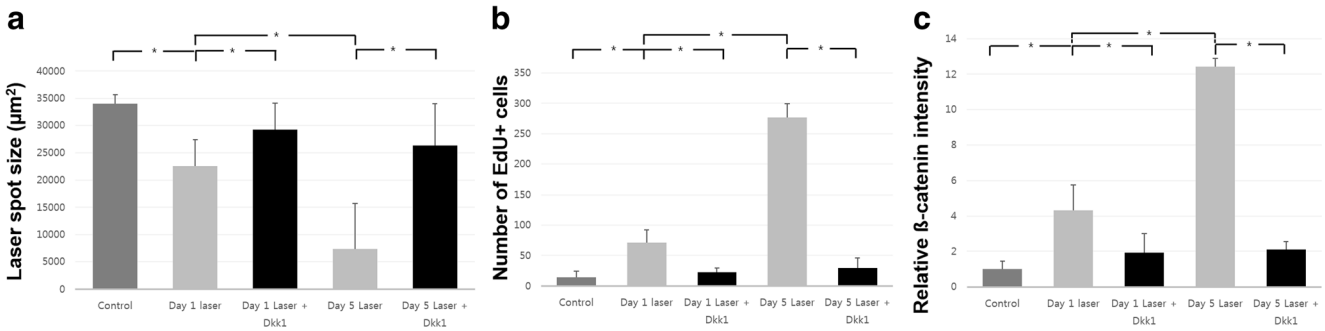

Fig. 6 Changes in laser spot size, number of EdU-positive cells, and relative β-catenin fluorescence intensity after laser photocoagulation. **a** The laser spot size is significantly decreased without Dkk-1 application but was not significantly decreased with Dkk-1 application ($p < 0.001$ and $p < 0.001$, respectively) compared to control at day 1 and day 5 after laser photocoagulation ($p = 0.745$ and $p = 0.421$, respectively). **b** The number of EdU-positive cells was significantly increased without Dkk-1 application but was not significantly increased with Dkk-1 application ($p < 0.001$ and $p < 0.001$, respectively) compared to control at day 1 and day 5 after laser photocoagulation ($p = 0.946$ and $p = 0.784$, respectively). **c** The relative β-catenin fluorescence intensity was significantly increased without Dkk-1 application but was not significantly increased with Dkk-1 application ($p < 0.001$ and $p < 0.001$, respectively) compared to control at day 1 and day 5 after laser photocoagulation ($p = 0.884$ and $p = 0.622$, respectively). There are significantly more EdU-positive cells and higher β-catenin fluorescence intensity at day 5 than at day 1 after laser photocoagulation ($p < 0.001$ and $p < 0.001$, respectively). *Statistically significant

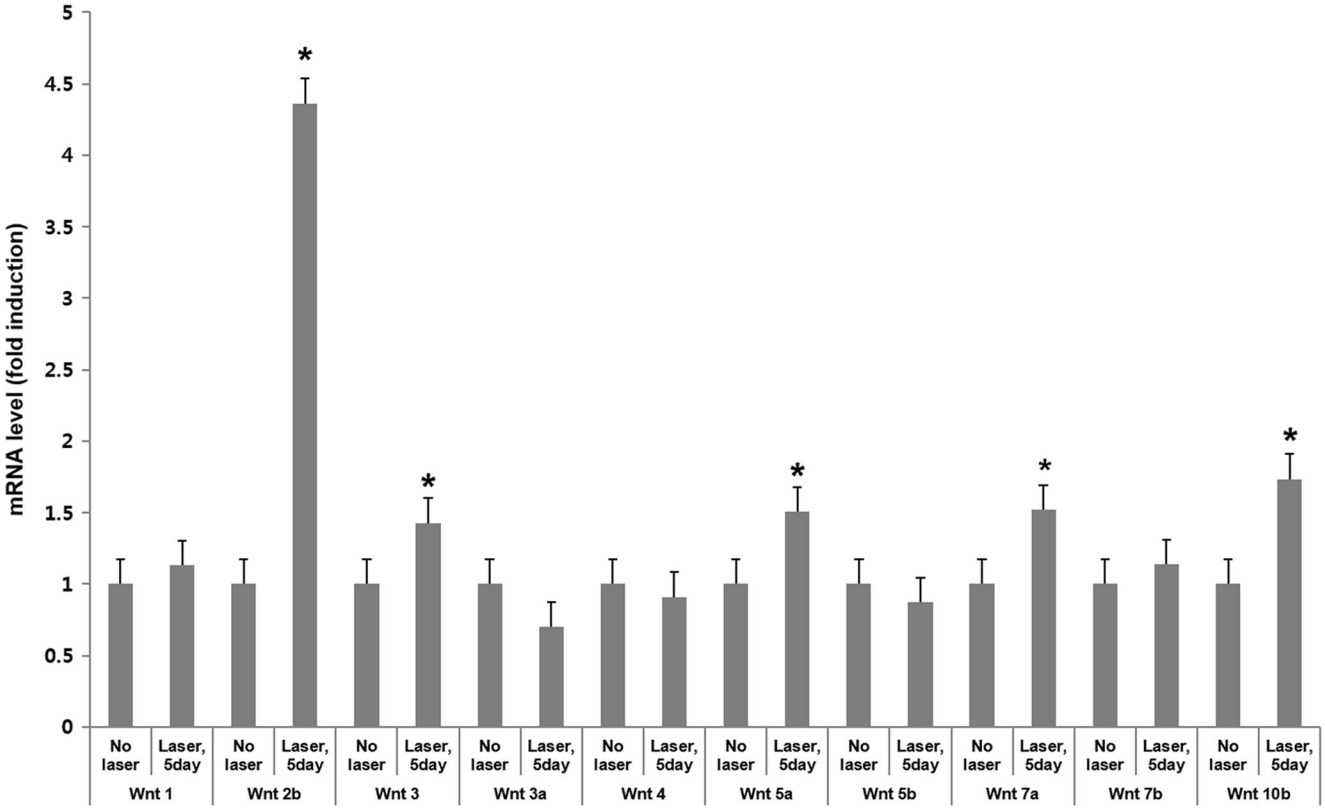

Fig. 7 mRNA levels of *Wnt* genes after laser photocoagulation. Levels of *Wnt*2b, *Wnt*3, *Wnt*5a, *Wnt*7a, and *Wnt*10b are increased at day 5 after laser photocoagulation in hiPSC-RPE compared to those in untreated hiPSC-RPE. *Statistically significant

Then, we chose day 5 to observe late changes because hiPSC-RPE took more time in the restoration of laser-treated area than ARPE-19 cells in our preliminary study. It is probably due to hiPSC-RPE are more differentiated than ARPE-19 cells.

The *Wnt*/β-catenin signaling pathway is activated by laser photocoagulation and induces cell proliferation in hiPSC-RPE. Beta-catenin was retained at the cell-to-cell border in control hiPSC-RPE; however, after laser photocoagulation, β-catenin accumulated in the cytoplasm and nucleus of cells. These results were consistent to those of our previous studies in normal mouse retina [9]. Most EdU-positive cells colocalized with β-catenin expression, and their number were decreased after application of *Wnt* antagonist, Dkk-1, suggesting that *Wnt*/β-catenin signaling could contribute to cell proliferation. At day 1 after laser photocoagulation, EdU-positive cells were mainly distributed around the laser-treated area suggesting the cells existing in the center of laser-treated area were derived from migration of cells. However, at day 5 after laser photocoagulation, most EdU-positive cells were located in the center of laser-treated area, suggesting that proliferation of cells had occurred.

Since the loss of cell-to-cell contact is a crucial step for initiating RPE proliferation [21], we further investigated ZO-1 expression after laser photocoagulation. ZO-1 expression was not decreased, but the number of EdU-positive cells was decreased with Dkk-1 application after laser photocoagulation. These findings suggested that the *Wnt*/β-catenin signaling pathway is associated with the loss of cell-to-cell contact and cell proliferation. However, it was not possible to elucidate whether the *Wnt*/β-catenin signaling pathway directly influenced cell-to-cell contact or cell proliferation through this study. Further experiments might be necessary to elucidate the precise relationship of the *Wnt*/β-catenin signaling pathway with the cell-to-cell contact and the proliferation of cells.

By quantitative analysis, we could confirm that the *Wnt*/β-catenin signaling pathway was activated by laser photocoagulation and induced proliferation of hiPSC-RPE cells. The laser spot size was not significantly decreased, and the number of EdU-positive cells and β-catenin expression was not significantly increased with Dkk-1 application after laser photocoagulation. At day 1 after laser photocoagulation, the laser spot size was significantly decreased compared to control, but the number of EdU-positive cells was significantly less than at day 5 with Dkk-1 application after laser photocoagulation. These findings suggest that proliferation than migration of cells might contribute to the restoration of laser-treated area at day 1 after laser photocoagulation. At day 5 after laser photocoagulation, significantly more EdU-positive cells were observed compared to day 1, suggesting that proliferation of cells might contribute to the restoration of laser-treated area.

Finally, we found that the mRNA levels of both the canonical pathway, including *Wnt*2b, *Wnt*3, *Wnt*7a, and *Wnt*10b, and the non-canonical pathway, including *Wnt*5a, were significantly increased at day 5 after laser photocoagulation in hiPSC-RPE. In particular, the increase in *Wnt*2b mRNA levels was most significant. *Wnt*2b is well known as a candidate gene for RPE specification and regulates differentiation of retinal progenitor cells [17, 24]. These results suggest that activation of genes involved in *Wnt* signaling, especially *Wnt*2b, has an important role in RPE cell specification after laser photocoagulation in hiPSC-RPE. It has been reported that *Wnt*7a leads to nuclear translocation of β-catenin and cytosolic β-catenin levels were elevated in cells expressing *Wnt*3 [25]. Increase in mRNA levels of *Wnt*3 and *Wnt*7a is consistent with the accumulation of β-catenin in the cytoplasm and nucleus after laser photocoagulation shown in the present study. The non-canonical pathway involving *Wnt*5a, which is known to activate β-catenin-independent pathway, was also upregulated after laser photocoagulation [26]. The β-catenin-independent pathway mainly regulates the cytoskeleton, thereby coordinating cell migration and polarity [26]. Thus, there is a possibility that *Wnt*5a is associated with migration of cells after laser photocoagulation. However, further studies are required to identify the exact role of the β-catenin-independent pathway in the restoration of hiPSC-RPE.

In conclusion, this study provides the first experimental evidence that the *W*nt/β-catenin signaling pathway has a crucial role in restoration of hiPSC-RPE after laser photocoagulation. Manipulation of the *W*nt/β-catenin signaling pathway and further elucidation of the RPE restoration mechanism might have therapeutic potential for the retinal degenerative diseases.

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