R-spondin1 Regulates Cell Proliferation of Corneal Endothelial Cells via the Wnt3a/β-Catenin Pathway

Naoki Okumura,[1,2] Takahiro Nakamura,[3] EunDuck P. Kay,[1] Makiko Nakahara,[1] Shigeru Kinoshita,[2] and Noriko Koizumi[1,2]

[1]Department of Biomedical Engineering, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan
[2]Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan
[3]Research Center for Tissue Engineering and Inflammation, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan

Correspondence: Noriko Koizumi, Department of Biomedical Engineering, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe 610-0321, Japan; nkoizumi@mail.doshisha.ac.jp.

Submitted: February 4, 2014
Accepted: September 17, 2014

Citation: Okumura N, Nakamura T, Kay EP, Nakahara M, Kinoshita S, Koizumi N. R-spondin1 regulates cell proliferation of corneal endothelial cells via the Wnt3a/β-catenin pathway. *Invest Ophthalmol Vis Sci*. 2014;55:6861–6869. DOI:10.1167/iovs.14-14091

Purpose. To evaluate the effect of Roof plate-specific spondin 1 (R-spondin1) on the proliferation of corneal endothelial cells (CECs) and to determine whether the Wnt/β-catenin pathway is involved in the activities of R-spondin1.

Methods. The proliferation of rabbit CECs (RCECs) and human CECs (HCECs) was measured by 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA. The effect of R-spondin1 on CEC density was evaluated in ex vivo organ-cultured rabbit and human corneal tissues. The cell density of HCECs cultured with R-spondin1 was also evaluated in vitro. The subcellular localization of function-associated markers of CECs (zona occludens 1 [ZO-1] and Na^+/K^+-ATPase) was determined by immunohistocytochemistry. The expression of cell cycle proteins and localization of β-catenin were determined by immunoblotting.

Results. The in vitro proliferation of RCECs and HCECs increased by 1.2- to 1.3-fold in response to R-spondin1. The CEC densities of rabbit and human corneal tissues were increased significantly by R-spondin1 treatment. Na^+/K^+-ATPase and ZO-1 were well preserved on the plasma membranes. When HCECs were maintained in the presence of R-spondin1 for up to 90 days, the maximum cell density was observed at approximately 50 days, and the cell density was maintained for up to 90 days. R-spondin1 facilitated the nuclear import of β-catenin in RCECs within 30 minutes, which subsequently upregulated cyclin D and downregulated p27, leading to G_1/S progression by hyperphosphorylation of the retinoblastoma protein.

Conclusions. The unique effects of R-spondin1 on the proliferation of CECs, regardless of species, indicate that R-spondin1 may play a key role in maintaining corneal endothelium homeostasis through the Wnt/β-catenin pathway.

Keywords: R-spondin, corneal endothelial cells, homeostasis

Roof plate-specific spondin (R-spondin) proteins were recently identified as consisting of four members, with conserved cysteine-rich furin-like and thrombospondin domains.[1–3] R-spondin1 was identified as a gene expressed in the developing spinal cord. The proteins are also expressed in mouse ovary during development, as well as in the developing dermis and kidney.[1–3] Injection of human R-spondin1 into mice demonstrated a specific proliferative effect of R-spondin1 on intestinal epithelial crypt cells.[4] R-spondin1 has also been reported to prevent chemotherapy- or radiotherapy-induced oral mucositis in mouse models.[4]

In terms of downstream signaling, R-spondins activate the canonical Wnt/β-catenin-dependent signaling pathway,[1–3,5–8] which is one of the key signaling pathways involved in cell proliferation, differentiation, and morphogenesis during embryogenesis and adulthood.[9–12] The mechanism by which the R-spondin family activates the Wnt/β-catenin signaling pathway is not fully understood. R-spondins interact with membrane receptors, such as lipoprotein receptor-related protein 5 (LRP5) and LRP6,[6] Frizzled,[5] RNF43/ZNRF3,[13,14] and Syndecan-4.[15] R-spondins were also recently reported to bind to leucine-rich repeat-containing G-protein-coupled receptors 4 to 6 (LGR4-6) and to mediate R-spondin-induced β-catenin signaling pathways.[16–20] Crystal structures showed that R-spondin1 binds to LGR5 and forms 2:2 LGR5-R-spondin1 complexes.[20] One interesting observation relevant to the present study is that LGR5 serves as a marker of resident stem cells in multiple adult tissues, including stomach, small intestine, colon, and hair follicles.[21–23] In addition, we recently reported that LGR5 is uniquely expressed in the peripheral region of human corneal endothelial cells (HCECs) and that LGR5-positive cells have some stem/progenitor cell-like characteristics.[24] These recent findings motivated us to study the effect of R-spondin1 on corneal endothelial cells (CECs).

The corneal endothelium (CE) is critical in maintaining homeostatic corneal transparency and clear vision. Restoration of clear vision lost because of endothelial disorders requires full-thickness corneal transplantation or endothelial keratoplasty. In addition to these surgical procedures, approaches employing regenerative medicine[25–30] and pharmaceutical therapy[31–34] have been extensively investigated as alternative procedures for treating CE dysfunction. Ironically, regenerative medicine and pharmaceutical therapy share a common obstacle: the poor proliferative capacity of HCECs. We

previously explored the usefulness of drug therapy for treating early-stage CE dysfunction and reported that the Rho kinase inhibitor increased the proliferative potency of the CE.[31,32] In addition, we recently demonstrated that corneal clarity and thickness were recovered following the administration of Rho-associated kinase inhibitor eye drops, especially in patients with the focal edema type of Fuchs' corneal dystrophy.[33,34] Full exploitation of the current developments in CE dysfunction therapy, however, will first require a better understanding of the mechanisms underlying the regulation of CE homeostasis and tissue regeneration.

The recent finding that LGR5-positive cells are stem/progenitor-like cells of CE[24] led us to investigate the effect of the LGR5 ligand, R-spondin1. In the present study, we evaluated the effect of R-spondin1 on cell proliferation and the functional phenotypes of CE. The effect of R-spondin1 on Wnt/β-catenin pathway was also evaluated.

Materials and Methods

Ethics Statement

The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. Written consent was acquired from the next of kin of all deceased donors regarding the donation of eyes for this research. All tissue was recovered under the Uniform Anatomical Gift Act (UAGA) of the particular state where consent for the eye donation was obtained and the eyes were recovered.

Cell Culture of Rabbit CECs

Rabbit eyes were purchased from the Funakoshi Corporation (Tokyo, Japan). Twenty rabbit eyes were used to culture rabbit CECs (RCECs). The RCECs were cultivated as described previously.[29,31] Briefly, Descemet's membrane with CECs was stripped and incubated in 0.6 U/mL Dispase II (Roche Applied Science, Penzberg, Germany) to release the CECs. After incubation for 60 minutes at 37°C, the CECs obtained from individual corneas were resuspended in culture medium and plated in one well of a six-well plate coated with FNC Coating Mix (Athena Environmental Sciences, Inc., Baltimore, MD, USA). All primary cell cultures and serial passages of CECs were performed in growth medium composed of Dulbecco's modified Eagle's medium (Life Technologies Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 ng/mL FGF-2 (Life Technologies Corp.). The RCECs were cultured in a humidified atmosphere at 37°C in 5% CO_2. The culture medium was changed every 2 days. When the cells reached confluency in 10 to 14 days, they were rinsed in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), trypsinized with 0.05% Trypsin-EDTA (Life Technologies Corp.) for 5 minutes at 37°C, and then passaged at ratios of 1:2-4. Cultivated CECs at passages 1 through 3 were used for all experiments. The proliferation effects of R-spondin1 (1, 10, and 100 ng/mL) (R&D Systems, Inc., Minneapolis, MN, USA), Wnt3a (10, 50, and 100 ng/mL) (R&D Systems, Inc.), and LiCl (3 mM) (Sigma-Aldrich Corp., St. Louis, MO, USA) were tested.

Cell Culture of HCECs

Six human donor corneas were obtained from SightLife (http://www.sightlife.org/, Seattle, WA, USA) for HCEC cultivation (Table). The HCECs were cultivated in a modified version of the protocol used for the RCECs, as described previously.[30] Briefly, the Descemet's membrane, including the CECs, was stripped and digested at 37°C for 2 hours with 1 mg/mL collagenase A (Roche Applied Science). After digestion at 37°C, the HCECs obtained from individual corneas were resuspended in culture medium and plated in one well of a 12-well plate coated with FNC Coating Mix (Athena Environmental Sciences, Inc.). The culture medium was prepared according to published protocols,[35] but with some modifications. Briefly, a basal medium was prepared containing OptiMEM-I (Life Technologies Corp.), 8% fetal bovine serum (FBS), 5 ng/mL epidermal growth factor (Sigma-Aldrich Corp.), 20 μg/mL ascorbic acid (Sigma-Aldrich Corp.), 200 mg/L calcium chloride (Sigma-Aldrich Corp.), 0.08% chondroitin sulfate (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 50 μg/mL gentamicin. The basal medium was conditioned by the cultivation of inactivated 3T3 fibroblasts, and then recovered as the culture medium for HCECs. Inactivation of the 3T3 fibroblasts was performed as described previously.[36,37] Briefly, confluent 3T3 fibroblasts were incubated with 4 μg/mL mitomycin C (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) for 2 hours at 37°C under 5% CO_2, and then trypsinized and plated onto plastic dishes at a density of 2×10^4 cells/cm^2. The HCECs were cultured in a humidified atmosphere at 37°C in 5% CO_2, and the culture medium was changed every 2 days. When the HCECs reached confluency in 14 to 28 days, they were rinsed in Ca^{2+}- and Mg^{2+}-free PBS, trypsinized with 0.05% Trypsin-EDTA for 5 minutes at 37°C, and then passaged at a 1:2 ratio. Cultivated HCECs at passages 2 through 5 were used for all experiments. The proliferative effect of R-spondin1 was tested by supplementing the HCEC culture medium with R-spondin1 (1, 10, and 100 ng/mL) and then culturing the HCECs. The cell density was evaluated using KSS-400EB software (Konan Medical, Inc., Hyogo, Japan).

Table. Numbers of Human Donor Corneas Used for the Study

Purpose of Experiments	Number of Donor Corneas
Cell culture of HCECs	6
Organ culture	4

BrdU ELISA

The RCECs or HCECs were cultured at a density of 5000 cells/well in a 96-well plate for 24 hours, and then incubated in the absence of serum for an additional 24 hours in the presence or absence of R-spondin1. DNA synthesis was detected as the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the Cell Proliferation Biotrak ELISA system, version 2 (GE Healthcare Life Sciences, Buckinghamshire, England), according to the manufacturer's instructions. Briefly, the RCECs or HCECs were incubated with 10 μM BrdU for 24 hours at 37°C and 5% CO_2 in a humidified atmosphere. The cultured cells were incubated with 10 μM BrdU labeling solution (Amersham Biosciences, Freiburg, Germany) for 2 hours and then incubated with 100 μL monoclonal antibody against BrdU for 30 minutes. The BrdU absorbance was measured directly using a spectrophotometric microplate reader at a test wavelength of 450 nm with a Veritas microplate luminometer (Promega Corporation, Madison, WI, USA).

Organ Culture

For rabbit organ culture experiments, 16 rabbit eyes were purchased from the Funakoshi Corporation. Cornea tissues were divided into four pieces and cultured with Dulbecco's modified Eagle's medium supplemented with R-spondin1 (1, 10, and 100 ng/mL) at 37°C. For human organ culture experiments, four independent human donor corneas were obtained from SightLife (Table). Cornea tissues were divided into two pieces and cultured with Minimum Essential Medium,

GlutaMAX Supplement (Life Technologies Corp.) supplemented with 100 ng/mL R-spondin1 at 37°C. The cell densities were quantified using KSS-400EB software following phalloidin staining.

Histological Examination

Rabbit or human CECs cultured on Lab-Tek chamber slides (NUNC A/S, Roskilde, Denmark) or whole corneal specimens were fixed in 4% formaldehyde for 10 minutes at room temperature and incubated for 30 minutes with 1% bovine serum albumin (BSA). The proliferation of the CECs was investigated by conducting immunohistochemical analyses of Ki67 staining when the cells reached 60% to 80% confluency, using a 1:400 dilution of anti-mouse Ki67 antibody (Sigma-Aldrich Corp.). The function-related proteins of the CECs were investigated using antibodies for Na^+/K^+-ATPase (Upstate Biotechnology, Inc., Lake Placid, NY, USA), the protein associated with pump function and zona occludens 1 (ZO-1) (Zymed Laboratories, Inc., South San Francisco, CA, USA), and tight junction-associated protein. The secondary antibody consisted of a 1:2000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies Corp.). The cellular morphology was evaluated by actin staining with a 1:400 dilution of Alexa Fluor 488-conjugated phalloidin (Life Technologies Corp.). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA, USA).The slides were then inspected by fluorescence microscopy (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany).

Cytoplasmic and Nuclear Protein Extractions

Rabbit CECs cultured in each culture condition on 60-mm culture dishes were washed twice with PBS, and cells were detached by scraping. Cells were transferred to a 1.5-mL tube and pelleted by centrifugation at 500*g* for 3 minutes. Cytoplasmic and nuclear proteins were extracted using a NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Briefly, supernatant was removed, 100 μL Cytoplasmic Extraction Reagent I (Thermo Fisher Scientific) was added, and the sample was incubated for 10 minutes on ice. Then, 5.5 μL Cytoplasmic Extraction Reagent I was added, and the sample was centrifuged at 16,000*g* for 5 minutes. The supernatant containing the cytosolic portion was transferred to a fresh tube and stored at –80°C until further use. The remaining pellet containing the nuclear portion was suspended Nuclear Extraction Reagent (Thermo Fisher Scientific). The samples were placed on ice and vortexed for 15 seconds every 10 minutes, for a total of 40 minutes, followed by centrifugation at 16,000*g* for 10 minutes. The supernatant containing the nuclear portion was transferred to a fresh tube and stored at –80°C until further use. The purity of the fractions was verified by immunoblotting the cytoplasmic or nuclear proteins with α-tubulin and lamin B antibodies. The cytoplasmic and nuclear proteins were used for further immunoblotting studies.

Protein Preparation and Immunoblotting

The RCECs were washed with ice-cold PBS and lysed with an ice-cold RIPA buffer (Bio-Rad Laboratories, Hercules, CA, USA) containing Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich Corp.) and Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were centrifuged at 15,000*g* for 10 minutes at 4°C to sediment the cell debris. The supernatant representing the total proteins was collected, and the protein concentration of the sample was determined with a BCA Protein Assay Kit (Takara Bio, Otsu, Japan). An equal amount of protein was fractionated by SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride membranes. The membranes were then blocked by 3% nonfat dry milk (Cell Signaling Technology, Inc., Danvers, MA, USA) in TBS-T buffer (50 mM Tris, pH 7.5, 150 mM NaCl2, and 0.1% Tween 20) for 1 hour at room temperature, followed by overnight incubation at 4°C with the following primary antibodies: β-catenin (1:3000; BD, Franklin Lakes, NJ, USA), α-tubulin (1:3000; MBL, Nagoya, Japan), lamin B (1:1000; Cell Signaling Technology), cyclin D1 (1:1000; Cell Signaling Technology), p27 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated p27 at threonine 187 (1:1000; Life Technologies Corp.), phosphorylated Rb protein at serine 807/811 (1:1000; Cell Signaling Technology), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:3000; Abcam, Cambridge, UK). The blots were washed and then incubated with horseradish peroxidase–conjugated secondary antibodies (1:5000: anti-rabbit IgG, anti-mouse IgG; Cell Signaling Technology). The blots were developed with luminal-enhanced chemiluminescence (ECL) using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ, USA), documented by LAS4000S (Fuji Film, Tokyo, Japan), and analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Statistical Analysis

Student's *t*-test was used to determine the statistical significance (*P* value) of the mean values of the two-sample comparison. The statistical significance determined by the comparison of multiple sample sets was analyzed using Dunnett's multiple-comparisons test. The values shown on the graphs represent the mean ± SEM.

Results

Effects of R-spondin1 on Cell Proliferation of CECs

The percentages of Ki67-positive cells were increased in a dose-dependent manner when the RCECs were treated with R-spondin1 (Fig. 1A). When the RCECs were treated with 100 ng/mL R-spondin1 for 24 hours, BrdU incorporation into DNA increased 1.3-fold (Fig. 1B). Human CECs treated with 100 ng/mL R-spondin1 also significantly increased BrdU incorporation by 1.2-fold (Fig. 1C). We then evaluated the effect of R-spondin1 on CEC proliferation in rabbit corneal tissue. After treating the cornea with R-spondin1 for 1 week, the tissue was stained with anti-Ki67 antibody. Because the peripheral area of the CE retains higher replication ability,[38] the proliferative response of peripheral CECs to R-spondin1 was compared to that of central CECs. A negligible increase was noted in the $Ki67^+$ cells in the central cornea, but a 4-fold increase in these cells occurred in the peripheral area of the CE (Fig. 2A). The cell densities of the central (0–2 mm from the center of the cornea) and peripheral (4–6 mm from the center of the cornea) areas of the CE were 2900 and 3600 cells/mm^2, respectively, when maintained in R-spondin1-free medium for 1 week. On the other hand, the cell densities of the central and peripheral areas after organ culture in the presence of 100 ng/mL R-spondin1 for 1 week were 3800 and 4300 cells/mm^2, respectively (Fig. 2B).When the corneas were cultured for 2 weeks, the cell densities of the central and peripheral areas increased to 4200 and 4600 cells/mm^2, respectively (Fig. 2C).

The effect of R-spondin1 on the morphology of the CECs was also determined using ZO-1 and observing the organization of F-actin at the cortex. The subcellular localization of actin fibers and monolayer sheet morphology were maintained in both the central and peripheral regions after treatment with R-spondin1 (Fig. 2D). Zona occludens 1 was expressed at the

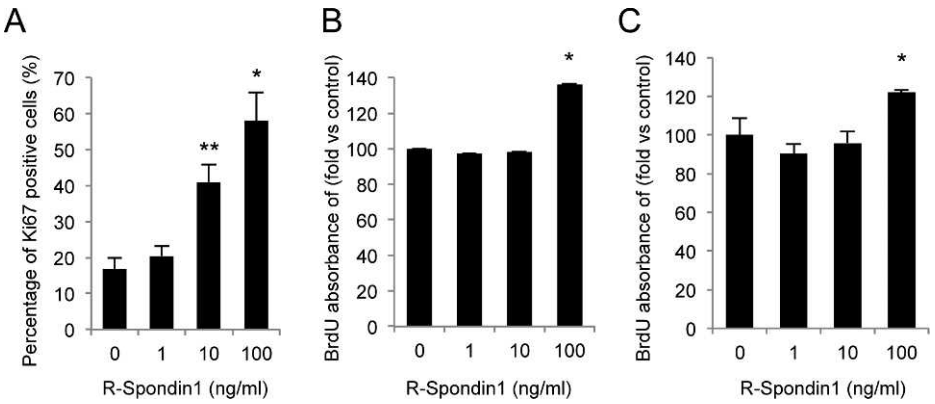

FIGURE 1. Effect of R-spondin1 on proliferation of cultured CECs. (**A**) Effect of R-spondin1 on the proliferation of cultured RCECs. The percentages of $Ki67^+$ cells cultured with 1, 10, or 100 ng/mL R-spondin1 for 24 hours were evaluated by fluorescence microscopy, and the data were then averaged and plotted ($n = 6$). (**B**) RCECs were cultured at a density of 5000 cells/well in a 96-well plate in the presence of 1, 10, or 100 ng/mL R-spondin1. The effect of R-spondin1 on the proliferation of the RCECs was evaluated by a 5-bromo-2′-deoxyuridine (BrdU) incorporation assay after 24 hours. (**C**) HCECs were cultured at a density of 5000 cells/well in a 96-well plate in the presence of 1, 10, or 100 ng/mL R-spondin1. The effect of R-spondin1 on the proliferation of the HCECs was evaluated by a BrdU incorporation assay after 24 hours. $^*P < 0.01$, $^{**}P < 0.05$. All experiments were performed in triplicate.

intercellular junction and maintained a hexagonal cell morphology after treatment with R-spondin1 (Fig. 2E).

Effect of R-spondin1 on Cell Proliferation and Phenotype of Human Corneal Endothelium

Because the replicative capacity of rabbit cornea differs from that of human CE, we also tested the effect of R-spondin1 on the proliferative capacity in HCECs. When we recovered HCECs from the donor corneas and cultured them in the presence of R-spondin1, we observed that the cell sizes were smaller than those of the control HCECs cultured without R-spondin1. The phase-contrast micrograph showed that HCECs maintained a monolayer cell sheet regardless of the presence or absence of R-spondin1 (Fig. 3A). The two functional marker proteins (Na^+/K^+-ATPase and ZO-1) were localized in the intercellular junctions of the monolayer of the hexagonal cells (Fig. 3B). We further determined the effect of R-spondin1 by maintaining serially passaged HCECs (passages 5–8) in the presence of R-spondin1. Interestingly, the cell density of HCECs was increased up to 49 days, after which the cell density was maintained in all conditions tested (Fig. 3C). We also evaluated the effect of R-spondin1 on cell proliferation in human corneal tissue. Similar to observations in rabbit cornea, the cell density of human CE was increased by R-spondin1: The cell density increased significantly from 2500 to 2700 cells/mm^2 in the central area and from 2800 to 3100 cells/mm^2 in the peripheral area after 2 weeks (Fig. 3D). The two functional proteins of the CE, Na^+/K^+-ATPase and ZO-1, were expressed and localized in the intracellular junctions of the HCECs, in both the central and peripheral regions (Figs. 3E, 3F). R-spondin1 stimulation had no effect on their subcellular localization. Similar to the findings observed in rabbit CE, the characteristic monolayer mosaic pattern of the cells was well preserved in the cells treated with R-spondin1. These findings indicate that HCECs and nonhuman CECs respond similarly to R-spondin1.

R-spondin1 Facilitates G_1/S Progression via Wnt3a/β-Catenin Signaling

R-spondins are known to activate the canonical Wnt/β-catenin-dependent signaling pathway.[1–3,5–8] We determined whether R-spondin1 stimulated cell proliferation of CECs through the Wnt/β-catenin-dependent pathway and whether a synergism existed between R-spondin1 and Wnt3a. The characteristic monolayer hexagonal pattern of RCECs was well preserved in all culture conditions: Wnt3a (10, 50, or 100 ng/mL), R-spondin1 (100 ng/mL), or a combination of Wnt3a (100 ng/mL) and R-spondin1 (100 ng/mL) (Fig. 4A). The results of the BrdU assay showed that an increase in the incorporation of BrdU into the DNA of the cells was stimulated with Wnt3a in a dose-dependent manner. The RCECs treated with 100 ng/mL Wnt3a demonstrated a 40% increase in BrdU incorporation, and the RCECs treated with 100 ng/mL R-spondin1 showed a 50% increase (Fig. 4B). Interestingly, simultaneous treatment of the RCECs with R-spondin1 and Wnt3a resulted in a 50% increase in BrdU incorporation into DNA, suggesting that R-spondin1 may exert its activity through the Wnt signaling pathway.

We then conducted tests to determine whether the action of R-spondin1 depended on the canonical Wnt/β-catenin pathway. In the absence of Wnt3a, β-catenin is present in the cytoplasm. Wnt activation inhibits glycogen synthase kinase 3β (GSK-3β) activity, preventing β-catenin degradation and facilitating its translocation from the cytoplasm to the nucleus.When the cells were treated with R-spondin1 for 30 or 60 minutes, R-spondin1 facilitated the nuclear import of β-catenin within 30 minutes (Fig. 4C). On the other hand, the cytoplasmic fraction of β-catenin was not altered with or without treatment with R-spondin1. Rabbit CECs treated with LiCl (an inhibitor of GSK-3β) served as a positive control, showing a degree of nuclear import of β-catenin similar to that observed in the R-spondin1-treated cells.

R-spondin1 Facilitates G_1/S Progression via Downregulation of p27 and Upregulation of Cyclin D

Phosphorylation, ubiquitination, and the subsequent degradation of p27 are the major mechanism of G_1/S progression in CECs.[39,40] A gradual, time-dependent decrease in p27 was seen in response to R-spondin1 stimulation (Fig. 4D). Phosphorylation of p27 at the Thr187 site was increased in the RCECs treated with R-spondin1 for 24 hours (Fig. 4D). This finding is similar to that of a previous report,[40] which clearly showed that the phosphorylation of p27 at Thr187 is a late G_1 event in the cell cycle. The hyperphosphorylation of Rb protein was observed in the RCECs treated with R-spondin1 for 24 hours. Note that the phosphorylation of Rb protein in the control

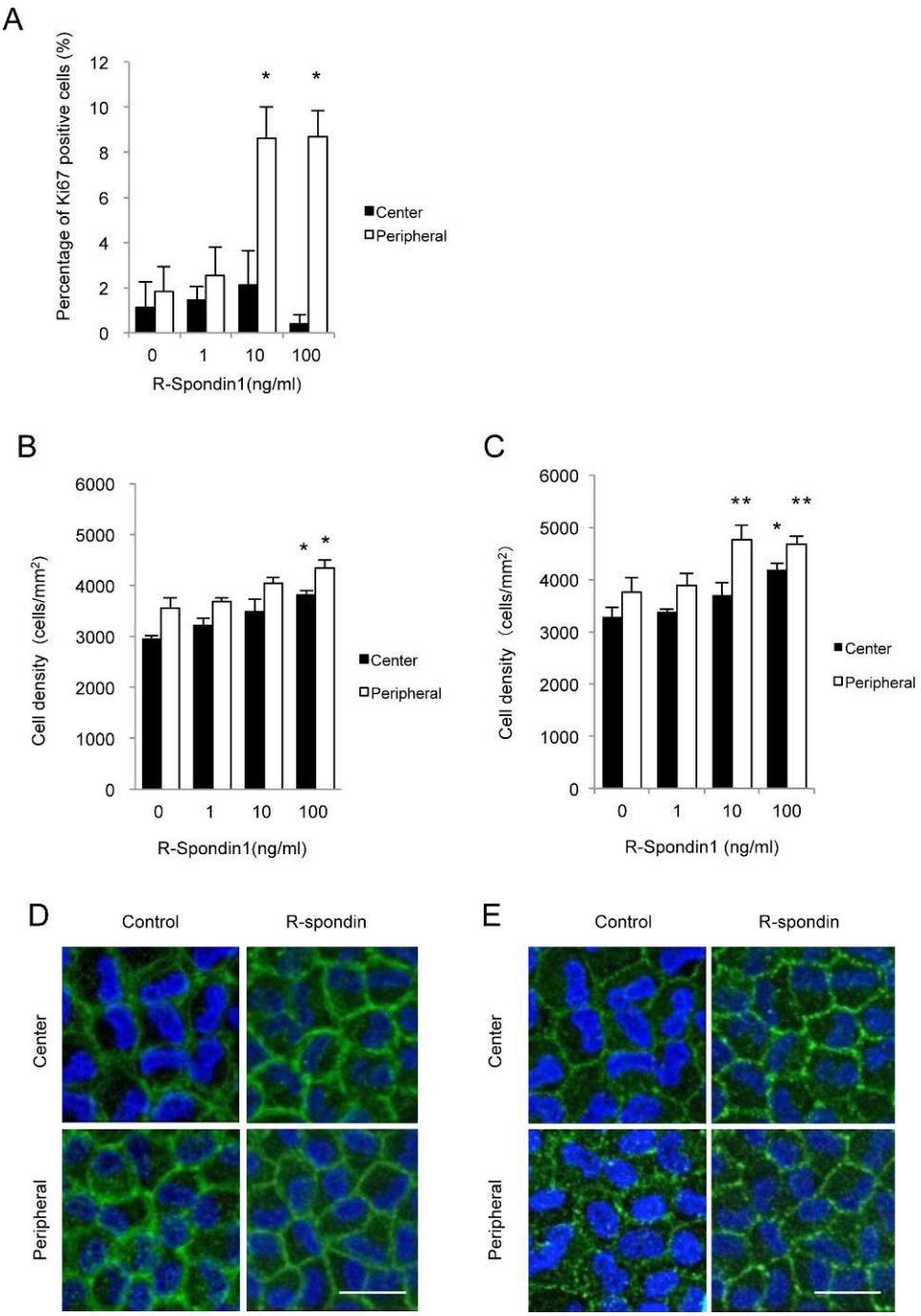

Figure 2. Effect of R-spondin1 on proliferation and cell density of rabbit CE. (**A**) A rabbit cornea was divided into four pieces and organ-cultured with DMEM supplemented with 1, 10, or 100 ng/mL R-spondin1. The percentages of $Ki67^+$ cells were evaluated after 1 week in culture, and the data were averaged and plotted ($n = 4$). (**B**, **C**) The cell density of the central (0–2 mm from the center of the cornea) and peripheral (4–6 mm from the center of the cornea) areas after organ culture with R-spondin1 for 1 week (**B**) and 2 weeks (**C**) was quantified by KSS-400EB software following phalloidin staining ($n = 4$). The cell densities were evaluated in an area > 1 mm away from the cut edges of the cornea. (**D**, **E**) The effect of R-spondin1 on the morphology of the CECs was determined by F-actin (**D**) and ZO-1 (**E**). All experiments were performed in triplicate. *Scale bars*: 25 μm.

cells was mediated by the growth medium, even in the absence of R-spondin1. In addition, the expression of cyclin D was enhanced for up to 24 hours in the presence of R-spondin1.

Discussion

Human CE is a physiologically important monolayer of the cornea. The simple but crucial role of the endothelium is to maintain cornea clarity.[41] The retention of the unique, contact-inhibited monolayer of the CE, through which the active pump and barrier functions of the tissue operate, is essential for maintaining the transparency of the entire cornea. The human CE has a limited wound healing capacity, in which residual CECs migrate and expand to cover the wound area. This unique wound repair process is explained in part by the fact that HCECs remain arrested at the G_1 phase of the cell cycle.[42]

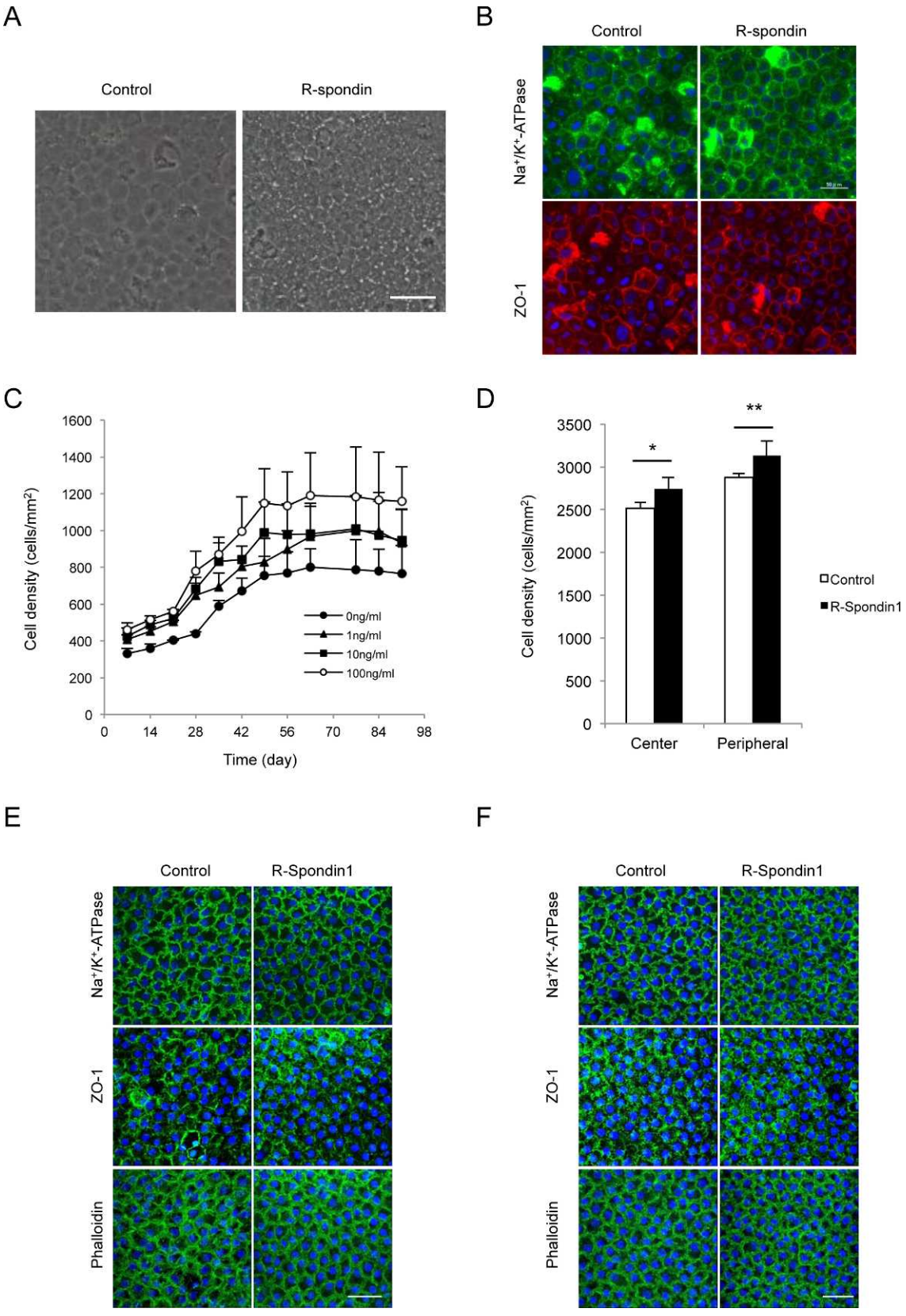

FIGURE 3. Effect of R-spondin1 on cell proliferation and phenotype of human CE. (**A**) HCECs were recovered from donor cornea and cultivated with culture medium supplemented with or without R-spondin1 (100 ng/mL). Representative phase-contrast images of the experiments performed in three independent donor corneas are shown. *Scale bar*: 200 μm. (**B**) The two functional proteins of the CE, Na^+/K^+-ATPase and ZO-1, were immunostained in primary cultured HCECs treated with or without R-spondin1. *Scale bar*: 50 μm. (**C**) Serial passaged HCECs (passages 5–8) were maintained in the presence of R-spondin1 at 1, 10, or 100 ng/mL for up to 91 days. The cell density was evaluated by KSS-400EB software ($n = 6$). The experiments were performed in three independent lots. (**D**) Four human donor corneas were each divided into two pieces and cultured with or

without R-spondin1 (100 ng/mL), respectively. The cell density was evaluated by KSS-400EB software after 2 weeks following phalloidin staining ($n = 4$); $^{*}P < 0.01$, $^{**}P < 0.05$. (**E**, **F**) The expression and localization of Na^{+}/K^{+}-ATPase, ZO-1, and F-actin were examined in ex vivo human corneal tissues ([**E**] center, [**F**] peripheral). *Scale bar*: 50 μm.

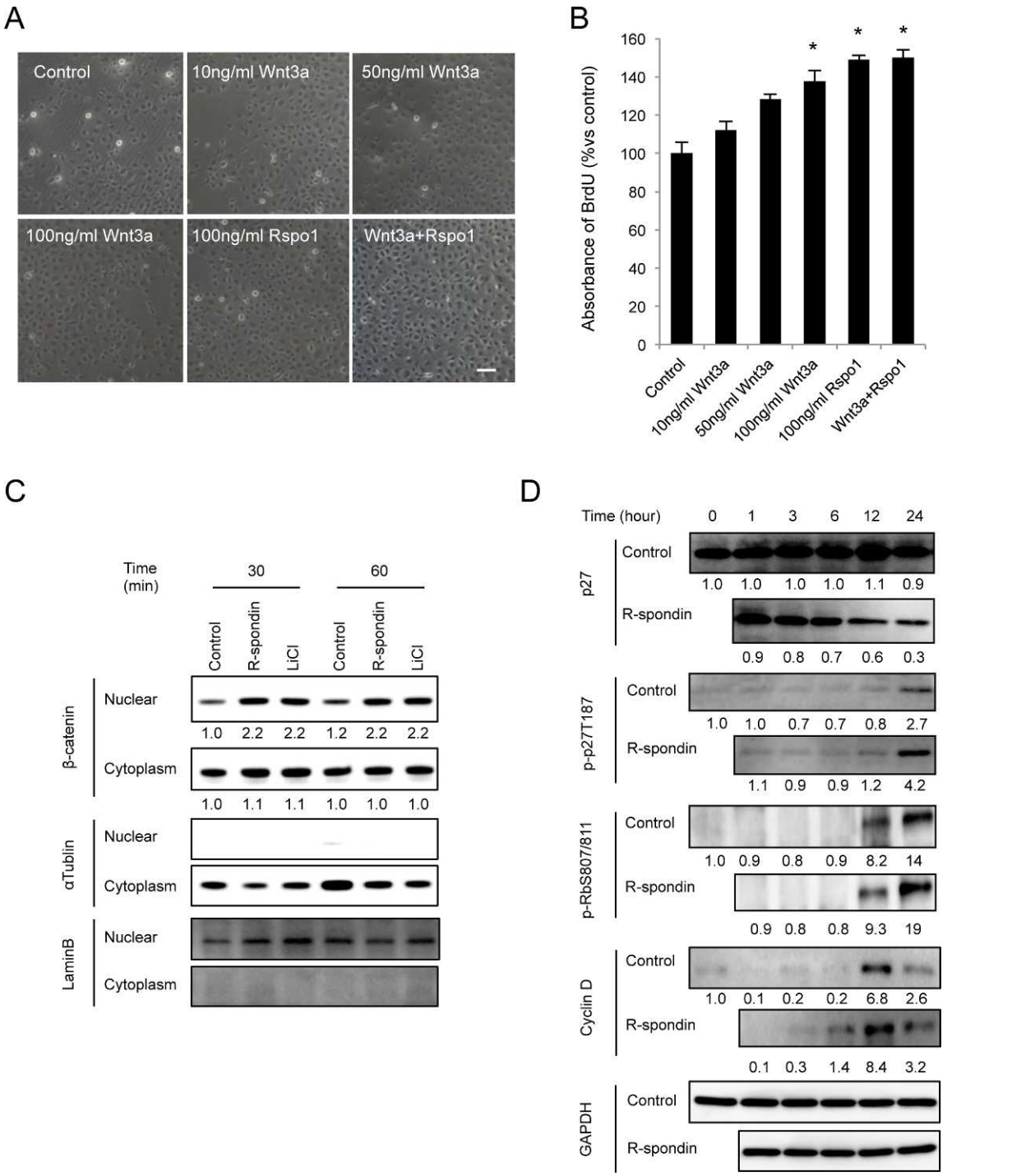

Figure 4. Involvement of Wnt3a/β-catenin signaling in the facilitation of G_1/S progression, mediated by R-spondin1. (**A**) Phase-contrast images of RCECs treated with Wnt3a (10, 50, or 100 ng/mL), R-spondin1 (100 ng/mL), or a combination of Wnt3a (100 ng/mL) and R-spondin1 (100 ng/mL) for 48 hours. *Scale bar*: 200 μm. (**B**) The effect of R-spondin1 on the proliferation of the RCECs was evaluated by a BrdU incorporation assay after 48 hours. $^{*}P < 0.01$. The experiments were performed in triplicate. (**C**) After activation of the cells with R-spondin1, the nuclear fraction and the cytosolic fraction were prepared, and the nuclear translocation of β-catenin was evaluated by Western blotting. Lamin B was used as a marker for the nuclear fraction, and α-tubulin was used as a marker for the cytosolic fraction. (**D**) Serum-starved RCECs were treated with or without R-spondin1 (100 ng/mL) for 1, 3, 6, 12, or 24 hours. Then p27, phosphorylation of p27, phosphorylation of Rb, and cyclin D were evaluated by Western blotting. The relative density of immunoblotted bands was determined using ImageJ software. The averages of relative fold differences from independent duplicate experiments were then compared with the values from the control.

Although the existence of CE stem cells is still controversial, a recent study suggested that CE at the extreme periphery[43] or in the transition zone between the endothelial edge and the trabecular meshwork might include stem cells and thereby have proliferative potency.[44,45] We recently reported that LGR5-positive cells of the human CE exhibit stem/progenitor-like cell characteristics[24]; LGR 5 has recently been demonstrated to function as a high-affinity receptor of R-spondins to activate Wnt/β-catenin signaling.[16,17,46] R-spondins are known agonists of Wnt signaling and are involved in stem cell regulation, such as survival and differentiation.[3,7,19] These findings indicate that HCECs in the peripheral region may retain proliferative potential and that a continuous supply of new cells may be available to maintain the CE Therefore, our aim was to determine whether R-spondin1 plays a key role in tissue homeostasis and tissue regeneration.

We demonstrated that R-spondin1 stimulated proliferation of both RCECs and HCECs in vitro and ex vivo. When the proliferative potential was compared following R-spondin1 stimulation, RCECs showed greater cell proliferative activity in the peripheral CE than in the central CE. Our results showed that approximately 8% of the Ki67-positive cells were observed in peripheral rabbit cornea, and these mitotic cells may increase peripheral cell density. However, despite the fact that limited numbers of Ki67-positive cells were observed in the central area, R-spondin1 increased the cell density in the center as well as in the peripheral area. One possible explanation may be that R-spondin1 predominantly enhances cell proliferation in the peripheral area, and the peripheral cells then proliferate and migrate toward the center where cells are not proliferating, resulting in a cell density increase in the central area. This cell migration from the periphery to the center is proposed as a CE homeostasis model in which cell clusters form niches in the extreme peripheral area and the peripheral CECs divide slowly and migrate toward the center.[45] Higher replication competence of the peripheral area during the wound healing process was also previously reported in an ex vivo system.[40] Further investigation is needed to clarify whether the response to R-spondin1 differs between LGR5-positive and -negative CECs,[24] but the greater potency of the proliferative activity of the peripheral CECs suggests that the response to R-spondin1 may be higher in peripheral LGR5-positive CECs.

We next evaluated the effect of R-spondin1 using human corneas, as the limitation of rabbit tissue for experiments on CE is that rabbit CE possesses an active proliferative ability that is not found in human corneas. For instance, rabbit CEC density was regenerated to its original level after cryoinjury in vivo.[47] Our findings that R-spondin1 promotes HCEC proliferation in human corneas agree with previous reports indicating that mechanical wounding or a combination of growth factors and release from contact inhibition promoted cell proliferation using ex vivo human cornea tissues.[48,49] However, the underlying mechanisms that explain how R-spondin1 enhances cell proliferation without mechanical wounding or contact inhibition release should be further studied.

We further demonstrated that R-spondin1 facilitated the nuclear import of β-catenin, which was subsequently involved in the transcription of cyclin D, one of the target genes of the β-catenin-involved LEF/TCF family of transcription factors.[9] Stimulation of the cells with R-spondin1 sequestered p27 from the cyclin D/Cdk4/Cdk6 complex to the cyclin E/Cdk2 complex, which subsequently phosphorylated p27, leading to the ubiquitination and degradation of p27. In this study, the phosphorylation of p27 by R-spondin1 aligns with previous reports showing that p27 plays a major role in G_1/S progression in the CE.[39,50,51] The present study suggested that R-spondin1 facilitates G_1/S progression through two G_1 regulators—cyclin D (a positive regulator) and p27 (a negative regulator)—via the canonical Wnt/β-catenin pathway.

Our findings are the first to provide evidence that the Wnt/β-catenin pathway is involved in cell proliferation and maintenance of the functional phenotype in CE cells. The progenitor cells of CECs ($LGR5^+$) in the peripheral region of the cornea may possibly proliferate in response to microenvironmental cues (in this case, R-spondin1). The unique tissue regenerative capacity of R-spondin1 may therefore be useful in the treatment of endothelial dysfunction.

Acknowledgments

The authors thank Eri Mizuhara, Shinichiro Nakano, Kazuya Kakutani, and Yuki Tsujimoto for their technical assistance and Kana Hirata-Tominaga for her kind assistance and invaluable advice about R-spondin.

Supported by the Funding Program for Next Generation World-Leading Researchers from the Cabinet Office in Japan (NK, LS117).

Disclosure: **N. Okumura**, P; **T. Nakamura**, P; **E.P. Kay**, None; **M. Nakahara**, None; **S. Kinoshita**, P; **N. Koizumi**, P

References

1. Kamata T, Katsube K, Michikawa M, Yamada M, Takada S, Mizusawa H. R-spondin, a novel gene with thrombospondin type 1 domain, was expressed in the dorsal neural tube and affected in Wnts mutants. *Biochim Biophys Acta*. 2004;1676:51–62.
2. Kazanskaya O, Glinka A, del Barco Barrantes I, Stannek P, Niehrs C, Wu W. R-Spondin2 is a secreted activator of Wnt/beta-catenin signaling and is required for Xenopus myogenesis. *Dev Cell*. 2004;7:525–534.
3. Yoon JK, Lee JS. Cellular signaling and biological functions of R-spondins. *Cell Signal*. 2012;24:369–377.
4. Zhao J, Kim KA, De Vera J, Palencia S, Wagle M, Abo A. R-Spondin1 protects mice from chemotherapy or radiation-induced oral mucositis through the canonical Wnt/beta-catenin pathway. *Proc Natl Acad Sci U S A*. 2009;106:2331–2336.
5. Nam JS, Turcotte TJ, Smith PF, Choi S, Yoon JK. Mouse cristin/R-spondin family proteins are novel ligands for the Frizzled 8 and LRP6 receptors and activate beta-catenin-dependent gene expression. *J Biol Chem*. 2006;281:13247–13257.
6. Wei Q, Yokota C, Semenov MV, Doble B, Woodgett J, He X. R-spondin1 is a high affinity ligand for LRP6 and induces LRP6 phosphorylation and beta-catenin signaling. *J Biol Chem*. 2007;282:15903–15911.
7. Kim KA, Wagle M, Tran K, et al. R-Spondin family members regulate the Wnt pathway by a common mechanism. *Mol Biol Cell*. 2008;19:2588–2596.
8. Han XH, Jin YR, Seto M, Yoon JKA. WNT/beta-catenin signaling activator, R-spondin, plays positive regulatory roles during skeletal myogenesis. *J Biol Chem*. 2011;286:10649–10659.
9. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem*. 2006;281:22429–22433.
10. Kikuchi A, Yamamoto H, Sato A. Selective activation mechanisms of Wnt signaling pathways. *Trends Cell Biol*. 2009;19:119–129.
11. Clevers H. Wnt/beta-catenin signaling in development and disease. *Cell*. 2006;127:469–480.
12. Holland JD, Klaus A, Garratt AN, Birchmeier W. Wnt signaling in stem and cancer stem cells. *Curr Opin Cell Biol*. 2013;25:254–264.

13. Hao HX, Xie Y, Zhang Y, et al. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature*. 2012;485: 195–200.
14. Peng WC, de Lau W, Madoori PK, et al. Structures of Wnt-antagonist ZNRF3 and its complex with R-spondin 1 and implications for signaling. *PloS One*. 2013;8:e83110.
15. Ohkawara B, Glinka A, Niehrs C. Rspo3 binds syndecan 4 and induces Wnt/PCP signaling via clathrin-mediated endocytosis to promote morphogenesis. *Dev Cell*. 2011;20:303–314.
16. Carmon KS, Gong X, Lin Q, Thomas A, Liu Q. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. *Proc Natl Acad Sci U S A*. 2011;108:11452–11457.
17. Glinka A, Dolde C, Kirsch N, et al. LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. *EMBO Rep*. 2011;12:1055–1061.
18. Xu K, Xu Y, Rajashankar KR, Robev D, Nikolov DB. Crystal structures of Lgr4 and its complex with R-spondin1. *Structure*. 2013;21:1683–1689.
19. Huch M, Dorrell C, Boj SF, et al. In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature*. 2013;494:247–250.
20. Peng WC, de Lau W, Forneris F, et al. Structure of stem cell growth factor R-spondin 1 in complex with the ectodomain of its receptor LGR5. *Cell Rep*. 2013;3:1885–1892.
21. Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*. 2007;449:1003–1007.
22. Barker N, Huch M, Kujala P, et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell Stem Cell*. 2010;6:25–36.
23. Jaks V, Barker N, Kasper M, et al. Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nat Genet*. 2008;40:1291–1299.
24. Hirata-Tominaga K, Nakamura T, Okumura N, et al. Corneal endothelial cell fate is maintained by LGR5 through the regulation of hedgehog and Wnt pathway. *Stem Cells*. 2013;31: 1396–1407.
25. Mimura T, Yamagami S, Yokoo S, et al. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. *Invest Ophthalmol Vis Sci*. 2004;45:2992–2997.
26. Ishino Y, Sano Y, Nakamura T, et al. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci*. 2004;45:800–806.
27. Sumide T, Nishida K, Yamato M, et al. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. *FASEB J*. 2006;20:392–394.
28. Mimura T, Yamagami S, Yokoo S, et al. Sphere therapy for corneal endothelium deficiency in a rabbit model. *Invest Ophthalmol Vis Sci*. 2005;46:3128–3135.
29. Koizumi N, Sakamoto Y, Okumura N, et al. Cultivated corneal endothelial cell sheet transplantation in a primate model. *Invest Ophthalmol Vis Sci*. 2007;48:4519–4526.
30. Okumura N, Koizumi N, Ueno M, et al. ROCK inhibitor converts corneal endothelial cells into a phenotype capable of regenerating in vivo endothelial tissue. *Am J Pathol*. 2012;181: 268–277.
31. Okumura N, Ueno M, Koizumi N, et al. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. *Invest Ophthalmol Vis Sci*. 2009;50:3680–3687.
32. Okumura N, Koizumi N, Ueno M, et al. Enhancement of corneal endothelium wound healing by Rho-associated kinase (ROCK) inhibitor eye drops. *Br J Ophthalmol*. 2011;95:1006–1009.
33. Okumura N, Koizumi N, Kay EP, et al. The ROCK inhibitor eye drop accelerates corneal endothelium wound healing. *Invest Ophthalmol Vis Sci*. 2013;54:2493–2502.
34. Koizumi N, Okumura N, Ueno M, Nakagawa H, Hamuro J, Kinoshita S. Rho-associated kinase inhibitor eye drop treatment as a possible medical treatment for Fuchs corneal dystrophy. *Cornea*. 2013;32:1167–1170.
35. Nakahara M, Okumura N, Kay EP, et al. Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. *PloS One*. 2013;8: e69009.
36. Koizumi N, Fullwood NJ, Bairaktaris G, Inatomi T, Kinoshita S, Quantock AJ. Cultivation of corneal epithelial cells on intact and denuded human amniotic membrane. *Invest Ophthalmol Vis Sci*. 2000;41:2506–2513.
37. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell*. 1975;6:331–343.
38. Mimura T, Joyce NC. Replication competence and senescence in central and peripheral human corneal endothelium. *Invest Ophthalmol Vis Sci*. 2006;47:1387–1396.
39. Lee JG, Kay EP. Two populations of p27 use differential kinetics to phosphorylate Ser-10 and Thr-187 via phosphatidylinositol 3-Kinase in response to fibroblast growth factor-2 stimulation. *J Biol Chem*. 2007;282:6444–6454.
40. Lee JG, Song JS, Smith RE, Kay EP. Human corneal endothelial cells employ phosphorylation of p27(Kip1) at both Ser10 and Thr187 sites for FGF-2-mediated cell proliferation via PI 3-kinase. *Invest Ophthalmol Vis Sci*. 2011;52:8216–8223.
41. Bourne WM. Clinical estimation of corneal endothelial pump function. *Trans Am Ophthalmol Soc*. 1998;96:229–239.
42. Joyce NC, Navon SE, Roy S, Zieske JD. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. *Invest Ophthalmol Vis Sci*. 1996;37:1566–1575.
43. He Z, Campolmi N, Gain P, et al. Revisited microanatomy of the corneal endothelial periphery: new evidence for continuous centripetal migration of endothelial cells in humans. *Stem Cells*. 2012;30:2523–2534.
44. Whikehart DR, Parikh CH, Vaughn AV, Mishler K, Edelhauser HF. Evidence suggesting the existence of stem cells for the human corneal endothelium. *Mol Vis*. 2005;11:816–824.
45. McGowan SL, Edelhauser HF, Pfister RR, Whikehart DR. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. *Mol Vis*. 2007;13:1984–2000.
46. de Lau W, Barker N, Low TY, et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature*. 2011;476:293–297.
47. Minkowski JS, Bartels SP, Delori FC, Lee SR, Kenyon KR, Neufeld AH. Corneal endothelial function and structure following cryo-injury in the rabbit. *Invest Ophthalmol Vis Sci*. 1984;25:1416–1425.
48. Senoo T, Joyce NC. Cell cycle kinetics in corneal endothelium from old and young donors. *Invest Ophthalmol Vis Sci*. 2000; 41:660–667.
49. Senoo T, Obara Y, Joyce NC. EDTA: a promoter of proliferation in human corneal endothelium. *Invest Ophthalmol Vis Sci*. 2000;41:2930–2935.
50. Joyce NC. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res*. 2003;22:359–389.
51. Kikuchi M, Zhu C, Senoo T, Obara Y, Joyce NC. p27kip1 siRNA induces proliferation in corneal endothelial cells from young but not older donors. *Invest Ophthalmol Vis Sci*. 2006;47: 4803–4809.

Investigative Ophthalmology & Visual Science