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研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

The ROCK Inhibitor Eye Drop Accelerates Corneal Endothelium Wound Healing

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Purpose. To evaluate the effect of Rho kinase (ROCK)-inhibitor eye drops on a corneal endothelial dysfunction primate model and human clinical case series of corneal endothelial dysfunction.

Methods. As a corneal-endothelial partially injured model, the corneal endothelium of seven cynomolgus monkeys was damaged by transcorneal freezing; 10 mM of ROCK inhibitor Y-27632 was then applied topically 6 times daily. The phenotype of the reconstructed corneal endothelium was evaluated by immunohistochemical analysis and noncontact specular microscopy. For clinical study, the effect of Y-27632 eye drops after transcorneal freezing was evaluated in eight corneal endothelial dysfunction patients: four central corneal edema patients and four diffuse corneal edema patients.

Results. Slit-lamp microscopy revealed that both Y-27632–treated and –nontreated corneas became hazy after transcorneal freezing, and then recovered their transparency within 4 weeks. ROCK inhibitor Y-27632 promoted recovery of corneal endothelial cell density and wound healing in terms of both morphology and function. The percentage of ZO-1 and Na^+/K^+-ATPase positive cells in the regenerated area in the Y-27632 group was significantly higher than in the controls. Noncontact specular microscopy revealed that corneal endothelial cell density was significantly higher in the Y-27632 group compared with the controls at 4 weeks; cell density reached approximately 3000 cells/mm^2, as opposed to 1500 cells/mm^2 in the control group. In addition to the animal study findings, the clinical study findings showed that Y-27632 eye drops effectively improved corneal edema of corneal endothelial dysfunction patients with central edema.

Conclusions. These findings show that ROCK inhibitor Y-27632 eye drops promote corneal endothelial wound healing in a primate animal model and suggest the possibility of Y-27632 as a novel therapeutic modality for certain forms of corneal endothelial dysfunction. (http://www.umin.ac.jp/ctr/ number, UMIN000003625.)

Keywords: corneal endothelial cells, rho kinase, ROCK inhibitor, wound healing

The corneal endothelium is critical in maintaining homeostatic corneal transparency. Human corneal endothelial cells (HCECs) show severely limited proliferative ability in vivo. As a result, pathological corneal endothelial cell (CEC) loss causes a concurrent compensatory migration and enlargement of the remaining endothelial cells to achieve the functional contact-inhibited monolayer. In corneal endothelial disorders such as Fuchs' corneal dystrophy, pseudophakic bullous keratopathy, or trauma-related injuries, severe impairments of the relevant functions of CECs, namely Na^+/K^+-ATPase pump and barrier functions, cause irreversible corneal haziness. In the United States, 42,642 corneal transplantations were performed in 2011.[1] Since corneal endothelial dysfunction is the major indication for performing corneal transplantations, endothelial keratoplasty represented over 40% of all corneal grafts performed in both 2009 and 2010.[2] However, several severe problems can arise associated with corneal transplantation, including allograft rejection, primary graft failure, and severe loss of cell density. To the best of our knowledge, no clinically practical medical therapy has been developed to effectively treat corneal endothelial dysfunction.

As an alternative to corneal transplantation, regenerative medical procedures might be a plausible path of therapy for treating severe corneal endothelial dysfunction. Several research groups, including ours, have reported transplantations of cultivated CECs in an animal model to establish a new clinical intervention for corneal endothelial dysfunction.[3–9] We recently reported the use of cell therapy to successfully achieve the recovery of corneal transparency in both rabbit and primate corneal endothelial dysfunction models.[9] However, in cases of early-stage corneal endothelial dysfunction, in which stem cells or progenitor cells are still maintained in the tissue, drug therapy may provide a less-invasive or antiprogression treatment. Our group, as well as several other groups, reported that pharmaceutical agents such as epidermal growth factor, platelet-derived growth factor, FGF-2, and small interfering RNA of connexin 43 showed the potent effect of enhancing the promotion of corneal endothelial cells, both in vitro and in

vivo.[10–12] However, a pharmaceutical agent has yet to be introduced into the clinical setting.[1,13] In a previous study, we demonstrated that a specific Rho kinase (ROCK)-inhibitor, Y-27632, increased the proliferative potential of cultivated primate CECs in vitro.[14] Previous studies have reported that the RhoA/ROCK pathway is involved in regulating the actin cytoskeleton, as well as cell migration, apoptosis, and proliferation.[15–19] In addition, Rho GTPases reportedly suppress cell-cycle progression in several cell systems.[15,19] We also reported that the topical administration of ROCK inhibitor Y-27632 enhanced corneal endothelial wound healing in an in vivo rabbit model in which the corneal endothelium was partially damaged.[13,20,21]

In the present study, the feasibility of using topically administered ROCK inhibitor Y-27632 eye drops for clinical application was evaluated using a primate corneal endothelial dysfunction model. Our findings demonstrated that the use of ROCK inhibitor Y-27632 in eye drop form promotes corneal endothelial wound healing of the primate CECs with respect to both endothelial morphology and endothelial functions. Furthermore, CEC density, which is the most crucial indicator in the context of the clinical setting, was found to be recovered to the normal level after administration of the ROCK inhibitor Y-27632 eye drops. Of importance, we performed a pilot clinical study and demonstrated the feasibility of ROCK inhibitor Y-27632 eye-drop treatment as a therapeutic modality for corneal endothelial dysfunction. These findings provide new insights that ROCK inhibitor eye drops can be developed and employed as a new pharmaceutical agent for the treatment or antiprogression therapy of corneal endothelial dysfunction.

Materials and Methods

Primate Corneal Endothelial Wound Model

The corneal endothelium of seven cynomolgus monkeys (3–5 years of age; estimated equivalent human age: 5–20 years) was damaged under general anesthesia by transcorneal freezing with a 7-mm–diameter probe for 15 seconds (Fig. 1A); the stainless-steel probe was immersed in liquid nitrogen for 3 minutes to stabilize its temperature at approximately −196°C. We confirmed that the transcorneal cryogenic injury damaged the central corneal endothelium in a round shape reproducibly. In all experiments, animals were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The monkey experiments were performed at the Research Center for Animal Life Science at Shiga University of Medical Science according to the protocol approved by the Animal Care and Use Committee of Shiga University of Medical Science (Approval No. 2008-10-5).

Histological Examination

To evaluate the effect of ROCK inhibitor Y-27632 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) on the morphological wound healing process, corneas obtained from one monkey euthanized 48 hours after the transcorneal freeze injury were examined by DAPI (Vector Laboratories, Burlingame, CA) staining. A 10-mM amount of Y-27632 was topically applied to one eye of each animal 6 times daily for the first 48 hours. PBS was applied to the fellow eye of each animal as a control. The wound area was then evaluated and calculated by use of Java-based image processing software (Image J; National Institutes of Health, Bethesda, MD).

To investigate the phenotype of reconstructed corneal endothelium obtained from four monkeys euthanized 4 weeks after transcorneal freezing in the presence or absence of Y-27632 treatment, immunohistochemical analyses of actin, Ki67, ZO-1, and Na^+/K^+-ATPase were performed. Actin staining was performed with 1:400 diluted AlexaFluor 488-conjugated phalloidin (Life Technologies Corporation, Carlsbad, CA). ZO-1 or Na^+/K^+-ATPase staining was performed with 1:200 diluted ZO-1 polyclonal antibody (Zymed Laboratories, South San Francisco, CA), or Na^+/K^+-ATPase monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY). Ki67 staining was performed using 1:400-dilution antimouse Ki67 antibody (Sigma-Aldrich Co., St. Louis, MO). AlexaFluor 488-conjugated goat antimouse IgG (1:2000; Life Technologies Corporation) was used as a secondary antibody. Each nucleus was stained with DAPI and examined under a confocal microscope (Leica TCS SPE; Leica Microsystems, Wetzlar, Germany).

Scanning Electron Microscopy

To evaluate the effect of Y-27632 on functional wound healing, corneal specimens were obtained from four monkeys at 4 weeks after the treatment. Excised corneas were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen buffer (pH 7.2 to 7.4) for at least 3 hours at room temperature. Following several washes in the buffer and postfixation with 1% aqueous osmium tetroxide, the corneas were dehydrated through an ascending ethanol series and transferred to hexamethyldisilazane (Agar Scientific, Stansted, UK), which was allowed to sublimate off. The samples were then mounted on stubs and sputter-coated with gold before being examined under a scanning electron microscope (model 5600; JEOL Ltd., Tokyo, Japan).

Evaluation of Corneal Appearance and Examination by Specular Microscopy

The corneal endothelium of seven cynomolgus monkeys was injured by transcorneal freezing as described above. Next, 10 mM of ROCK inhibitor Y-27632 diluted in PBS (50 μL) was topically applied in one eye of each animal 6 times daily for 2 days, while PBS was applied in the animal's fellow eye as a control. One animal was euthanized at 48 hours after cryoinjury to determine the morphological wound healing process, while the in vivo corneal endothelium of the surviving animals was examined by use of a noncontact specular microscope (Noncon ROBO SP-8800; Konan Medical, Nishinomiya, Japan). Four weeks after transcorneal injury, four animals were euthanized and processed for immunohistological analysis and electron microscopy. The other two animals were kept alive for long-term observation.

Clinical Trial of ROCK Inhibitor Eye Drops

The clinical trial performed in this study was conducted in accordance with the tenets set forth in the Declaration of Helsinki. This study was performed according to a protocol approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (approval number: C-626-2). Prior to this, a phase 1 clinical study of ROCK inhibitor Y-27632 eye drops involving 10 healthy volunteers was conducted (approval number: C-626-1), which confirmed that 10 mM of Y27632 applied 6 times daily for 7 days caused no systemic or local side effects. Clinical trial registration was obtained from the Japanese Infrastructure for Academic Activities, University hospital Medical Information Network (UMIN) (No. UMIN000003625; in the public domain at http://www.umin.ac.jp/english/). After proper informed consent was obtained from all subjects, eight eyes of 8 patients scheduled for Descemet's Stripping Automated Endothelial

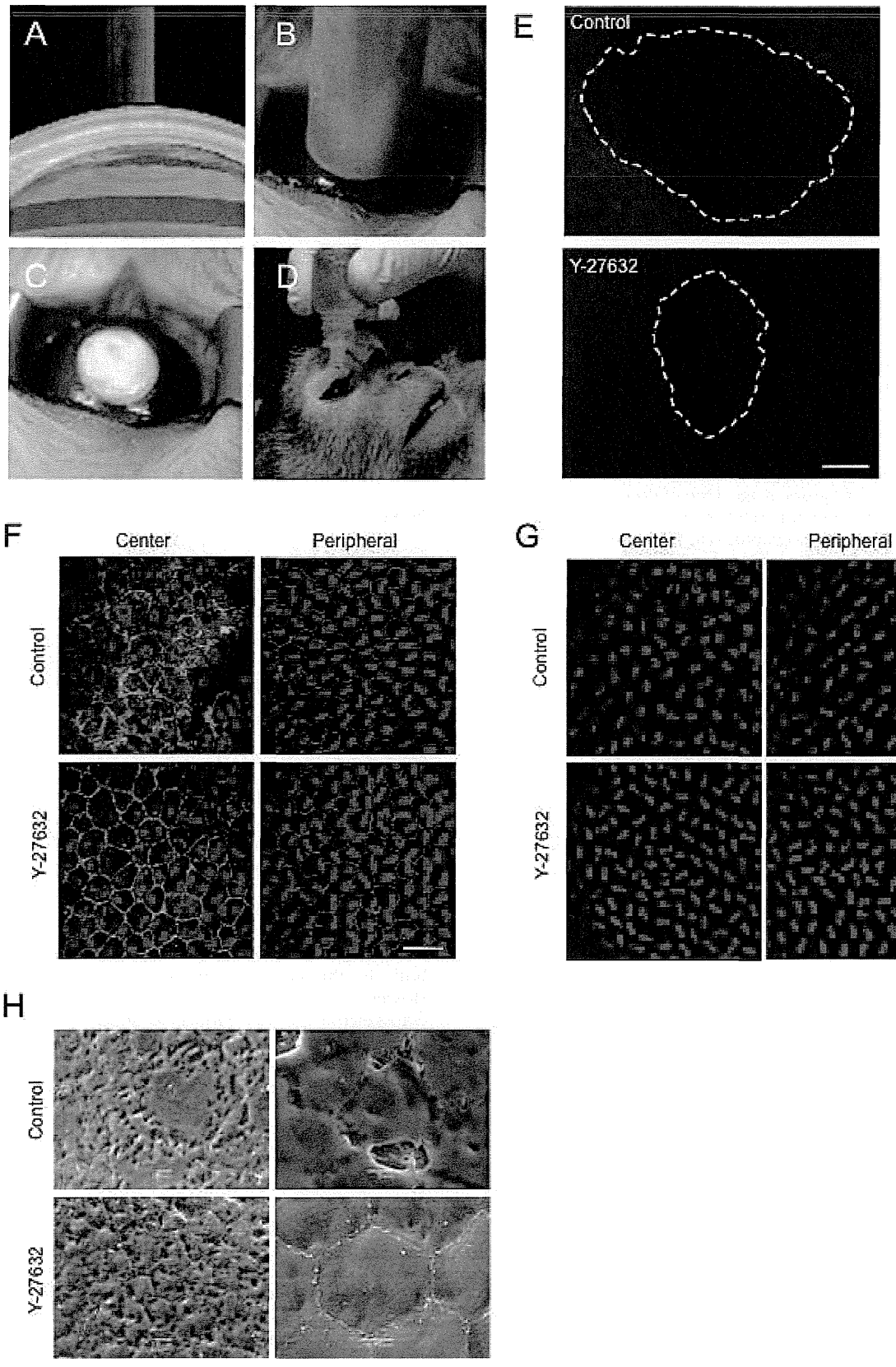

Figure 1. Promotion of wound healing by ROCK inhibitor Y-27632 in a primate model. (**A**–**C**) Central corneal endothelium was partially damaged by transcorneal cryogenic injury. (**D**) A 10-mM amount of Y-27632 (50 μL) was topically applied in one eye of each animal six times daily for 2 days. (**E**) DAPI staining shows the corneal endothelial wound. The wound area of the Y-27632–treated eye was smaller than that of the control eye after 48 hours. The *white dotted line* indicates the wound area. *Scale bar*: 500 μm. (**F**) Flatmount examination of the posterior side of the corneal tissue 4 weeks after Y-27632 treatment. *Green* fluorescein indicates F-actin staining (phalloidin) and *blue* indicates nuclear staining (DAPI). In the central area of the corneal endothelium, the actin cytoskeleton is organized at the cortex of the hexagonal cells in the Y-27632–treated eyes and exhibits a

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monolayer cell shape, while the actin cytoskeleton is disrupted in the control eyes. In the peripheral area, actin filaments are organized at the cortex, both in the Y-27632-treated eyes and the control eyes. *Scale bar*: 100 μm. (**G**) Immunohistochemical staining for Ki67, a cell proliferation marker, in the regenerated CECs. Both the Y-27632-treated eyes and the control eyes showed no Ki67-positive cells 4 weeks after cryoinjury. *Scale bar*: 100 μm. (**H**) Morphological scanning electron microscopy (SEM) analysis of the regenerated corneal endothelium of the central area revealed that ROCK inhibitor Y-27632 promotes morphological recovery. There is a large variation in cell size with some giant endothelial cells in the control eye, while the Y-27632-treated cornea shows fairly normal endothelial morphology without a large variation in cell size (*left*). CECs in the control eyes exhibited poorly formed cell junctions, while those in the Y-27632-treated eye exhibited fairly normal junctions with a hexagonal cell shape (*right*).

Keratoplasty (DSAEK) surgery were enrolled in this study, which ran from May to August 2010.

The patients were divided into two groups consisting of four patients per group according to the severity and extent of their corneal edema. In the first group, categorized as "diffuse corneal edema," patients presented with widespread corneal edema throughout the central and peripheral cornea. In the other group of patients, categorized as "central corneal edema," more peripheral corneal regions were clear. All of those four patients were clinically diagnosed as "late-onset Fuchs corneal dystrophy" in our cornea clinic based on the typical findings of multiple guttae and corneal edema that progressed after 40 years of age. One of these Fuchs' dystrophy cases (case 1) was recently reported in another manuscript as a case report.[22] The demographic data and pretreatment clinical manifestations of the patients are summarized in the Table.

Treatment Procedures

Prior to the administration of ROCK inhibitor Y-27632 eye drops, corneal endothelial cells were removed from the central part of cornea by transcorneal freezing according to the previous reports, yet with some modifications.[13] In brief, a 2-mm-diameter stainless steel rod was immersed in liquid nitrogen for 3 minutes to stabilize its temperature at approximately −196°C. Under topical anesthesia using oxybuprocaine hydrochloride (Santen Pharmaceutical Co., Ltd., Osaka, Japan), the frozen rod was pressed gently onto the central cornea for 15 seconds in order to damage diseased corneal endothelial cells. After the frozen rod was removed and the cornea had thawed, 10 mM of Y-27632 dissolved in 50 μL of PBS was topically applied in eye drop form 6 times daily for 7 days. Gatifloxacin hydrate eye drops (0.3%; Senju Pharmaceutical Company, Ltd., Osaka, Japan) were also applied 4 times daily to prevent ocular surface infection. Slit-lamp examination, noncontact specular microscopy, anterior segment optical coherence tomography (OCT; Visante; Carl Zeiss Meditec, Tokyo, Japan), and intraocular pressure measurements were performed daily for the first 7 days. Thereafter, the eyes were examined every week up to 1 month, and every 4 weeks up to 6 months. Prior to treatment, and at 3 and 6 months following treatment, the eyes were examined to elucidate any systemic side effects related to the ROCK inhibitor eye drop application.

Statistical Analysis

Statistical analysis was performed by use of a commercial spreadsheet program (Excel; Microsoft Corporation, Redmond, WA). The statistical significance (*P* value) in mean values of the two-sample comparison was determined with the Student's *t*-test. A *P* value of <0.05 was considered statistically significant. Values shown represent the mean ± SEM.

Results

ROCK Inhibitor Enhanced Morphological Recovery of Corneal Endothelium

In a previous report, we demonstrated that ROCK inhibitor Y-27632 facilitated wound healing in a rabbit animal model.[13] However, rabbit corneal endothelium has the ability to proliferate in vivo. Thus, we attempted to confirm that Y-27632 eye drops promote wound healing of primate corneal endothelium, in which cell proliferation is barely observed in vivo. In order to test this, we created partial corneal endothelial wounds by transcorneal freezing in the eyes of cynomolgus monkeys. Following cryoinjury, 10 mM of Y-27632 was topically applied to the eyes in eye drop form 6 times daily (Figs. 1A–D). Consistent with the rabbit findings,[13] the wounded area of the corneal endothelium following Y-27632 treatment was significantly reduced in comparison with that of the control eye (Fig. 1E). After treating the monkey eyes with ROCK inhibitor for 4 weeks, an actin cytoskeleton was observed at the cortex in the peripheral undamaged area, both in the control eyes and the Y-27632-treated eyes. However, in the control eyes, organization of the actin cytoskeleton of the corneal endothelium in the damaged central region was greatly disturbed and the cell shapes were severely altered (Fig. 1F). On the other hand, the Y-27632-treated eyes showed actin cytoskeleton at the cell cortex of hexagonal cells in the central area (Fig. 1F). Ki67-positive cells were not observed, in both the control eyes and the Y-27632-

Table. Demographic Data of the Patients Involved in the ROCK Inhibitor Eye Drop Clinical Trial

						Central Corneal Thickness, μm		BCVA, logMar		
N	Eye	Sex	Age	Type of Edema	Cause of Endothelial Decompensation	Pre	6M	Pre	6M	Ocular Complications
1	L	M	52	Central	Fuchs' dystrophy	703	568	0.7	−0.18	None
2	R	F	75	Central	Fuchs' dystrophy	809	(722:DSAEK)	1	0.7	Cataract
3	L	F	57	Central	Fuchs' dystrophy	682	663	0.52	0.52	None
4	L	F	62	Central	Fuchs' dystrophy	759	687	0.7	0.52	Myopic CRA
5	L	F	76	Diffuse	Laser iridotomy	683	506	1	1.7	Cataract
6	L	F	70	Diffuse	Laser iridotomy	920	920	0.7	1.52	Cataract
7	L	F	72	Diffuse	Laser iridotomy	827	827	0.7	0.7	Cataract
8	R	F	72	Diffuse	Pseudoexfoliation syndrome	721	757	0.4	1	Cataract

CRA, chorioretinal atrophy.

treated eyes, regardless of the region (Fig. 1G). Thus, it is likely that the proliferation of the CECs after administration of Y-27632 was blocked when CECs covered the wound area and formed contact-inhibited phenotypes. Analysis by scanning electron microscopy further confirmed the effect of Y-27632 on morphological recovery after 4 weeks of treatment (Fig. 1H). CECs in the Y-27632-treated eyes showed the characteristic hexagonal morphology of CECs, which have similar cell sizes. On the other hand, cells in the control eyes showed a large variation of cell sizes and the presence of enlarged cells. In addition, the cell junctions in the control eyes were poorly formed, whereas the cell junctions in the Y-27632-treated eyes were fairly normal among the hexagonal-shape cells. These findings demonstrate that the topical administration of ROCK inhibitor Y-27632 eye drops greatly enhances morphological recovery during the corneal endothelial wound repair process.

ROCK Inhibitor Enhanced Functional Recovery of Corneal Endothelium

During the regenerative wound healing process, there was a compensatory migration and enlargement of the neighboring corneal endothelial cells that migrated into the injury site to restore the contact-inhibited monolayer. After full recovery of the contact-inhibited monolayer of the corneal endothelium, the CECs were then able to exert their characteristic barrier and pump functions. We examined the fully recovered wound sites as divided into three areas as follows: the recovered central area that was previously damaged, the peripheral undamaged area, and the transition zone between the two areas (Figs. 2A, 2B). When cells were stained with anti-ZO-1 (the barrier-function-associated protein marker) antibody, the subcellular localization of ZO-1 in the control eyes was greatly disturbed in the central area and in the transition zone (Figs. 2A, 2B). On the other hand, the characteristic plasma-membrane staining pattern was observed in the Y-27632-treated eyes in all three of the examined areas (Figs. 2A, 2B). To confirm these findings, the histological phenotypes were further determined using confocal microscopy. The regenerated corneal endothelial cells of the Y-27632-treated eyes demonstrated plasma-membrane staining of ZO-1 in both the central and peripheral areas, while the staining pattern of ZO-1 was greatly disturbed in the central area of the control eye as opposed to the normal phenotypes of the peripheral region of the same cornea (Fig. 2C). Similar findings were observed when corneal endothelium was stained for Na^+/K^+-ATPase (the pump-function-associated protein marker). Y-27632 promoted the authentic subcellular localization of the of Na^+/K^+-ATPase at the plasma membrane of the regenerated cells in the central area, whereas the distribution of Na^+/K^+-ATPase was greatly disturbed in the cells of the central area in the absence of Y-27632 (Fig. 2D). The percentages of ZO-1- and Na^+/K^+-ATPase-positive cells in the central area were significantly higher in the Y-27632-treated eyes than those in the control eyes (Figs. 2E, 2F), suggesting that Y-27632 rapidly enhances functional recovery as well as morphological recovery.

ROCK Inhibitor Eye Drop Enabled Recovery of CEC Density in a Primate Model

Slit-lamp microscopy examination revealed that both Y-27632-treated and -nontreated corneas became hazy immediately after the corneal endothelial damage induced by transcorneal freezing; their transparency was recovered within 1 month (Fig. 3A). No severe side effects such as irreversible corneal haze and persistent corneal epithelial defect were observed during the wound healing process following cryoinjury. To test whether or not Y-27632 actually promoted the recovery of endothelial cell density, which is the most important clinical indicator, endothelial cell density in the monkey eyes was determined by use of noncontact specular microscopy. Since monkey CECs (MCECs) barely proliferate in vivo, corneal endothelial damage induced a compensatory migration and enlargement of the remaining neighboring endothelial cells. Consistent with this finding, in the control group, noncontact specular microscopy showed enlarged CECs, with a cell density of approximately 1500 cells/mm^2, 1 week after the injury (Figs. 3B, 3C). However, the corneal endothelium of the Y-27632-treated group was reconstructed without the compensatory enlargement of cell size, and the cell density reached approximately 3000 cells/mm^2, which is in the high range of endothelial cell density (Figs. 3B, 3C). The fact that the CEC density was significantly higher in the Y-27632-treated group compared with that of the controls suggests that ROCK inhibitor eye drops may stimulate the peripheral undamaged cells to proliferate, subsequently resulting in the recovery of functional corneal endothelium with a normal high cell density.

ROCK Inhibitor Eye Drops Restored the Corneal Thickness of Human Corneal Endothelial Dysfunction

Finally, we tested whether or not ROCK inhibitor eye drops enhance the proliferation of HCECs in vivo. This clinical trial involved four patients with central corneal edema and four patients with diffuse corneal edema. The patients were treated with the ROCK inhibitor eye drops 6 times daily for 7 days. Corneal thickness and best corrected visual acuity (BCVA) data of these 8 patients before and 6 months after treatment are shown in the Table.

A representative case of a central corneal edema patient is shown in Figures 4A to 4D. Before treatment, central corneal edema accompanied by the epithelial bulla was detected in patient No. 1. (Figs. 4A, 4B). The corneal edema was significantly reduced and BCVA recovered from logMAR 0.7 to −0.18 (Figs. 4C, 4D) at 6 months after treatment. A representative case of a diffuse corneal edema patient is shown in Figures 4E to 4H. Before treatment, diffuse corneal edema due to argon laser iridotomy-induced bullous keratopathy (ALI-BK) was observed (Figs. 4E, 4F). The corneal edema persisted and recovery of visual acuity was not obtained at 6 months after treatment (Figs. 4G, 4H). Specular microscopic examination was found to be difficult to perform on most of these patients due to corneal edema. We found some portion of relatively healthy corneal endothelial cells in the paracentral area before treatment in patient no. 1, though no analyzable image was obtained from the center cornea. Moreover, we confirmed the remodeling of corneal endothelial cells both of the center and paracentral area in this patient after treatment (Fig. 4I). In the central corneal edema patients, central corneal thickness was reduced 6 months after treatment compared to pretreatment levels (Fig. 4J). In contrast, there was no reduction of central corneal thickness in the eyes with diffuse corneal edema (Fig. 4J). Although no statistical significance was shown from this small cohort, reduction of corneal thickness may indicate recovery of corneal endothelial function in patients with central edema. In terms of the visual acuity, not much improvement was seen even in the patients with central edema, except in patient No. 1, due to the presence of preexisting senile cataract or macular degeneration. However, vision was kept at the same level or was slightly improved during the 6-month observation period. In all patients, no complications such as intraocular pressure elevation or systemic complications were detected in relation to the transcorneal freezing or the ROCK inhibitor eye drop application.

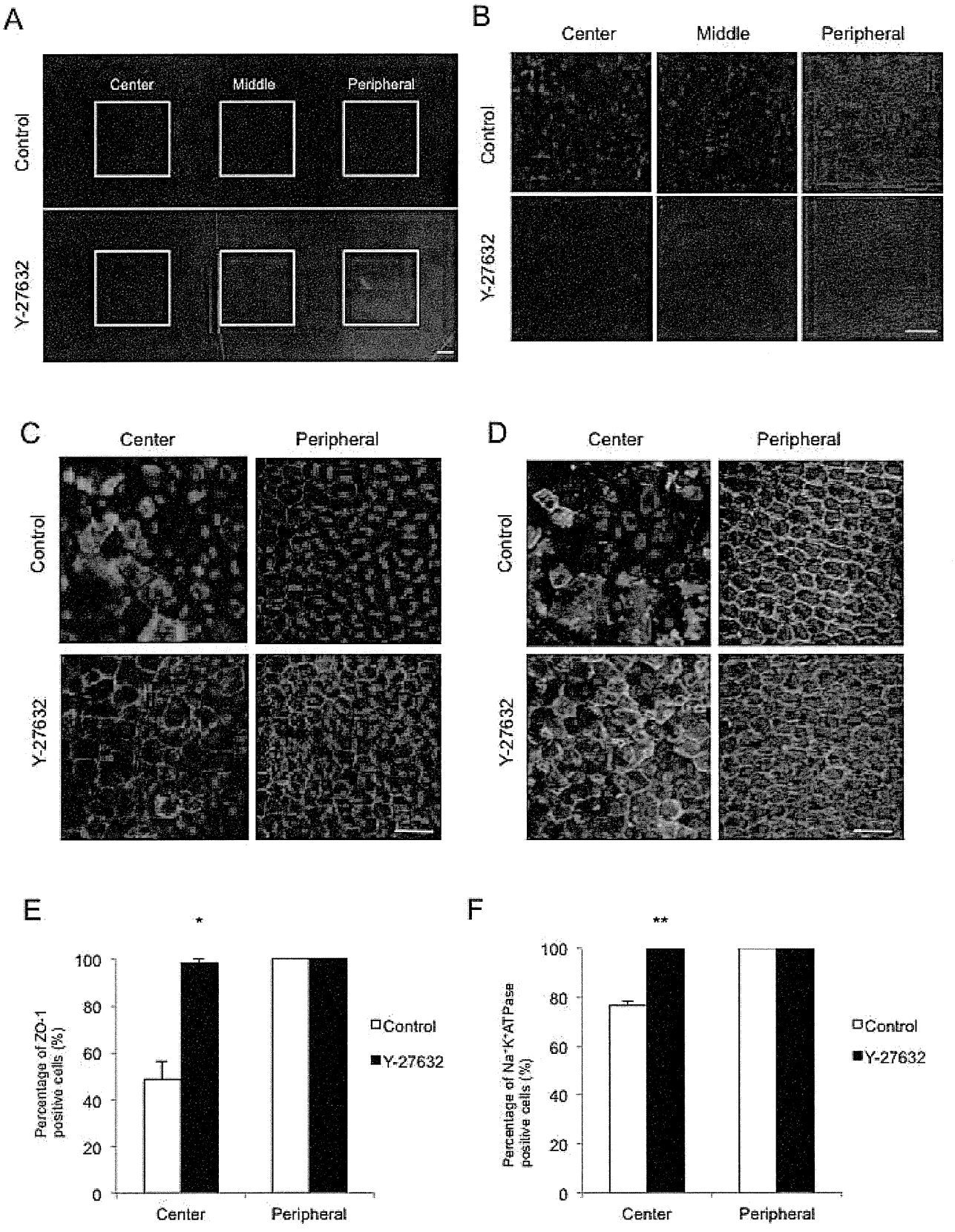

Figure 2. ROCK inhibitor Y-27632 promoted the functional recovery of regenerated corneal endothelium. (**A**, **B**) Subcellular localization of ZO-1 was disturbed in the central area of the control eyes, while the subcellular localization of ZO-1 in the Y-27632–treated eye was demonstrated at the plasma membrane, the physiological location. *Scale bar*: 500 μm. (**C**, **D**) In the Y-27632-treated eye, all regenerated cells in the wounded central area expressed ZO-1 and Na^+/K^+-ATPase. On the other hand, the expression of ZO-1 and Na^+/K^+-ATPase was decreased and their subcellular location was greatly disturbed in the control eyes. *Scale bar*: 100 μm. (**E**, **F**) The percentages of ZO-1 and Na^+/K^+-ATPase–positive cells in the wounded area are significantly higher in the Y-27632–treated eye than in the control eye. $^*P < 0.01$, $^{**}P < 0.05$.

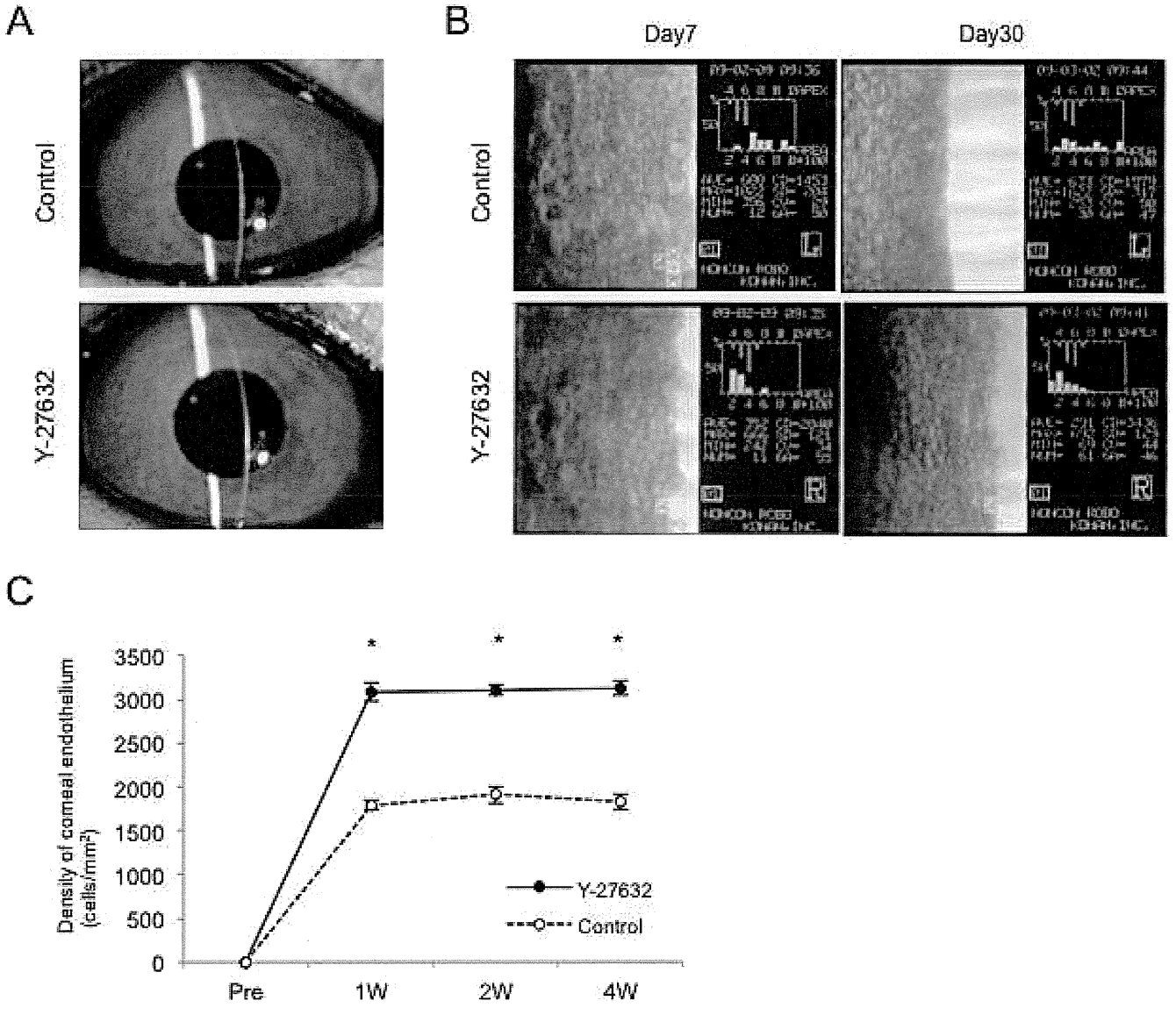

FIGURE 3. ROCK inhibitor Y-27632 eye drops promoted the recovery of cell density in a corneal-endothelial partially damaged primate model. (**A**) Slit-lamp microscopy examination revealed that both Y-27632–treated and –nontreated corneas recovered their transparency 1 month after cryoinjury. (**B**) In the control group, noncontact specular microscopy shows enlarged corneal endothelium migrating into the damaged area at the density of approximately 1500 cells/mm^2 1 week after the injury. However, corneal endothelium of the Y-27632–treated group was reconstructed without compensatory enlargement with a normal cell density of approximately 3000 cells/mm^2. (**C**) Noncontact specular microscopy analysis revealed that the CEC density was significantly higher in the Y-27632–treated group than in the control group throughout the 4-week observation period (*$P < 0.01$).

DISCUSSION

In most tissues, the wound repair process consists of cell migration and cell proliferation. Unlike such a generalized mechanism of wound healing, the regenerative wound repair observed in human corneal endothelium is accomplished by cell migration and attenuation of neighboring cells adjacent to the injury site, with limited involvement of cell proliferation. Of interest, species-specific differences exist in regard to cell proliferation ability during wound healing (e.g., rabbit, mouse, and bovine CECs exhibit proliferative ability, while the proliferative ability of human, monkey, and cat CECs is severely limited).[23–27]

The corneal endothelium is critical for maintaining homeostatic-corneal transparency; corneal endothelium has to retain sufficient cell density to maintain the contact-inhibited monolayer, which is crucial to perform the ionic pump and barrier functions. If the corneal endothelium fails to retain sufficient cell density due to either the aging process or severe injury, the result of this abnormality is an increase of overall cell size and an alteration of the cell shape to a pleomorphic shape. Enlarged corneal endothelial cells, as well as cells with abnormal morphology, are closely associated with endothelial dysfunction. Therefore, it has been widely studied to trigger the proliferation of CECs in vivo in the absence of pathological complications leading to another ocular dysfunction. Of importance, HCECs are arrested at the G_1 phase of the cell cycle,[27] suggesting that they are not terminally differentiated but do possess proliferative potential.

In our search for a biological tool that could proliferate CECs, we investigated the efficacy of the selective ROCK inhibitor Y-27632. We recently published that Y-27632

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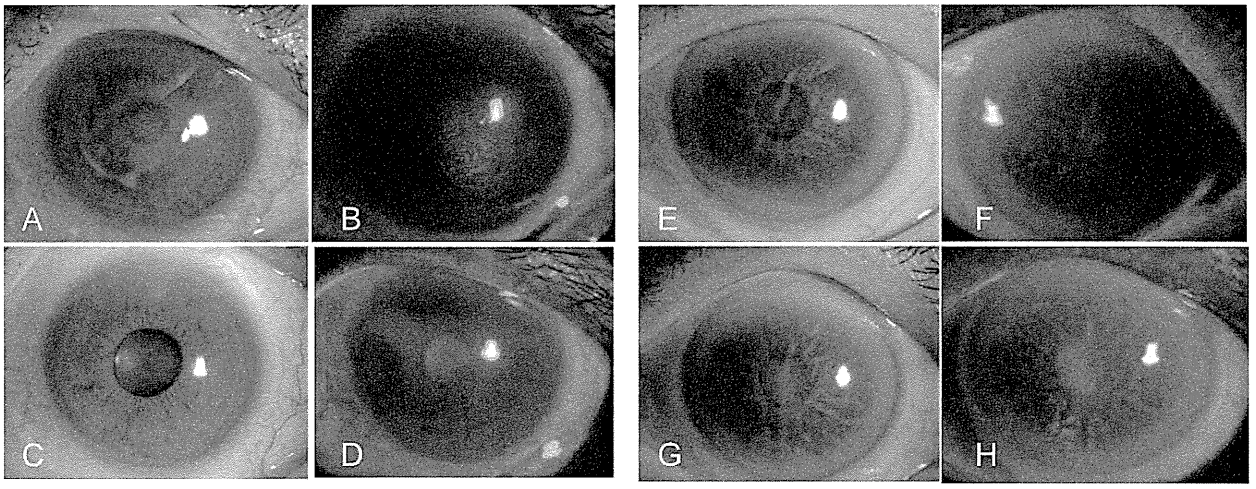

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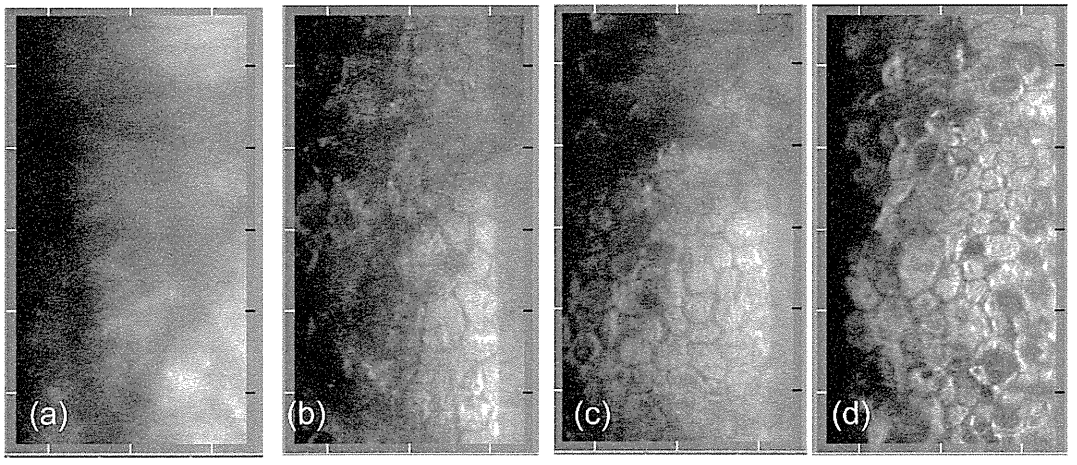

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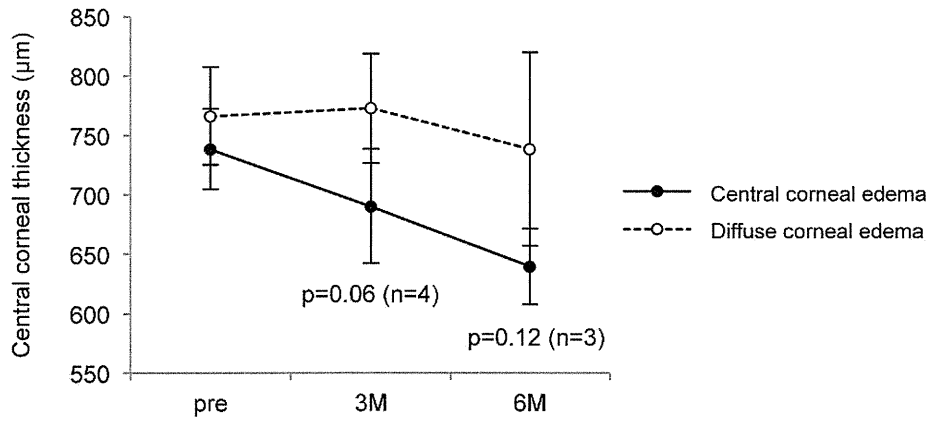

Figure 4. Clinical trial of ROCK inhibitor Y-27632 eye drops for treating patients with central corneal edema and diffuse corneal edema. **(A, B)** Representative case of a central corneal edema patient is shown. Before treatment, central corneal edema was detected in patient 1. **(C, D)** Six months after treatment, the corneal edema was significantly reduced and visual acuity recovered from logMAR 0.70 to −0.18. **(E, F)** Representative case of a diffuse corneal edema patient is shown. Before treatment, diffuse corneal edema due to argon laser iridotomy-induced bullous keratopathy (ALI-BK) was observed. **(G, H)** Six months after treatment, the corneal edema persisted and recovery of visual acuity was not obtained. **(I)** The corneal endothelium of case 1 observed by noncontact-specular microscopy before **(A, B)** and 6 months after treatment **(C, D)**. Before treatment,

we could not obtain clear image of corneal endothelium from the center part of cornea due to corneal edema (**A**). In contrast, some endothelial cells with guttae were observed para-central area of the same eye (**B**). Six months after treatment, specular microscopic images were obtained from both of center (**C**) and peripheral cornea (**D**). Approximate cell density after treatment was 1200 to 1500 cells/mm^2 in both areas. (**J**) In the central corneal edema patients, central corneal thickness was reduced 6 months after treatment compared to pretreatment levels. In contrast, the central corneal thickness did not reduce in eyes with diffuse corneal edema.

inhibited dissociation-induced apoptosis and promoted the adhesion and proliferation of MCECs.[14] Moreover, we reported that the topical application of Y-27632 promoted corneal endothelial wound healing in a rabbit model.[20,21] In this present study, we attempted to establish a new pharmacological intervention in the form of Y-27632 eye drops that would promote corneal endothelial wound healing. Using a primate corneal endothelial dysfunction model, we demonstrated that: the topical application of Y-27632 eye drops greatly enhanced wound healing of corneal endothelium; that the regenerated corneal endothelium of the central damaged region demonstrated physiological hexagonal cell morphology and resumed the characteristic adhesion profiles (ZO-1 and Na^+/K^+-ATPase) and actin cytoskeleton when treated with Y-27632 eye drops; and that Y-27632 eye drops greatly enhanced cell density to the normal level. These important findings suggest that the administration of ROCK inhibitor Y-27632 eye drops enhances both the functional and morphological recovery. In addition, we demonstrated that Y-27632 eye drops proved effective for the recovery of corneal transparency and the gradual reduction of corneal thickness for up to 6 months in human patients who had central edema due to endothelial dysfunction. Those findings suggest that the use of Y-27632 eye drops may be clinically beneficial to a certain group of patients with central edema caused by endothelial dysfunction (i.e., Fuchs' corneal dystrophy patients). Moreover, it has been reported that spontaneous remodeling may take place in Y-27632–treated patients with central edema, similar to that observed in HCECs after Descemet's stripping procedures,[28,29] likely due to the existence of corneal endothelial precursors with higher proliferative ability in the peripheral cornea. However, our data (Figs. 3B, 3C) using primate CECs, whose proliferative behavior is similar to that of HCECs, supports the finding that ROCK inhibitor greatly stimulates the proliferation of MCECs.

Rho GTPases members (RhoA, Rac1, and Cdc42) reportedly play an important role at many aspects of the cell cycle.[19] Earlier studies have shown that Rho contributes to cell cycle progression and that inactivation of Rho by C3 blocks G_1/S progression in Swiss 3T3 fibroblast.[15,30] Unlike these findings, we revealed that inhibition of Rho/ROCK signaling by selective ROCK inhibitor Y-27632 promotes the proliferation of cultured CECs.[14] Therefore, Rho/ROCK activity on the cell cycle may be cell-type dependent.[31] However, the mechanism by which ROCK inhibitor promotes corneal endothelial cell proliferation has yet to be elucidated. In regard to the clinical application, Fasudil, one of the ROCK inhibitors, has already been approved for clinical use and has been administered in over 124,000 cases in Japan.[32] Moreover, ROCK inhibitors have been developed for a wide range of diseases such as cardiovascular disease, pulmonary disease, and cancer.[32] In the field of ophthalmology, another ROCK inhibitor (Y-39983) has been developed for treating glaucoma and is currently undergoing clinical trials.[33] It is likely that the application of ROCK inhibitor might also be of clinical benefit for the treatment of corneal endothelial dysfunction. Thus, we hypothesize that the combined treatment regimen consisting of partial denudation of diseased CECs via transcorneal freezing and the topical application of Y-27632 in eye drop form may be useful to promote the proliferation of corneal endothelium in patients with central cornea edema. However, there are several concerns to be addressed before advancing the current findings toward clinical application; they include which endothelial diseases, stage of disease, method of performing the transcorneal freezing, and the duration and dosage of the ROCK inhibitor eye drops. Such issues will influence the healing pattern. Nevertheless, the current pilot study offers encouragement that some patients with endothelial dysfunction, especially those with Fuchs' corneal dystrophy, might be good candidates for transcorneal freezing/ROCK inhibitor eye drop treatment as an alternative to graft surgery.

In summary, our results demonstrate that ROCK inhibitor Y-27632 promotes corneal endothelium wound healing in a primate animal model. Furthermore, this is the first report of a case series demonstrating a pharmaceutical agent being successfully used to treat corneal endothelial dysfunction in human eyes in the absence of other ocular complications, and our findings encourage us to further develop ROCK inhibitor eye drops as a novel therapy for certain forms of corneal endothelial dysfunction.

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Corneal Endothelial Cell Fate Is Maintained by LGR5 Through the Regulation of Hedgehog and Wnt Pathway

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Key Words. Cornea • Endothelium • Stem cell • LGR5 • Hedgehog • Wnt

ABSTRACT

Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a target of Wnt signaling, is reportedly a marker of intestine, stomach, and hair follicle stem cells in mice. To gain a novel insight into the role of LGR5 in human corneal tissue, we performed gain- and loss-of-function studies. The findings of this study show for the first time that LGR5 is uniquely expressed in the peripheral region of human corneal endothelial cells (CECs) and that LGR5$^{(+)}$ cells have some stem/progenitor cell characteristics, and that in human corneal endothelium, LGR5 is the target molecule and negative feedback regulator of the Hedgehog (HH) signaling pathway. Interestingly, the findings of this study show that persistent LGR5 expression maintained endothelial cell phenotypes and inhibited mesenchymal transformation (MT) through the Wnt pathway. Moreover, R-spondin-1, an LGR5 ligand, dramatically accelerated CEC proliferation and also inhibited MT through the Wnt pathway. These findings provide new insights into the underlying homeostatic regulation of human corneal endothelial stem/progenitor cells by LGR5 through the HH and Wnt pathways. STEM CELLS *2013;31:1396–1407*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

In most vertebrates, including humans and other primates, the majority of external information is gained through eyesight, and the cornea is a very important avascular tissue related to the maintenance of this vision system. The cornea consists of a stratified surface epithelial cell layer, a thick collagenous stroma, and an inner single-cell-layered endothelium. Through the combination of these three cell layers, corneal tissue is kept optically clear, and ocular homeostasis and integrity are maintained. According to the World Health Organization, an estimated 25-million people worldwide are affected by cornea-related blindness [1]. Therefore, it is important to understand the underlying mechanisms by which corneal integrity is maintained.

From the medical standpoint, corneal endothelial cells (CECs) represent the most important component of the cornea, as they are crucial for maintaining corneal integrity [2]. CECs, which are derived from the neural crest, play an essential role in the maintenance of corneal transparency through their barrier and pump functions. Although human CECs are mitotically inactive and are arrested at the G1 phase of the cell cycle in vivo [3], they retain the capacity to proliferate in vitro [4]. However, a recent study has shown that to date, culturing human CECs for a long period of time is extremely difficult [5]. In view of these findings, it is now understood that the molecular mechanism, including the stem cell biology of corneal endothelial behavior, is an important research subject to explore to better understand the role and function of the cornea, as well as to elucidate the most effective means by which to reconstruct damaged corneal tissue.

It is well known that stem cells facilitate the maintenance of self-renewing tissues and organs [6–8]. With regard to corneal tissue, various studies indicate that corneal epithelial stem cells reside in the basal layer of the peripheral cornea in the limbal zone [9–11]. In contrast, even though it has been reported that CECs from the peripheral area of the cornea retain higher replication ability [12], the corneal endothelial stem cells have yet to be specifically identified and their exact locations are also not fully understood owing to the lack of unique markers and the absence of stem cell assay [13–15].

Recently, genetic mouse models have allowed for the visualization, isolation, and genetic marking of leucine-rich

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repeat G protein-coupled receptor 5 (LGR5)-positive cells and have provided evidence that there are stem cells in the stomach, small intestine, colon, and hair follicles of those mice [16–18]. LGR5 reportedly is expressed downstream of Hedgehog (HH) signaling in basal cell carcinoma, and $LGR5^{high}$ cells in hair follicles reportedly show active HH signaling [16, 19]. To gain more insights on the mechanism of corneal stem cells, we performed Affymetrix Microarray (Affymetrix, Inc., Santa Clara, CA) analyses using holoclone-type human corneal keratinocytes, and LGR5 was identified as a potential marker for human corneal keratinocyte stem/progenitor cells (data not shown). These findings have led us to an interesting hypothesis that a common stem cell marker exists between developmentally distinct tissues, yet to date, there have been no reports regarding the role and function of LGR5 in CECs.

In this study, we show for the first time that LGR5 is uniquely expressed in the peripheral region of human CECs and that $LGR5^{+}$ cells have some stem/progenitor cell characteristics. In addition, the findings of this study show that LGR5 is a key molecule for maintaining the integrity of CECs and is mainly regulated by HH and Wnt signaling. Moreover, R-spondin-1 (RSPO1), an LGR5 ligand, was found to dramatically influence the maintenance of CECs. Thus, our data provide new insights into the underlying homeostatic regulation of corneal endothelial stem/progenitor cells by LGR5.

Materials and Methods

Tissues

All human donor cornea tissues were obtained from SightLife (Seattle, WA) eye bank, and all corneas were stored at 4°C in storage medium (Optisol; Bausch&Lomb, Rochester, NY, http://www.bausch.com). A total of 80 donor corneas were used for all experiments (donor age: 61.8 ± 8.6 years (mean ± SD); mean time to preservation: 7.6 ± 5.6 hours; mean endothelial cell density: $2{,}757 \pm 221$ mm^2; mean storage time: 6.0 ± 0.9 days). All experiments were performed in accordance with the tenets set forth in the Declaration of Helsinki. Eight corneas obtained from cynomolgus monkeys (donor age: 7.1 ± 4.5 years (mean ± SD); estimated equivalent human age: 15–42 years) housed at NISSEI BILIS Co., Ltd., Koka, Japan and Eve Bioscience, Co., Ltd., Japan, respectively, were used for this study. For other research purposes, the monkeys were given an overdose of sodium pentobarbital for euthanization intravenously according to the approval by the Laboratory Animal Use and Ethics Committee of the Shiga Laboratory, NISSEI BILIS Co., Ltd. and the institutional animal care and use committee of Eve Bioscience, Co., Ltd., respectively. The corneas of the cynomolgus monkeys were harvested after confirmation of cardiopulmonary arrest by veterinarians, and were then provided for our research. All corneas were stored at 4°C in Optisol storage medium for less than 24 hours before the experiment. All animals were housed and treated in accordance with the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies and Reagents

For immunohistochemistry and flow cytometry, the following rabbit polyclonal antibodies were used: anti-C-terminal domain of human LGR5 (71143; GeneTex Inc., San Antonio, TX) and anti-ZO1 (40-2200; Zymed Laboratories Inc., South San Francisco, CA, http://www.invitrogen.com/content.cfm?pageid11356). The following mouse monoclonal antibodies were used: anti-Na^{+}/K^{+} ATPase (05-369; EMD Millipore Corporation, Billerica, MA http://www.emdmillipore.com), anti-Ki67, and anti-β-catenin (556003, 610153; BD Biosciences, Franklin Lakes, NJ http://www.bdbiosciences.com/home.jsp). Secondary antibodies were Alexa Fluor-488 goat anti-rabbit or mouse IgG (A11034, A11029; Molecular Probes Inc., Eugene, OR, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) and Cy3 anti-mouse IgG (715-165-150; Jackson Immunoresearch Laboratories, Inc., West Grove, PA, http://www.jacksonimmuno.com). For Western blotting, the following rabbit polyclonal antibodies were used: anti-LRP6 and p-LRP6 (3395, 2568; Cell Signaling Technology, Inc., Beverly, MA, http://www.cellsignal.com). The following mouse monoclonal antibodies were used: β-catenin (BD Biosciences) and β-actin (A5441; Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Secondary antibodies were horse radish peroxide (HRP)-conjugated anti-rabbit or mouse IgG (NA934, NA931; GE Healthcare, Piscataway, NJ, http://www.gehealthcare.com). Recombinant human sonic HH (SHH), purmorphamine, cyclopamine, and RSPOs were purchased from R&D Systems Inc. (Minneapolis, MN, http://www.rndsystems.com).

Cell Culture

The human and monkey CECs were cultured using the method of our previously reported system [2, 20–22]. Briefly, the Descemet's membrane including CECs was stripped and digested with 2 mg/ml collagenase A (Roche Applied Science, Penzberg, Germany, http://www.roche-applied-science.com) at 37°C. After incubation for 3 hours, the CECs (individual cells and cell aggregates) obtained from individual corneas were resuspended in culture medium containing Opti-MEM-I (Invitrogen), 5% fetal bovine serum (FBS), 50 μg/ml gentamicin, and 10 μM Y-27632 (Calbiochem, LA Jolla, CA) and then plated in one well of a 12-well plate coated with FNC Coating Mix (Athena Environmental Sciences, Inc., Baltimore, MD, http://www.athenaes.com). The CECs were cultured in a humidified atmosphere at 37°C in 5% CO_2. The culture medium was changed every 2 days. When cells reached subconfluence, they were rinsed in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS), trypsinized with TrypLE Select (Life Technologies) for 10 minutes at 37°C, and passaged at ratios of 1:2–4.

Immunohistochemistry

Immunohistochemical studies followed our previously described method [23, 24]. Briefly, 8-μm-thin sections and whole-mount sections prepared by peeling the Descemet's membrane from cornea tissues were placed on silane-coated slides, air dried, and fixed in 100% acetone at 4°C for 15 minutes. After washing in PBS containing 0.15% TRITON X-100 surfactant (The Dow Chemical Company, Midland, MI, http://www.dow.com) at room temperature (RT, 24°C) for 15 minutes, sections were incubated with 1% bovine serum albumin (Sigma-Aldrich) at RT for 30 minutes to block nonspecific binding. Sections were then incubated with primary antibody at RT for 1 hour and washed three times in PBS containing 0.15% TRITON X-100 for 15 minutes. Control incubations were conducted with the appropriate normal mouse and rabbit IgG at the same concentration as the primary antibody, and the primary antibody for the respective specimen was omitted. The sections were then incubated with the appropriate secondary antibodies at RT for 1 hour. After being washed three times with PBS, the sections were then coverslipped using glycerol-containing propidium iodide (PI) (Nacalai Tesque, Inc., Kyoto, Japan, https://www.nacalai.co.jp), and examined under a confocal microscope (FluoView; Olympus Corporation, Tokyo, Japan, http://www.olympus.co.jp).

Table 1. Sequences for PCR and shRNA

LGR5 (NM_003667), Forward 5′-GAGGATCTGGTGAGCCTGAGAA-3′
Reverse 5′-CATAAGTGATGCTGGAGCTGGTAA-3′
SHH (NM_000193.2), Forward 5′-ACGGCCCAGGGCACCATTCT-3′
Reverse 5′-GGACTTGACCGCCATGCCCA-3′
Ptch1 (NM_000264.3), Forward 5′-TCGCTCTGGAGCAGATTTCCAAGGG-3′
Reverse 5′-GCAGTCTGGATCGGCCGGATTG-3′
Smo (NM_005631.4), Forward 5′-GTGAGTGGCATTTGTTTTGTGGGC-3′
Reverse 5′-CAGGCATTTCTGCCGGGGCA-3′
Gli1 (NM_005269.2), Forward 5′-GCCCCCATTGCCCACTTGCT-3′
Reverse 5′-TGCAGGGGACTGCAGCTCC-3′
Gli2 (NM_005270.4), Forward 5′-GGCCGCCTAGCATCAGCGAG-3′
Reverse 5′-CACCGCCAGGTTGCCCTGAG-3′
β-Actin (NM_ 001101), Forward 5′-GGACTTCGAGCAAGAGATGG-3′
Reverse 5′-ATCTGCTGGAAGGTGGACAG-3′
sh LGR5, 5′-CCGGGCTCTACTGCAATTTGGACAACTCGAGTTGTCCAAATTGCAGTAGAGCTTTTT-3′
sh NT, 5′-CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3′

Abbreviations: LGR5, leucine-rich repeat G protein-coupled receptor 5; PCR, polymerase chain reaction; Ptch1, protein patched homolog one receptor molecule; SHH, sonic Hedgehog; sh NT, short hairpin nontarget; shRNA, short hairpin RNA; Smo, smoothened receptor molecule.

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was performed following our previously described method [19]. To prepare the samples, we first separated the central cornea from the peripheral cornea using an 8-mm trephine. We then stripped the Descemet's membrane including CECs using micro forceps under a microscope to separate the corneal epithelium, stroma, and endothelium in the central and peripheral cornea, respectively. We then separated the corneal epithelial cells from the corneal stroma using dispase treatment (37°C for 1 hour). All samples were homogenized in lysis buffer (Buffer RLT; QIAGEN, Inc., Valencia, CA http://www.qiagen.com) and total RNA was eluted by use of the RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. The relative abundance of transcripts was detected by use of SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA http://www.appliedbiosystems.com) according to the manufacturer's instructions. The primers that were used are shown in Table 1.

Flow Cytometry

For the cell sorting of $LGR5^{high}$ cells, monkey CECs prepared as described above were passaged in 1:2 dilutions and cultured to subconfluence. The CECs were dissociated to single cells by use of TrypLE Select. We then performed the following two experiments. First, the CECs were fixed in 70% (wt/vol) ethanol at 4°C for 2 hours, washed with PBS, and incubated at RT for 15 minutes with 1% FBS. The CECs were then incubated with 1:100-diluted anti-rabbit LGR5 and 1:100-diluted anti-mouse Ki67, washed, and incubated with 1:1500-diluted Alexa Fluor 488-conjugated goat anti- rabbit IgG (Life Technologies) and 1:1000-diluted Cy3 anti-mouse IgG (Jackson Immunoresearch Laboratories). Flow cytometric analyses were then performed with FACS Aria II (BD Biosciences).

Second, the CECs were washed with PBS, and then incubated at RT for 15 minutes with 1% FBS. They were then incubated with 1:100-diluted anti-rabbit LGR5 at RT for 20 minutes, washed, and incubated with 1:1500-diluted Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies). $LGR5^{high}$ and $LGR5^{low}$ cells were isolated by use of fluorescence activated cell sorting (FACS) Aria II, and the resultant cells were then cultured on an eight-well chamber slide with poly-L-lysine (Sigma-Aldrich). After 5 days of culture, those cells were immunostained by anti-mouse Ki67 as described above, and the $Ki67^{high}$ cells in each group were then counted ($n = 4$).

Measurement of Cell Area

Each isolated cell fraction was centrifuged and resuspended in culture medium. Cells (approximately 100 cells/ml) were placed in a six-well plate and photographed under an inverted microscope. Cell areas were measured randomly (200 cells/fraction) using Scion Image software and statistically analyzed [23].

RNA Interference

Short hairpin RNA (shRNA) was purchased from Sigma-Aldrich. The *LGR5* shRNA targeted sequences and the nontarget (NT) shRNA sequences are shown in Table 1. The lentivirus plasmid DNA was transfected to the HEK293T cells along plasmid packaging plasmid mixture (MISSION Lentiviral Packaging Mix; Sigma-Aldrich) using a commercially available transfection reagent (FuGENE HD; Roche Diagnostics Corporation, Indianapolis, IN, http://www.roche-diagnostics.com). After 18 hours, the media was aspirated off and replaced with complete medium. The quantity of lentiviral particles was assessed by HIV-1 p24 Antigen ELISA (ZeptoMetrix Corp., Buffalo, NY, http://www.zeptometrix.com) according to the manufacturer's instructions.

Construction of Lentivirus Plasmid Vector for Gene Expression

For the construction of the lentivirus plasmid vector that expresses the introduced gene, *LGR5*, a commercially available lentiviral vector (pLenti6.3_V5-TOPO; Life Technologies) was used. cDNAs were amplified with a primer pair (Forward Primer: CTACTTCGGGCACCA TGGACACCT, Reverse Primer: CACATATTAATTAGAGACATGGGA) encompassing an entire coding sequence of *LGR5*, gel-purified, and then ligated into the lentivirus plasmid vector.

The expression lentivirus Production and Infection were in a modified version of our protocol used for the shRNA [25]. Briefly, the lentivirus plasmid DNA was transfected to the HEK293T cells along with the plasmid packaging plasmid mixture ViraPower Lentiviral Packaging Mix (Life Technologies) which contains pLP1, pLP2, and pLP/VSVG plasmids and FuGENE HD as the transfection reagent. After 18 hours, the media was aspirated off and replaced with complete

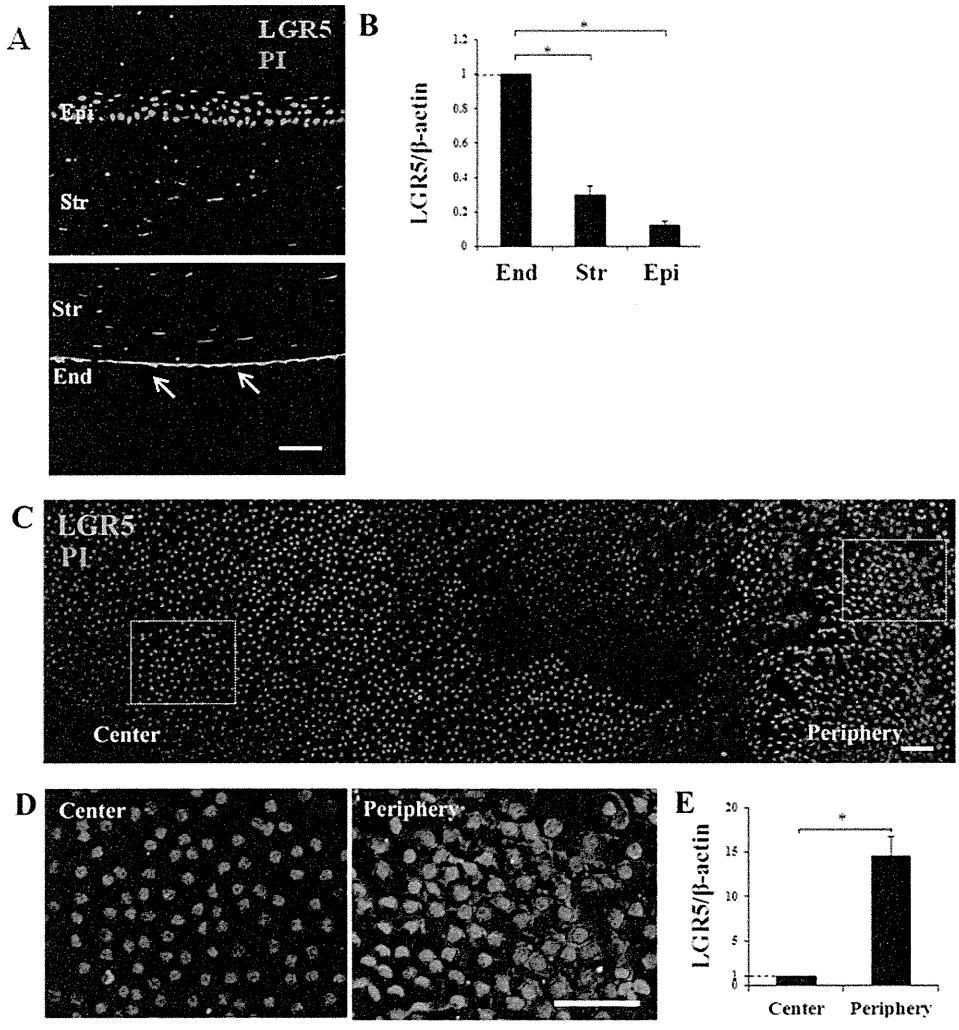

Figure 1. Unique expression pattern of leucine-rich repeat G protein-coupled receptor 5 (LGR5) in human corneal endothelial cells (CECs). **(A):** Immunostaining of LGR5 in a human cornea. Arrows point to CECs. Scale bar = 100 μm. **(B):** Real-time polymerase chain reaction (PCR) for *LGR5* in the cornea. Mean ± SEM. *, $p < .05$. $n = 4$. **(C):** Whole-mount immunostaining of LGR5 in human CECs. Scale bar = 100 μm. **(D):** Higher magnification of boxed areas in **(C)**. Scale bar = 100 μm. **(E):** Real-time PCR for *LGR5* in the central and peripheral CECs. Mean ± SEM. *, $p < .05$. $n = 3$. Abbreviations: LGR5, leucine-rich repeat G protein-coupled receptor 5; PI, propidium iodide.

medium and the quantity of lentiviral particles was then assessed.

Gene Transfer

The culture supernatant containing the infection-competent virus particle was harvested to human CECs at 5,000 cells/well in a six-well plate with FNC Coating Mix for 24 hours (Multiplicity of infection (MOI) = 1) using the culture medium described above. The supernatant was applied onto cultivated CECs in the presence of 4 μg/ml polybrene. As puromycin-resistant colonies (shRNA experiment) and blasticidin-resistant colonies (overexpression model) were collected, cells were cultured in the presence of 0.4 μg/ml of puromycin and 2 μg/ml of blasticidin, with the media being changed every 2 days.

Western Blotting

The cultivated human CECs were washed with PBS and then lysed with lysis buffer containing PBS, 1% TRITON X-100, 0.5 M EDTA, Phosphatase Inhibitor Cocktail two (Sigma-Aldrich), and Protease Inhibitor Cocktail (Roche Diagnostics). Detection of activated β-catenin (nonmembrane bound) was performed according to the previously reported protocol [26]. Briefly, cell lysates treated with Con A Sepharose 4B (GE Healthcare) were incubated at 4°C for 1 hour. After centrifugation at 4°C for 10 minutes, the supernatants were transferred to new tubes and Con A Sepharose was added to each tube and incubated at 4°C for 1 hour. Finally, after a brief centrifugation, the supernatants were transferred to new tubes and their protein concentration was determined.

The proteins were then separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were then blocked with 1% ECL Advance Blocking Reagent (GE Healthcare) in Tris Buffered Saline with Tween 20 (TBS-T) buffer and were incubated with primary antibody at 4°C overnight. After being washed three times in TBS-T buffer, the polyvinylidene fluoride (PVDF) membranes were incubated with appropriate HRP-conjugated anti-rabbit or mouse IgG secondary antibody at RT for 1 hour. The membranes were exposed by use of the ECL Advance Western Blotting Detection Kit (GE Healthcare), and then examined by use of the LAS-3000 (FujiFilm Corporation, Tokyo, Japan, http://www.fujifilm.com) imaging system.

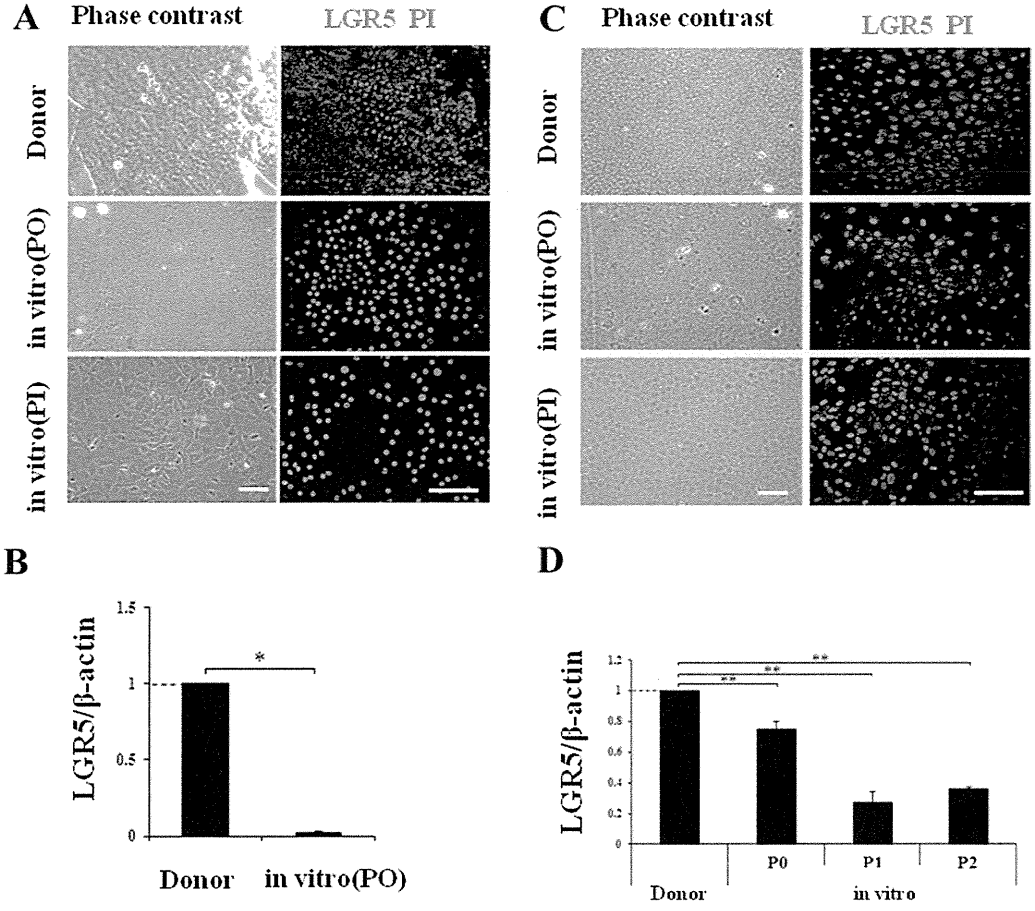

Figure 2. Downregulation of leucine-rich repeat G protein-coupled receptor 5 (LGR5) in cultivated corneal endothelial cells (CECs). **(A):** Phase contrast image and immunostaining of LGR5 in donor and in vitro human CECs. Scale bars = 100 μm. **(B):** Real-time polymerase chain reaction (PCR) for *LGR5* in donor and in vitro human CECs. Mean ± SEM. *, $p < .05$. $n = 4$. **(C):** Phase contrast image and immunostaining of LGR5 in donor and in vitro monkey CECs. Scale bars = 100 μm. **(D):** Real-time PCR for *LGR5* in donor and in vitro monkey CECs. Mean ± SEM. **, $p < .01$. $n = 3$. Abbreviations: LGR5, leucine-rich repeat G protein-coupled receptor 5; PI, propidium iodide; PO, passage 0.

Results

Unique Expression Pattern of LGR5 in Human Donor CECs

The expression pattern of LGR5 in human CECs was investigated by indirect immunofluorescence. On examination of the CECs of those tissues, intensive LGR5 expression was observed, especially in the peripheral area. However, LGR5 was only minimally expressed in the corneal epithelium and stroma (Fig. 1A). Real-time PCR showed that compared with stroma and epithelium, mean *LGR5* messenger RNA (mRNA) expression was significantly upregulated in the CECs ($*p < .05$, $n = 4$, mean age: 60 years) (Fig. 1B). Thus, among the corneal tissues, the expression of LGR5 was found to be most prominent in the CECs.

Next, we examined the location pattern of LGR5 using whole-mount immunofluorescence ($n = 3$, mean age: 64 years). The expression of LGR5 was observed in the peripheral-region CECs, yet its level gradually decreased in CECs located towards the central region (Fig. 1C, 1D). Real-time PCR clearly showed that the expression of *LGR5* in the peripheral regions was upregulated in comparison with the central region (8-mm diameter) ($*p < .05$, $n = 3$, mean age: 70 years) (Fig. 1E). These findings indicate that in corneal tissue, LGR5 is uniquely expressed in the peripheral CECs.

Downregulation of LGR5 in In Vitro Culture Conditions

It is well known that the proliferative potential of CECs varies among species [27]. To date, it is extremely difficult to consistently culture human CECs which retain a healthy morphology and high cell density. In contrast, we previously reported that under the proper in vitro conditions, monkey and rabbit CECs can proliferate reasonably well [2, 20–22]. Thus, to gain an insight into the molecular mechanism that underlies the varying proliferative potentials of CECs, we examined the expression of LGR5 in vitro.

Phase contrast microscopy photographs of human peripheral donor CECs revealed that they exhibited a confluent monolayer of smaller-size homogeneously hexagonal cells (Fig. 2A). In contrast, cultured CECs (P0, P1) were found to be enlarged and not homogeneously hexagonal (Fig. 2A). Immunostaining showed that LGR5 was well-expressed in the peripheral donor CECs (Fig. 2A). Worthy of note, the expression of LGR5 was only minimally observed in the cultured CECs in vitro (P0, P1) (Fig. 2A). Real-time PCR showed that the mean *LGR5* mRNA expression was significantly downregulated in in vitro CECs as compared to that in peripheral donor CECs ($*p < .05$) (Fig. 2B).

Phase contrast photographs of monkey CECs showed that both the peripheral donor and the in vitro (P0, P1) cells exhibited a confluent monolayer of smaller-size