proteins at the plasma membrane. Furthermore, it causes induction of fibrillar proteins such as type I collagen and fibronectin. In this present study, we demonstrated that the fibroblastic phenotypes of cultivated CECs greatly lost the endothelial characteristics; expression of Na^+/K^+-ATPase and ZO-1 was markedly reduced and their subcellular localization was in the cytosol rather than the authentic plasma membrane location. Furthermore, fibroblastic phenotypes markedly enhance the production of fibrillar ECM proteins (type I collagen, fibronectin, and integrin α5) rather than basement membrane phenotypes (type IV and VIII collagens). The presence of such undesirable cells will greatly hamper the success of transplantation of cultivated cells in the clinical setting. Therefore, it is crucial to determine what causes the phenotypic changes and how to intervene in such endothelial-mesenchymal transformation processes of the cultivated CECs. The fact that phosphorylation of Smad2/3 was greatly enhanced in the fibroblastic phenotypes led us to conclude that the fibroblastic phenotypes in both primate and HCECs are mediated by TGF-β signaling. Therefore, we employed a specific inhibitor to the TGF-β receptor (SB431542) [45] to block the endothelial-mesenchymal transformation process observed in the fibroblastic phenotypes. SB431542 completely abolished the undesirable cellular changes, and when either primate or HCEC cultures were treated with SB431542, the prerequisite change of cells to fibroblastic phenotypes was completely abolished. Simultaneously, the characteristic subcellular location of ZO-1 and Na^+/K^+-ATPase is resumed at the plasma membrane and the expression of the two proteins is greatly increased at both mRNA and protein levels, suggesting that the barrier and pump functions in these cultures is intact. Moreover, we found that the production of fibrillar ECM proteins was greatly reduced. We further tested the effect of BMP-7, a well-known anti-EMT agent [31,34], to reverse the fibroblastic phenotypes of HCECs. BMP-7 also reversed the fibroblastic phenotypes to the normal endothelial cells with contact-inhibited monolayer and characteristic endothelial adhesion. Taken together, both SB431542 and BMP-7 can be powerful tools to maintain the normal endothelial phenotypes of the cultivated CECs, thus leading to a successful subsequent transplantation.

In conclusion, our findings showed that the use of the inhibitor to TGF-β receptor (SB431542) and/or anti-EMT molecules (BMP-7) enables HCECs to grow with maintaining normal physiological function (i.e., barrier and pump function). Although more extensive future studies would be beneficial, we have not observed any obvious adverse effects of continuous SB431542 or BMP-7 treatment on morphology and functions, even after several numbers of passages. This present study may prove to be the substantial protocol to provide the efficient *in vitro* expansion of HCECs. In addition, this novel strategy of inhibition of fibroblastic change during cultivation may ultimately provide clinicians with a new therapeutic modality in regenerative medicine, not only for the treatment of corneal endothelial dysfunctions, but also for a variety of pathological diseases in general.

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Author Contributions

Conceived and designed the experiments: NO EPK MN JH SK NK. Performed the experiments: NO MN. Analyzed the data: NO EPK MN JH SK NK. Contributed reagents/materials/analysis tools: NO SK NK. Wrote the paper: NO EPK NK.

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Letter to the Editor

Relationship between frequent swimming pool use and lacrimal duct obstruction

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Editor,

In our clinical practice, we often encounter frequent swimming pool users and swimming instructors to be among patients with acquired lacrimal duct obstruction (LDO). Only one previous report of 45 patients with primary acquired nasolacrimal duct obstruction has noted that history of swimming pool exposure may be associated with the development of LDO (Ohtomo et al. 2013). To examine whether or not frequent swimming pool use is associated with LDO, we conducted a questionnaire survey of patients who underwent treatment for LDO and compared the results with an age- and gender-matched control group.

The questionnaire survey was conducted in 332 patients who visited the Department of Ophthalmology at Kyoto Prefectural University of Medicine, Kyoto, Japan, and underwent surgical treatment (dacryocystorhinostomy or silicone tube insertion) for LDO between April 2003 and March 2009. LDO was defined as the complete obstruction of the nasolacrimal duct, canaliculus or both by syringing and probing during surgery. A questionnaire survey on the frequency of swimming pool use was given to 332 LDO patients, of whom 227 completed the questionnaire (adopted LDO group; 68.4%, 45 males and 182 females, mean age: 65.4 ± 12.8 years), and a control group of 625 patients without LDO (159 males and 466 females, mean age: 63.3 ± 15.3 years), recruited from the outpatient clinic and matched for age and gender. Statistical analysis was performed on the correlation between LDO and frequency of swimming pool use and the frequency between the two groups. Frequent swimming pool use was defined as the use of the pool one or more times per month for a period of at least 6 months. Individuals who had onset of symptoms prior to commencing swimming pool use were not defined as frequent users. Individuals unable to use swimming pools for medical reasons were excluded from the study.

The percentage of frequent swimming pool use differed significantly and statistically between the LDO group (35 of 227 patients, 15.4%) and the control group (20 of 625 subjects, 3.2%). In the LDO group, the average age of patients with frequent swimming pool use (60.1 years old) significantly differed from the patients without frequent use (66.4) (Table 1). In the LDO group, patient-age-related percentages of frequent swimming pool use were 100% (2/2, ages 10–19), 20% (1/5, ages 20–29), 0% (0/4, ages 30–39), 40% (4/10, ages 40–49), 18.9% (7/37, ages 50–59), 18.4% (14/76, ages 60–69), 7% (5/71, ages 70–79) and 9% (2/22, ages 80–89).

Table 1. Percentage of frequent swimming pool use.

Frequent swimming pool use yes or no, *n* (%)	Adopted LDO group (*n* = 227)	Control group (*n* = 625)
Yes	35 (15.4%)***	20 (3.2%)***
Mean age (SD), years	60.1 (15.9)*	64.7 (12.3)
Range	18–86	30–88
Men	7 (20.0%)	4 (20.0%)
Women	28 (80.0%)	16 (80.0%)
No	192 (84.6%)	605 (96.8%)
Mean age (SD), years	66.4 (11.9)*	63.2 (15.4)
Range	21–87	11–89
Men	38 (19.8%)	155 (25.6%)
Women	154 (80.2%)	450 (74.4%)

LDO = lacrimal duct obstruction, SD = standard deviation.
* $p < 0.05$, *** $p < 0.0001$ (Chi square test).

Previous studies have reported epidemiological evidence that bathing or swimming in polluted waters is a potential health risk (Seyfried et al. 1985). In most swimming pools, microbiological control is performed by disinfection via the addition of chlorine. The disinfection properties of free chlorine are linked to its oxidant capacity, and chlorination of swimming pool water leads to the formation of disinfection by-products, including combined chlorine or trihalomethanes, which are associated with some types of illness (Florentin et al. 2011). The phenomenon of a swimming-induced rhinitis in elite swimmers in chlorinated pools has been reported (Alves et al. 2010), and allergic rhinitis may have a role in primary acquired LDO (Eriman et al. 2012). Thus, it is possible that combined chlorine in water is one of the factors potentially involved in LDO among swimming pool users, and our results suggest that frequent swimming pool use may be a risk factor for LDO. Further investigation is needed to provide a more detailed analysis of the relationship between LDO and the quality of swimming pool water.

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Involvement of Cyclin D and p27 in Cell Proliferation Mediated by ROCK Inhibitors Y-27632 and Y-39983 During Corneal Endothelium Wound Healing

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Purpose. To investigate the molecular mechanism of Rho-associated kinase (ROCK) inhibitors Y-27632 and Y-39983 on corneal endothelial cell (CEC) proliferation and their wound-healing effect.

Methods. The expression of G_1 proteins of the cell cycle and expression of phosphorylated Akt in monkey CECs (MCECs) treated with Y-27632 were determined by Western blotting. The effect of Y-39983 on the proliferation of MCECs and human CECs (HCECs) was evaluated by both Ki67 staining and incorporation of BrdU. As an in vivo study, Y-39983 was topically instilled in a corneal-endothelial partially injured rabbit model, and CEC proliferation was then evaluated.

Results. Investigation of the molecular mechanism of Y-27632 on CEC proliferation revealed that Y-27632 facilitated degradation of p27Kip1 (p27), and promoted the expression of cyclin D. When CECs were stimulated with Y-27632, a 1.7-fold increase in the activation of Akt was seen in comparison to the control after 1 hour. The presence of LY294002, the PI 3-kinase inhibitor, sustained the level of p27. When the efficacy of Y-39983 on cell proliferation was measured in a rabbit model, Y-39983 eye-drop instillation demonstrated rapid wound healing in a concentration range of 0.095 to 0.95 mM, whereas Y-27632 demonstrated rapid wound healing in a concentration range of 3 to 10 mM.

Conclusions. These findings show that ROCK inhibitors employ both cyclin D and p27 via PI 3-kinase signaling to promote CEC proliferation, and that Y-39983 may be a more potent agent than Y-27632 for facilitating corneal endothelium wound healing.

Keywords: corneal endothelial cells, ROCK inhibitor, bullous keratopathy, cell proliferation

It is well known that healthy corneal endothelium is vital for maintaining homeostatic corneal transparency and clear vision.[1] To date, full-thickness corneal transplantation or endothelial keratoplasty have been the only therapeutic choices available for the restoration of clear vision lost due to endothelial disorders.[2] In fact, more than 40,000 corneal transplantations were performed in 2011 in the United States alone.[2] In both 2009 and 2010, more than 40% of the corneal transplantation surgeries performed worldwide were endothelial keratoplasty,[2] thus suggesting that the primary disorder requiring corneal grafting is corneal endothelial dysfunction. Despite the high incidence of endothelial keratoplasty surgeries being performed, problems associated with corneal transplantation, such as allograft rejection, primary graft failure, and continuous loss of cell density, have yet to be resolved.[2–4]

As an alternative to corneal transplantation, transplantations of cultivated human corneal endothelial cells (HCECs) by a tissue engineering technique[5–10] or drug therapies[11–13] are expected to provide new therapeutic pathways for the treatment of corneal endothelial dysfunction. The applications of those two therapeutic approaches, as well as the purposes for which they are specifically intended, are distinct from one another (i.e., drug therapy may be a powerful tool in cases of early-stage corneal endothelial dysfunction in which stem cells or progenitor cells[14,15] are still maintained in the tissue, whereas transplantation of cultivated HCECs may be useful for the treatment of a fully progressed corneal endothelial dysfunction).[13]

In our previous study, we demonstrated that Y-27632, a specific Rho-associated kinase (ROCK), increased the proliferative potential of cultivated primate CECs in vitro.[16] We also reported that the topical administration of ROCK inhibitor Y-27632 enhanced corneal endothelial wound healing in an in vivo rabbit model, as the inhibitor facilitated cell proliferation as one of the major mechanisms.[11] In addition, we recently reported that the administration of ROCK-inhibitor Y-27632 eye drops recovers corneal clarity and thickness, especially in some patients with focal-edema-type Fuchs' corneal dystrophy.[12,13] Surprisingly, the best-corrected visual acuity of one bullous keratopathy patient that we reported recovered from logMAR 0.7 to −0.18, and with a completely transparent cornea, thus prompting us to cancel a corneal transplantation that was previously scheduled for that patient.[12]

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In this present study, we investigated the molecular mechanism by which ROCK inhibitor Y-27632 stimulates the proliferation of CECs. Our results show that Y-27632 employs phosphatidylinositol 3-kinase (PI 3-kinase) signaling that subsequently regulates two proteins of the G_1 phase of the cell cycle: upregulation of cyclin D, and downregulation of p27Kip1 (p27); both activities being required for G_1/S progression. In addition, we investigated the novel, selective ROCK inhibitor Y-39983, an inhibitor with a reportedly higher potency than Y-27632 for inhibiting ROCK activity.[17,18] We then compared Y-27632 and Y-39983 with regard to their action on the proliferation of CECs, both in vitro and in vivo. We found that a lower concentration of Y-39983 (0.3 μM or 3.0 μM) stimulates the proliferation of CECs to the same level stimulated by 10 μM of Y-27632. Furthermore, our findings demonstrated that the topical administration of Y-39983 enhances corneal endothelial wound healing associated with cell proliferation in an in vivo rabbit model. Those results suggest that Y-39983 may be a highly effective drug candidate for the treatment of corneal endothelial dysfunction.

Materials and Methods

Materials

FNC Coating Mix was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). Collagenase A was purchased from Roche Applied Science (Penzberg, Germany). Dulbecco's modified Eagle's medium, fibroblast growth factor 2 (FGF-2), Trypsin-EDTA, OptiMEM-I, Alexa Fluor 488-conjugated goat anti-mouse IgG, and Click-iT EdU Imaging Kits were purchased from Life Technologies Corp. (Carlsbad, CA). Y-27632, LY-294002, chondroitin sulfate, and Alizarin red S were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Epidermal growth factor (EGF), ascorbic acid, calcium chloride, anti-mouse Ki67 antibody, and Phosphatase Inhibitor Cocktail 2 were purchased from Sigma-Aldrich Co. (St. Louis, MO). The 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Burlingame, CA); CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega Corporation (Madison, WI); Cell Proliferation Biotrak ELISA System, version 2 was purchased from GE Healthcare Life Sciences (Buckinghamshire, England); BrdU labeling solution was purchased from Amersham Biosciences (Freiburg, Germany); and RIPA buffer was purchased from Bio-Rad Laboratories (Hercules, CA).

Protease Inhibitor Cocktail was purchased from Nacalai Tesque (Kyoto, Japan). Nonfat dry milk, Cyclin D1, Cyclin D3, Akt1, phosphorylated Akt, and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Cdc25A and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Abcam (Cambridge, UK).

Animal Experiment Approval

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 rabbit experiments were performed at Doshisha University (Kyoto, Japan) according to the protocol approved by the Animal Care and Use Committee (Approval No. 1224) of the university. The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. For all eye donations from deceased donors, written consent to use the eyes for research was obtained from the next of kin. All donor tissue was obtained under the tenets of the Uniform Anatomical Gift Act (UAGA) of the particular state where both the donor consent and tissue were obtained.

Cell Culture of Monkey CECs

CECs used to produce the monkey CEC (MCEC) culture were obtained from eight corneas of four cynomolgus monkeys (3 to 5 years of age; estimated equivalent human age: 5 to 20 years), respectively housed at Nissei Bilis and the Keari Co., Ltd., Osaka, Japan. The MCECs were cultivated in a modified protocol as described previously.[9,16,19] Briefly, Descemet's membrane, including corneal endothelium, was stripped and digested at 37°C for 2 hours with 1 mg/mL collagenase A. After digestion, the MCECs were resuspended in culture medium and plated in one well of a six-well plate coated with FNC Coating Mix. All primary cell cultures and serial passages of the MCECs were performed in growth medium composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 ng/mL FGF-2. The cells were then cultured in a humidified atmosphere at 37°C in 5% CO_2, with the culture medium being changed every 2 days. The MCECs were then trypsinized with 0.05% Trypsin-EDTA for 5 minutes at 37°C, and passaged at the ratio of 1:2 to 4 once they had reached confluence. Cultivated MCECs at passages 2 through 5 were used for all experiments. Y-27632 (a selective inhibitor of Rho kinase) and LY-294002 (a PI 3-kinase inhibitor) were tested for their cell proliferation and antiproliferation effects. In addition, ROCK-inhibitor Y-39983 (obtained from Mitsubishi Pharma Corporation, Osaka, Japan) was tested for its effect on cell proliferation.

Cell Culture of HCECs. The HCECs were cultivated using the recently reported protocol.[13] Briefly, Descemet's membrane, including CECs, was stripped and digested with 1 mg/mL collagenase A, and the HCECs were then resuspended in culture medium. The culture medium was prepared from basal medium after conditioning by inactivated NIH-3T3 fibroblasts. The inactivated NIH-3T3 was maintained by basal medium for 24 hours. Then, the medium was collected, filtered, and used as the culture medium for the HCECs. Basal medium was composed of OptiMEM-I supplemented with 8% FBS, 5 ng/mL EGF, 20 μg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, and 50 μg/mL gentamicin. Inactivation of the 3T3 fibroblasts was performed as described previously.[20,21] The HCECs were cultured in a humidified atmosphere at 37°C in 5% CO_2, with the culture medium being changed every 2 days. Cultivated HCECs at passages 2 through 5 were used for all experiments.

In Vitro Wound Healing Assay

The MCECs were further maintained in culture for 14 days after reaching confluence so as to form a contact-inhibited hexagonal layer. Scrape wounds were then produced using a plastic pipette tip to create six linear defect sites in each culture dish. The culture medium was then replaced with fresh medium containing 10 μM of Y-27632, while control cells were maintained in the absence of Y-27632. The percentage of Ki67-positive ($Ki67^+$) cells among the proliferating and migrating cells in the wounded area was determined after 48 hours of incubation. All experiments were performed in duplicate.

In Vivo Wound Healing After Y-27632 Treatment

As an in vivo wound model, the corneal endothelium of nine Japanese white rabbits was damaged in a modified protocol as described previously.[11,22,23] Briefly, a stainless-steel 7-mm-

diameter probe was immersed in liquid nitrogen for 3 minutes to stabilize its temperature at approximately −196°C, and the probe was then placed onto the rabbit cornea for 15 seconds under general anesthesia. Care was taken to confirm that this procedure did not induce complete blindness or any severe general adverse effect. Next, 1, 3, or 10 mM of Y-27632 diluted in PBS (50 μL) was topically instilled in eye eye of each rabbit six times daily, while PBS alone was instilled in the fellow eye of each rabbit as a control. After 48 hours of treatment, the rabbits were euthanized and the $Ki67^+$ cells located at the edge of the original corneal endothelium wound (3.5-mm distant from the center of the cornea) were then evaluated.

In Vivo Wound Healing After Y-39983 Treatment

The corneal endothelium of 27 Japanese white rabbits was damaged by transcorneal freezing as described above. Next, 0.095 mM (0.003%), 0.32 mM (0.01%), or 0.95 mM (0.03%) of Y-39983 diluted in PBS (50 μL) was topically instilled in one eye of each rabbit six times daily, while PBS alone was instilled in the fellow eye of each rabbit as a control. After 48 hours of treatment, the anterior segment of each eye was assessed by use of a slit-lamp microscope and the rabbits were then euthanized. The corneal endothelium wound area was then evaluated by use of Alizarin red staining after enucleation. Briefly, corneas were stained with 0.5% Alizarin red for 1 minute, fixed in 4% formaldehyde, and then examined under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The residual wound areas shown in the Alizarin red staining images were then evaluated by use of Image J (National Institutes of Health, Bethesda, MD) software. In addition, $Ki67^+$ cells located at the edge of the original corneal endothelium wound in the same specimens were evaluated.

Ki67 Immunostaining

MCECs or HCECs cultured on Lab-Tek Chamber Slides (NUNC A/S, Roskilde, Denmark), or flat-mounted whole corneal specimens, were fixed in 4% formaldehyde for 10 minutes at room temperature (RT), and then incubated for 30 minutes with 1% bovine serum albumin (BSA). To investigate the proliferation of the CECs, immunohistochemical analyses of Ki67 staining was performed. Samples were incubated with a 1:400 dilution of anti-mouse Ki67 antibody overnight at 4°C, washed three times in PBS, and then incubated with a 1:2000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG for 2 hours at RT. Cell nuclei were stained with DAPI. The slides were then examined under a fluorescence microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany).

Effect of Y-39983 on the MCECs in Culture

MCECs were seeded at a density of 5.0×10^3 cells/cm^2 per well on a 96-well plate for 24 hours, and then subjected to serum starvation for an additional 24 hours in the presence or absence of Y-39983 (0.03 μM, 0.3 μM, and 3.0 μM). The MCECs were then examined under a phase-contrast microscope (Leica), and the number of viable cells was determined by use of the CellTiter-Glo Luminescent Cell Viability Assay performed in accordance with the manufacturer's recommended protocol. The number of MCECs at 24 hours after stimulation with Y-39983 was measured by use of the Veritas Microplate Luminometer (Promega). Five samples were prepared for each group.

EdU-Labeling Assay

MCECs seeded at a density of 5.0×10^4 cells/cm^2 on micro cover glass (Matsunami Glass Ind., Ltd., Osaka, Japan) in a 24-well plate were maintained for 24 hours, and then incubated in the absence of serum for an additional 24 hours with or without Y-39983 (0.03 μM, 0.3 μM, and 3.0 μM). DNA synthesis was determined by use of Click-iT EdU Imaging Kits using the recommended protocol. Briefly, the cells were incubated for an additional 24 hours with a 20 μM EdU-labeling reagent. The cells were then washed in PBS, fixed with 4% formaldehyde for 20 minutes at RT, and washed with 3% BSA. The cells were then incubated for 30 minutes at RT with a Click-iT reaction cocktail, washed 3 times in PBS, and mounted on glass slides with anti-fading mounting medium containing DAPI. The slides were then examined under the TCS SP2 AOBS fluorescence microscope.

BrdU ELISA

MCECs or HCECs were seeded at the density of 5000 cells per 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 Y-39983 ($n = 5$). DNA synthesis was detected as incorporation of 5-bromo-2′-deoxyuridine (BrdU) into the Cell Proliferation Biotrak ELISA system, version 2, according to the manufacturer's instructions. Briefly, MCECs or HCECs were incubated with 10 mol/L BrdU for 24 hours in a humidified atmosphere at 37°C in 5% CO_2. The cultured cells were incubated with 10 μM BrdU labeling solution for 2 hours, and then incubated with 100 μL of monoclonal antibody against BrdU for 30 minutes. The BrdU absorbance was measured directly using a spectrophotometric microplate reader (Promega) at a test wavelength of 450 nm.

Immunoblotting

The MCECs were washed with ice-cold PBS, and then lysed with ice-cold RIPA buffer containing Phosphatase Inhibitor Cocktail 2 and Protease Inhibitor Cocktail. The lysates were centrifuged at 15,000 rpm for 10 minutes at 4°C to sediment the cell debris. The supernatant representing total proteins was collected and the protein concentration of the sample was assessed by use of the BCA Protein Assay Kit (Takara Bio, Inc., Otsu, Japan). An equal amount of protein was fractionated by SDS-PAGE; proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 3% nonfat dry milk in TBS-T buffer (50 mM Tris, pH 7.5, 150 mM $NaCl_2$, and 0.1% Tween20) for 1 hour at RT, followed by an overnight incubation at 4°C with the following primary antibodies: Cdc25A (1:1000), Cyclin D1 (1:1000), Cyclin D3 (1:1000), p27 (1:1000), Akt1 (1:2000), phosphorylated Akt (1:2000), and GAPDH (1:3000). The blots were washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000). The blots were then developed with luminal for enhanced chemiluminescence using the ECL Advanced Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ), documented using an LAS4000S (Fuji Film, Tokyo, Japan) cooled charge-coupled-device camera gel documentation system, and analyzed with Image J software.

Statistical Analysis

The statistical significance (P value) in mean values of the two-sample comparison was determined with the Student's t-test. The statistical significance in the comparison of multiple sample sets was analyzed by use of the Dunnett's multiple-comparisons test. Values shown on the graphs represent the mean ± SEM.

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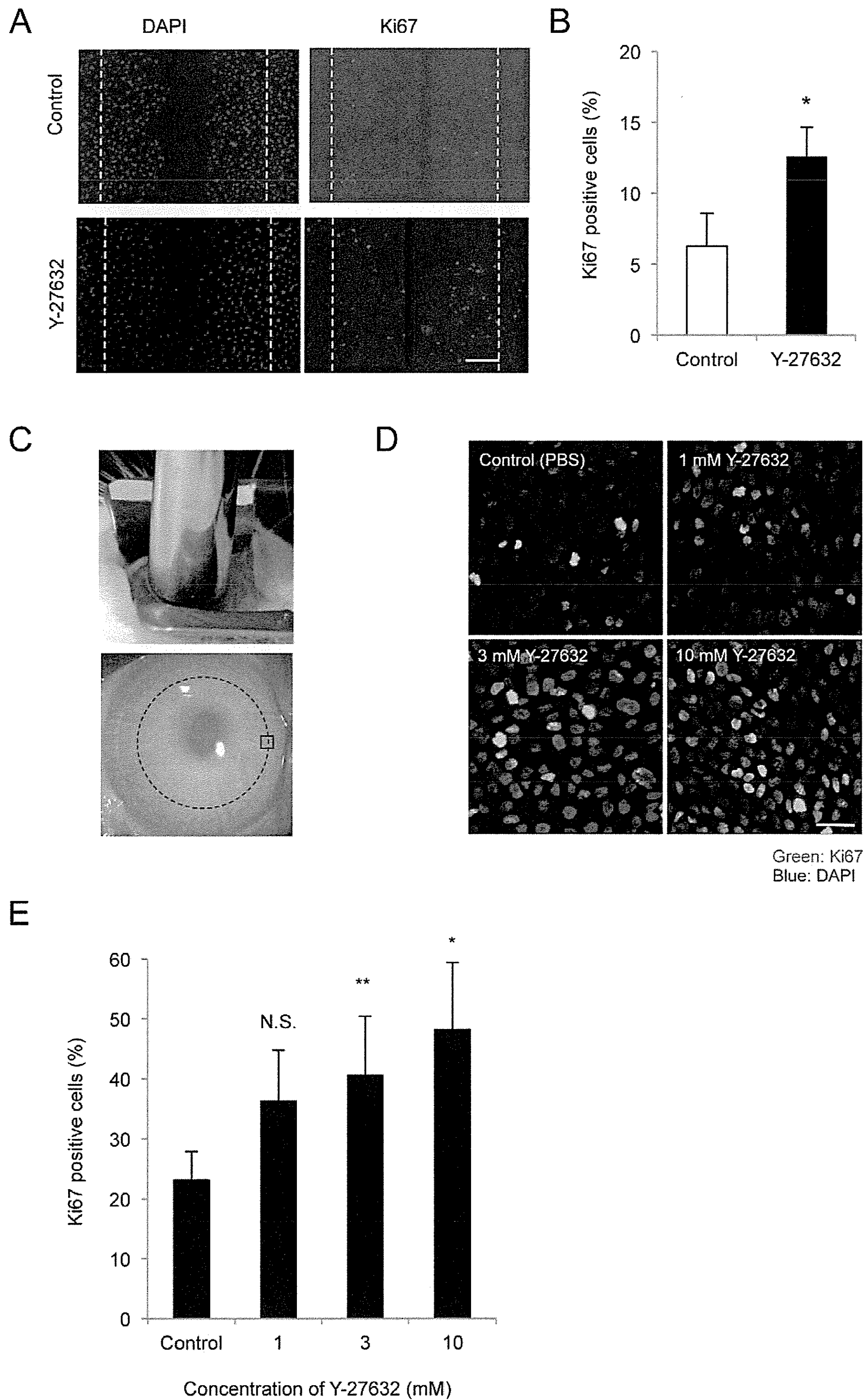

FIGURE 1. Effect of ROCK-inhibitor Y-27632 on cell proliferation during in vitro and in vivo wound healing. (**A**) Effect of Y-27632 on the proliferation of cultured MCECs. Representative Ki67 staining images obtained 48 hours after scrape wounding treated with 10 μM Y-27632. *Scale bar*: 500 μm. (**B**) In the absence of Y-27632, approximately 6% of the cell population in the injury sites was found to be composed of $Ki67^+$ cells. However, in the cells treated with Y-27632, 13% of the cells in the injury site were $Ki67^+$ cells. The $Ki67^+$ cells were counted in the wounded area ($n = 4$). The images are representative of two independent experiments. (**C**) Central rabbit corneal endothelium was partially damaged by transcorneal cryogenic injury. *Dotted line*: original wounded area; *solid line*: edge of the original wounded area. Y-27632 (50 μL) was topically applied in one eye of each animal six times daily for 2 days. (**D**, **E**) $Ki67^+$ cells at the edge of the original wounded area were counted in the absence or presence of Y-27632 eye drops in three different concentrations (1, 3, or 10 mM), and the mean data were then plotted ($n = 6$). Y-27632 eye-drop instillation increased the number of $Ki67^+$ cells at the edge of the original wounded site in a dose-dependent manner. *Scale bar*: 100 μm. *$P < 0.01$, **$P < 0.05$.

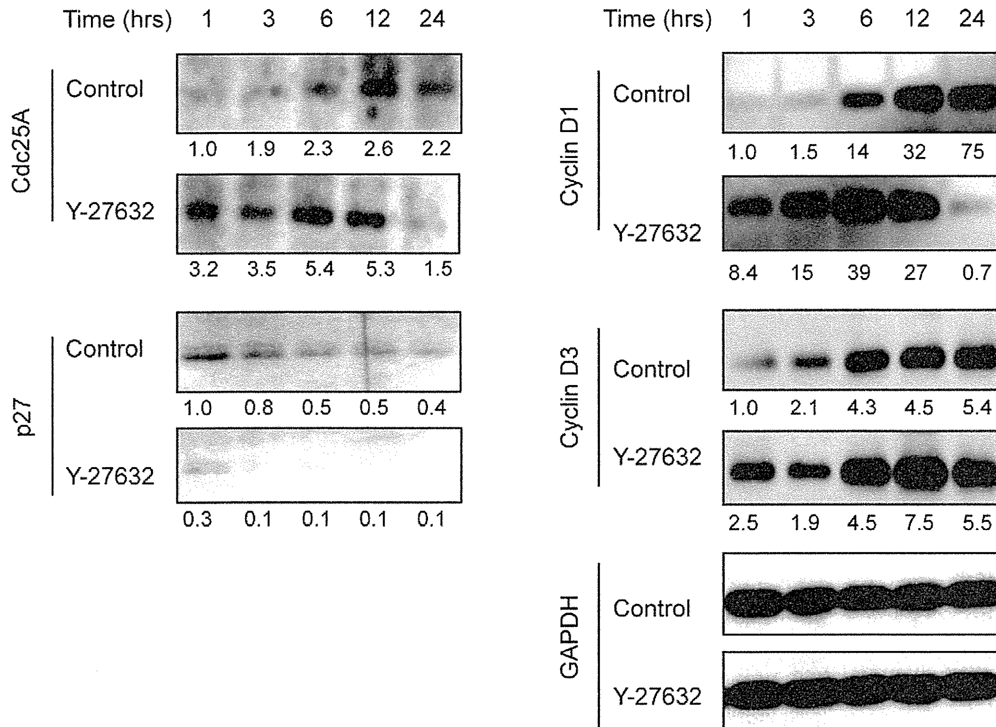

Figure 2. Involvement of ROCK-inhibitor Y-27632 on G_1/S progression. MCECs were serum starved for 24 hours prior to the treatment of cells with growth medium containing 10 μM of Y-27632. After 1, 3, 6, 12, or 24 hours, Cdc25A, p27, cyclin D1, and cyclin D3 were analyzed at the protein levels. Y-27632 produced a 3.2-fold increase in the expression of Cdc25A within 1 hour, and maintained that expression up to 12 hours. Y-27632 stimulation produced a 0.3-fold reduction of p27 at 1 hour, and an 8.4-fold and 2.5-fold increase in the expression of cyclin D1 and D3, respectively, was observed within 1 hour. The relative density of the immunoblot bands was determined by Image J software. Relative fold differences were compared with the values of the controls at 1 hour. All experiments were performed in triplicate.

Results

Effect of Y27632 on Cell Proliferation During in Vitro and in Vivo Wound Healing

A directional scrape wound was introduced to the cultured confluent MCECs to test whether or not Y-27632 facilitated wound healing via cell proliferation. Immediately following the wounding, cells were treated with 10 μM Y-27632 for 48 hours, and the $Ki67^+$ cells in the wounded area were then counted. In the absence of Y-27632, approximately 6% of the cell population in the injury sites was found to be composed of $Ki67^+$ cells. However, in the cells treated with Y-27632, 13% of the cells in the injury site were $Ki67^+$ cells (Figs. 1A, 1B). Such proliferative effect of Y-27632 was further confirmed in in vivo rabbit corneas injured by transcorneal freezing (Fig. 1C). $Ki67^+$ cells were counted 48 hours after cryo injury in the absence or presence of Y-27632 eye drops in three different concentrations (1, 3, or 10 mM). In the control eyes, 23% of the cells observed in the injury site were $Ki67^+$ cells, whereas there was a dose-dependent increase of $Ki67^+$ cells in the presence of Y-27632; 50% of the cells present at the edge of the original wounded site (Fig. 1C) were $Ki67^+$ cells when treated with 10 mM Y-27632 (Figs. 1D, 1E).

Involvement of ROCK Inhibitor on G_1/S Progression

Although ROCK is involved in many cellular activities, such as proliferation, differentiation, apoptosis, and oncogenic transformation, the particular mechanism related to each cellular activity has yet to be fully elucidated. Therefore, we investigated the molecular mechanism of cell proliferation facilitated by ROCK inhibitors using Y-27632. MCECs were serum starved for 24 hours before treatment of the cells with growth medium containing 10 μM Y-27632. Serum was removed to avoid any effect caused by the serum; however, we confirmed that a similar result was obtained even when the serum was not removed (data not shown). After 1, 3, 6, 12, or 24 hours, two classes of G_1 proteins of the cell cycle were analyzed at the protein levels: (1) Cdc25A was chosen for its activity on cyclin-dependent kinase 2 (Cdk2), which subsequently phosphorylates p27, a prerequisite event for degradation of p27,[24] and (2) the D class of cyclin (D1 and D3) for its positive regulatory activity on G_1/S progression. In the absence of Y27632, 6 to 12 hours was required to obtain the maximum expression of Cdc25A, whereas Y-27632 produced a 3.2-fold increase in the expression of Cdc25A within 1 hour and maintained the expression up to 12 hours (Fig. 2). On the other hand, there was a 0.3-fold reduction of p27 production at 1 hour after treating the cells with Y-27632, after which p27 levels were barely detectable in the cells treated for 12 hours (Fig. 2). However, an 8.4-fold and 2.5-fold increase in the expression of cyclin D1 and D3, respectively, was observed within 1 hour and for up to 12 hours (cyclin D1 and D3) or 24 hours (cyclin D3) in the presence of Y-27632 (Fig. 2).

Involvement of PI 3-Kinase Signaling in Y-27632-Mediated p27 Degradation and Upregulation of Cyclin D

PI 3-kinase signaling reportedly plays a key role in the cell proliferation of both HCECs and nonhuman CECs by degrading p27.[25,26] We tested whether Y-27632 employs PI 3-kinase signaling to remove p27 from the cell cycle. Serum-starved

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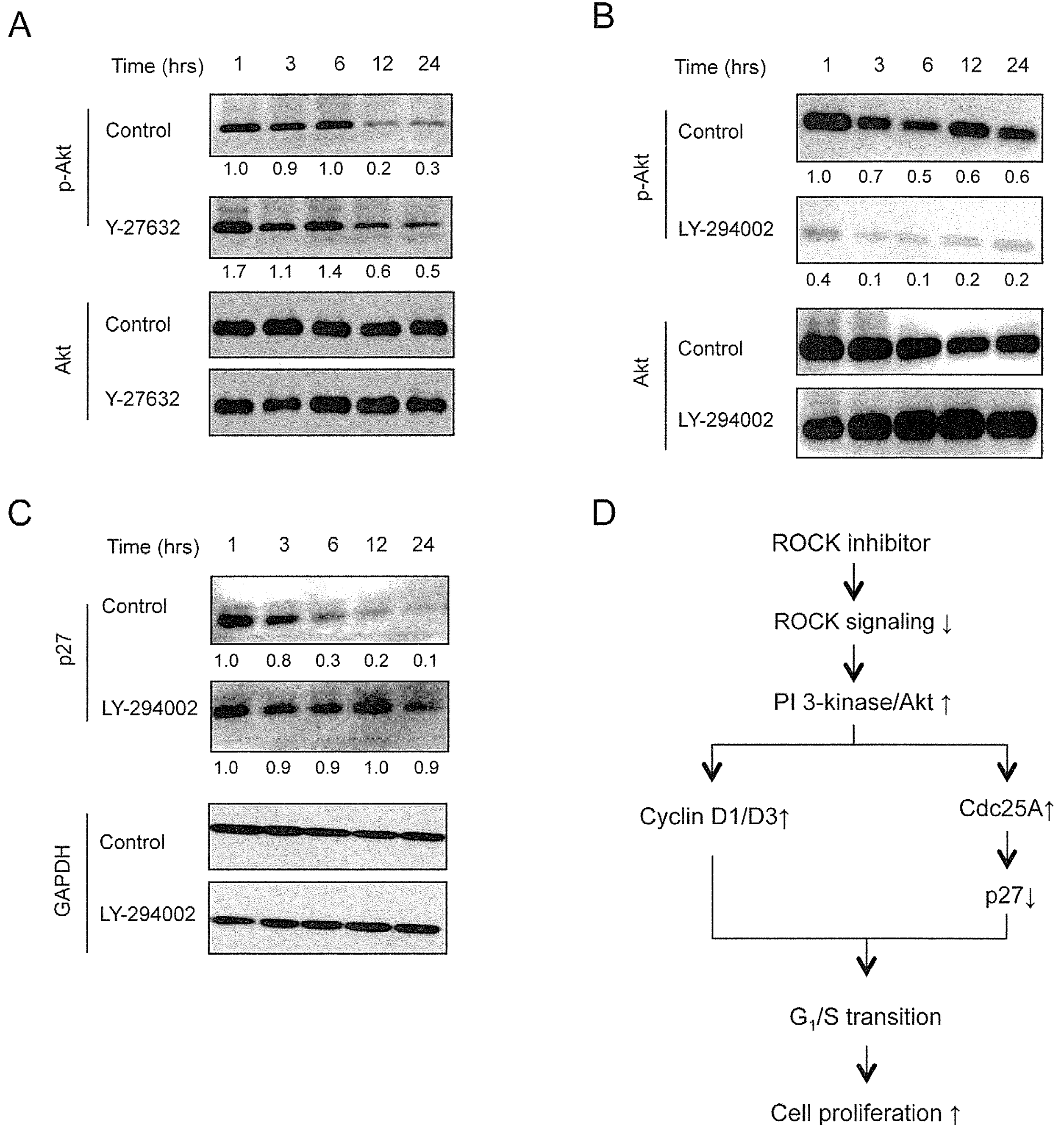

Figure 3. Involvement of PI 3-kinase signaling in Y-27632–mediated p27 degradation and upregulation of cyclin D. (A–C) Serum-starved MCECs were treated with or without Y-27632 for 1, 3, 6, 12, or 24 hours. Phosphorylation of Akt, total Akt, and p27 was evaluated by Western blotting. The phosphorylation of Akt was sustained 1.7-fold higher in the Y-27632-treated cells than in the control cells at 1 hour. LY294002 abolished the phosphorylation of Akt and maintained the p27 level. The relative density of immunoblot bands was determined by Image J software. Relative fold differences were compared with the values of the control at 1 hour. All experiments were performed in triplicate. (D) Schema illustrating our theory that ROCK inhibitor activates PI 3-kinase signaling, thus triggering the following two pathways for G_1/S transition: (1) upregulation of cyclin D1 and D3, and (2) removal of p27 through Cdk2 activated by Cdc25A.

MCECs were treated with or without Y-27632 for 1, 3, 6, 12, or 24 hours. Y-27632 produced a 1.7-fold increase in the phosphorylation of Akt, a serine/threonine protein kinase, in 1 hour, after which, the phosphorylation was found to decrease in a time-dependent manner. The phosphorylation of Akt was sustained higher in the Y-27632–treated cells than in the control cells (Fig. 3A). LY294002, the PI 3-kinase inhibitor, abolished phosphorylation of Akt (Fig. 3B), and p27 levels were maintained up to 24 hours in the presence of LY294002 (Fig. 3C). Thus, those findings show that the activities of the regulatory proteins of the G_1 phase of the cell cycle allow for G_1/S transition in the presence of ROCK inhibitor (Fig. 3D).

Effect of Y-39983 on Cell Proliferation of MCECs and HCECs

Fasudil, also known as HA-1077, is a selective ROCK inhibitor, and it has been successfully used for the treatment of cerebral

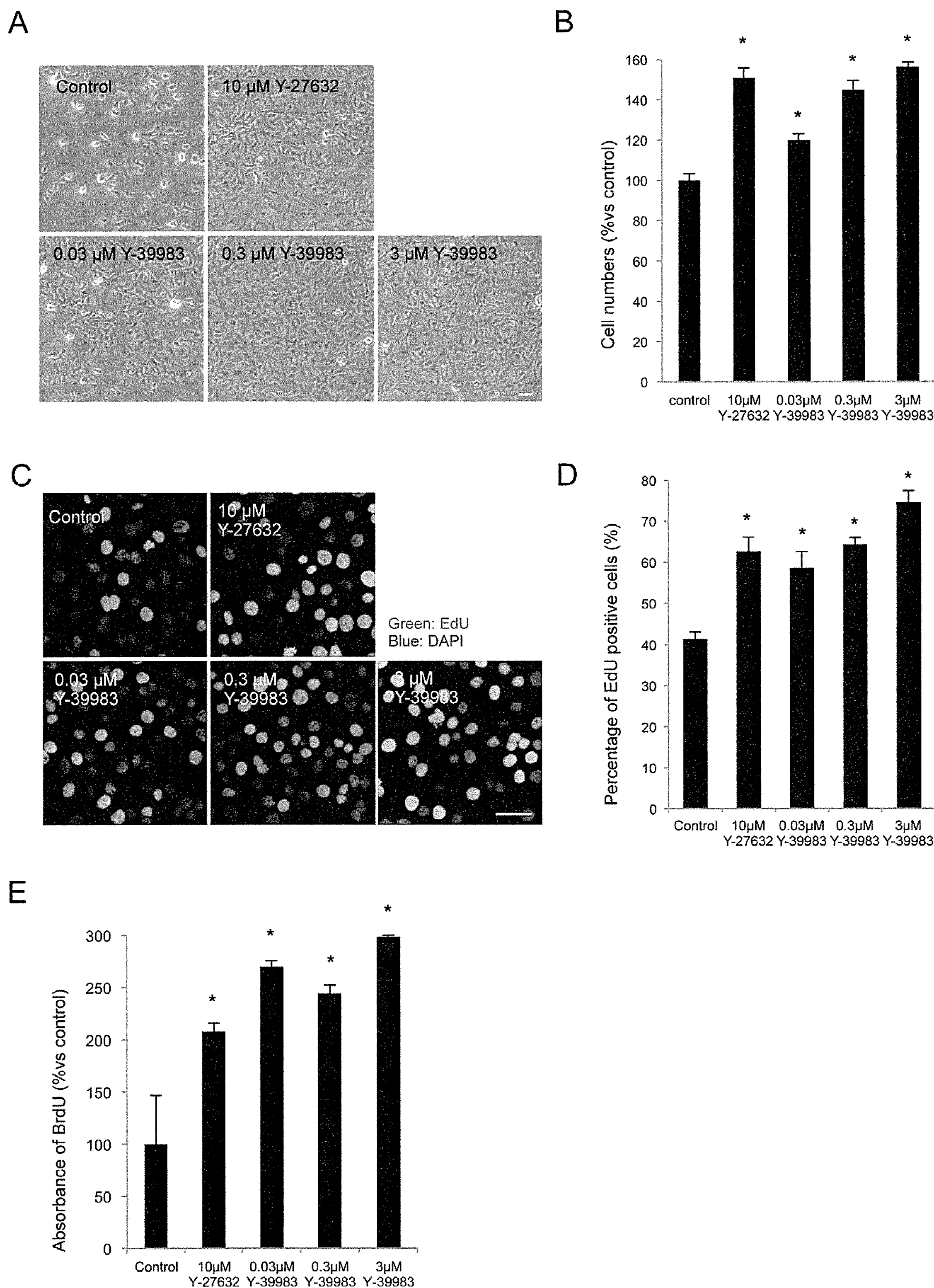

FIGURE 4. Effect of ROCK-inhibitor Y-39983 on the proliferation of MCECs. (**A**, **B**) MCECs were seeded at a density of 5.0×10^3 cells/cm^2 for 24 hours and then incubated with serum starvation for an additional 24 hours in the presence or absence of Y-39983. The MCECs were inspected by phase-contrast microscopy. The numbers of MCECs increased from 1.2- to 1.5-fold following stimulation with Y-39983 for 24 hours. Five samples were prepared for each group, and the experiments were performed in duplicate. *Scale bar*: 200 μm. (**C**, **D**) MCECs seeded at a density of 5.0×10^4 cells/cm^2 were maintained for 24 hours, followed by serum starvation for an additional 24 hours in the presence or absence of Y-39983. The percentage of EdU-positive cells was evaluated by fluorescence microscopy, and the data were then averaged and plotted ($n = 6$). EdU-positive proliferating cells increased following the treatment with Y-39983 (0.03–3.00 μM). *Scale bar*: 200 μm. (**E**) The effect of Y-39983 on the proliferation of MCECs was evaluated by BrdU incorporation assay. BrdU incorporation was enhanced 240% to 300% by Y-39983 (0.03–3.00 μM), while it was enhanced 200% by 10 μM Y-27632. $^*P < 0.01$. All experiments were performed in duplicate.

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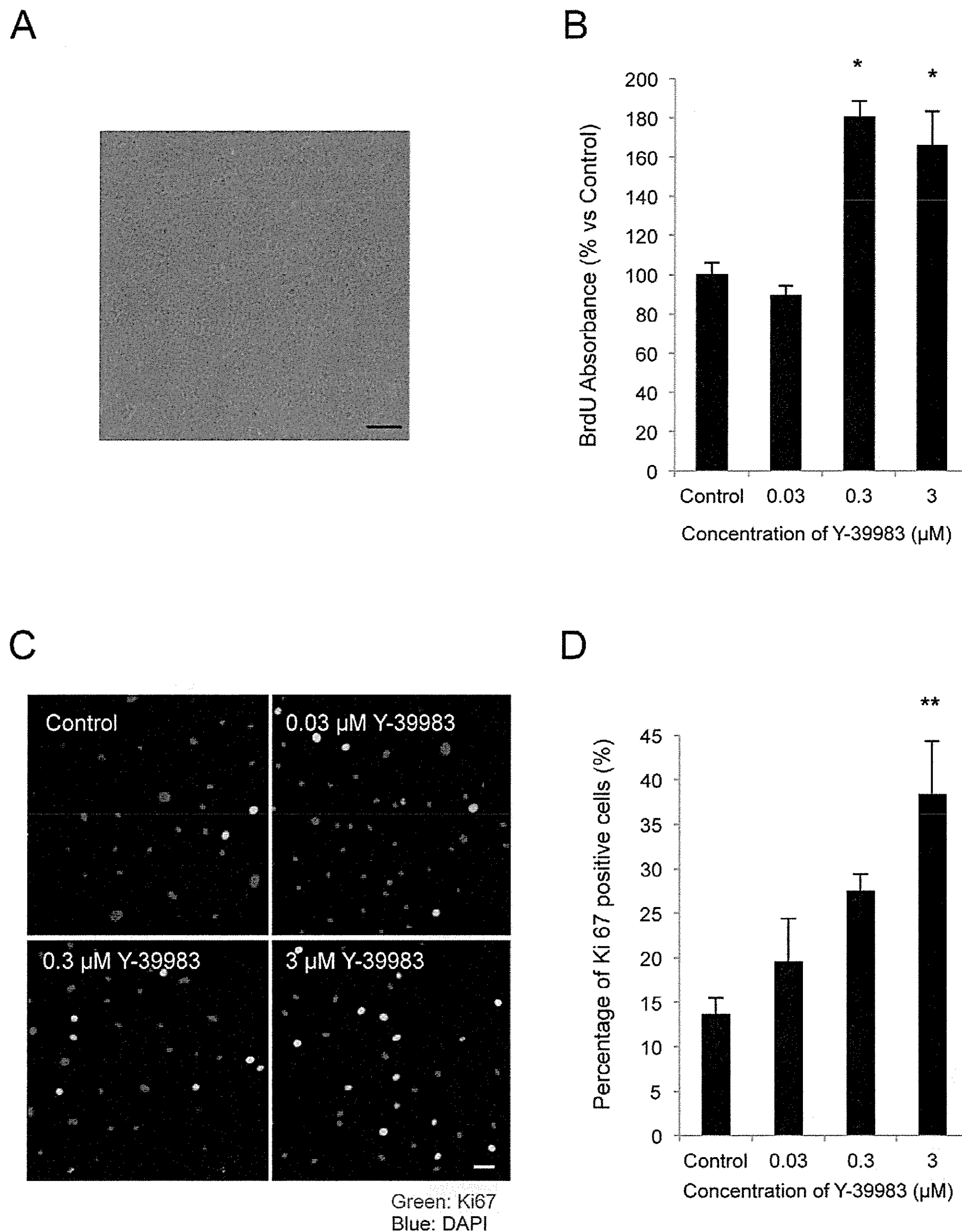

FIGURE 5. Effect of Y-39983 on the proliferation of HCECs. (A) Representative phase-contrast image of cultured HCECs. *Scale bar*: 50 μm. (B) HCECs were cultured, and the effect of Y-39983 on the proliferation of HCECs was evaluated by BrdU incorporation assay. BrdU incorporation into the newly synthesized DNA was increased from 1.6- to 1.8-fold at the concentration of 0.3 and 3.0 μM of Y-39983. (C, D) HCECs were subjected to serum starvation for an additional 24 hours in the presence or absence of Y-39983. The percentage of $Ki67^+$ cells was evaluated by fluorescence microscopy. Y-39983 increased the percentage of $Ki67^+$ HCECs in a dose-dependent manner. *Scale bar*: 200 μm. $^*P < 0.01$, $^{**}P < 0.05$. All experiments were performed in duplicate.

vasospasm in Japan.[27,28] Although Fasudil has been used in the clinical setting to target the ROCK pathway,[28,29] it was created as a compound to inhibit protein kinase A and protein kinase C. It was subsequently determined that Fasudil was significantly more potent for ROCK, as its half maximal inhibitory concentration (IC_{50}) is at least 10-fold lower than for other kinases.[28,29] Similarly, Y-27632 has been shown to inhibit additional kinases, and it is not available in good manufacturing practice (GMP) grade.[18] Therefore, we tested the effect of Y-39983, another novel ROCK inhibitor that is available in GMP grade, on the proliferation of MCECs, and compared it with the proliferation effect produced by Y-27632. Three concentrations of Y-39983 were used to examine its effect on cell proliferation, whereas Y-27632 was used at the concentration of 10 μM, as it is reportedly the most commonly used concentration[30] and most potent concentration to enhance the proliferation of CECs.[16] Evaluation of the cell numbers demonstrated that the proliferation of MCECs was 1.2- to 1.5-fold greater in the presence of ROCK inhibitors (Figs. 4A, 4B). When cell proliferation was examined with EdU or BrdU incorporation into the newly synthesized DNA, the activities differentiated between Y-27632 and Y-39983; for example, even 0.03 μM of Y-39983 produced stimulation of EdU or BrdU incorporation into the DNA (Figs. 4C–E). We further confirmed the effect of Y-39983 on cell proliferation in HCECs (Fig. 5A). Although contradictory findings have been reported,[31] our results revealed that Y-39983 at the concentrations of 0.3 and 3.0 μM produced a 1.6- to 1.8-fold increase of BrdU incorporation

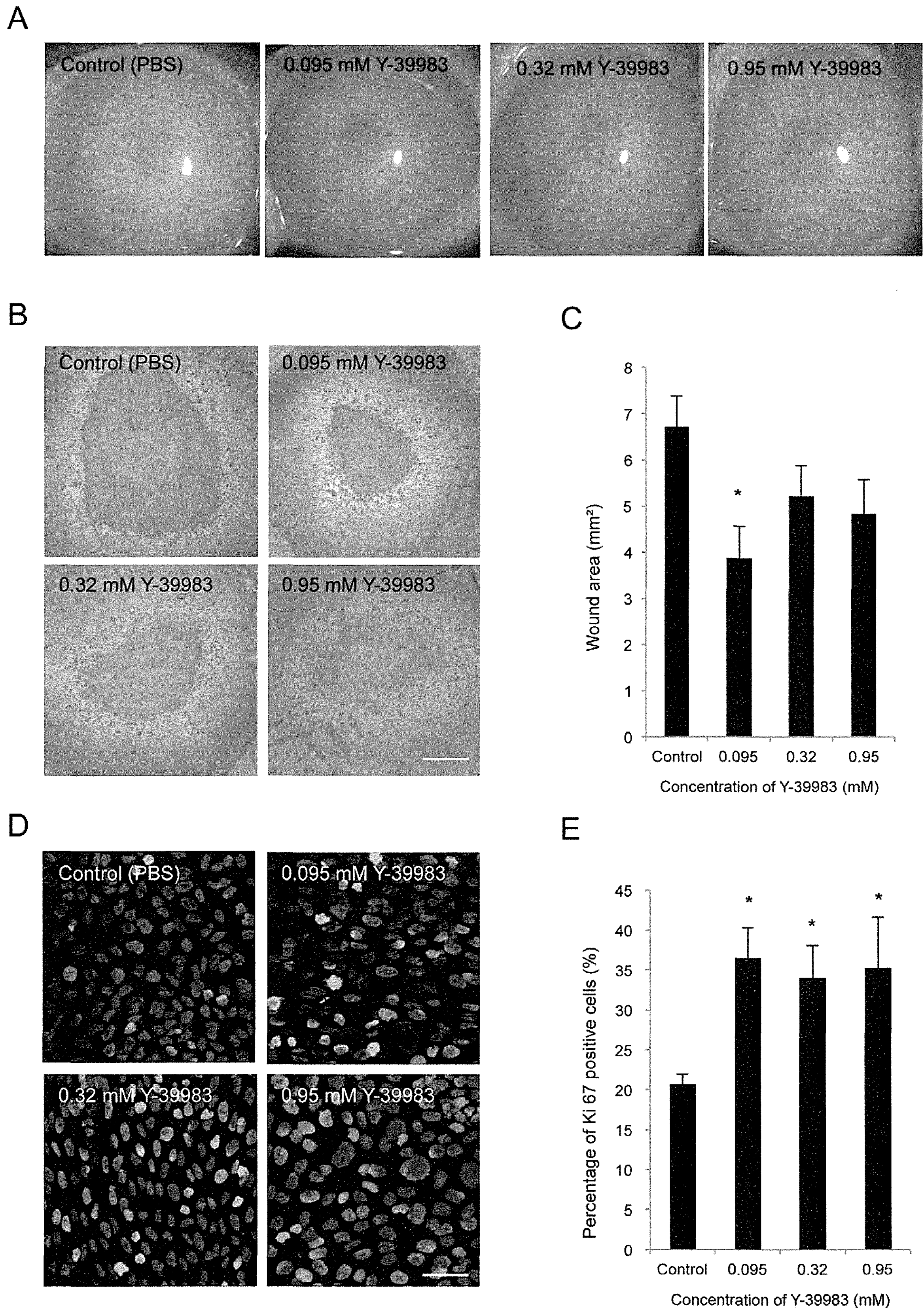

FIGURE 6. The effect of Y-39983 eye-drop instillation on wound healing and cell proliferation in an in vivo rabbit model. (A) The corneal endothelium of 27 Japanese white rabbits was damaged by transcorneal freezing. Then, 0.095, 0.32, or 0.95 mM of Y-39983 was topically instilled in one eye of each animal six times daily, while PBS was applied in the fellow eye as a control. Corneal clarity was examined by slit-lamp microscopy at 48 hours after treatment. (B, C) The rabbits were euthanized after 48 hours of treatment, and the wound area of the corneal endothelium was evaluated by Alizarin red staining. The wounded area of the corneal endothelium following the 0.095 mM treatment of Y-39983 was reduced (43%)when compared with that of the control eye. *Scale bar*: 200 µm. (D, E) The number of $Ki67^{+}$ cells among the undamaged peripheral corneal

←
endothelium was evaluated in the same specimens. The percentages of $Ki67^+$ cells at the edge of the original wounded area were evaluated by fluorescence microscopy, and the data were then averaged and plotted ($n = 6$). Approximately 35% of the cells in the Y-39983-treated groups were $Ki67^+$ cells in all concentrations tested, whereas 20% of the cells in the control group were $Ki67^+$ cells. *Scale bar*: 200 μm.

into the newly synthesized DNA (Fig. 5B). Moreover, Y-39983 increased the percentage of $Ki67^+$ HCECs in a dose-dependent manner (Figs. 5C, 5D).

Effect of Y-39983 on Cell Proliferation in In Vivo Wound Healing

Finally, we examined the effect of Y-39983 on wound healing using an in vivo rabbit model. Rabbit corneas were subjected to transcorneal freezing, and the wound areas were measured 48 hours after the topical administration of 0.095 mM (0.003%), 0.32 mM (0.01%), or 0.95 mM (0.03%) of Y-39983. There was a tendency for the Y-39983-treated corneas to be clearer than the untreated control eyes (Fig. 6A). When the wound areas were measured, the wounded area of the corneal endothelium following the 0.095-mM treatment of Y-39983 was significantly reduced (43% reduction) when compared with that of the control eye. The mean reduction of the wound area tended to be 30% in the corneas treated with 0.32 mM Y-39983 and 37% in the corneas treated with 0.95 mM Y-39983 (Figs. 6B, 6C). The above-described wound closure appeared to have been achieved by cell proliferation; 20% of the cells in the control group were $Ki67^+$ cells, whereas approximately 35% of the cells in the Y39983-treated groups were $Ki67^+$ cells, regardless of the concentration of Y-39983 that was tested (Figs. 6D, 6E).

Discussion

In most tissues, the wound repair process consists of cell proliferation and migration. 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. In humans, CEC density reportedly decreases linearly 0.3% to 0.6% per year throughout life.[32] Moreover, CEC density is known to decrease rapidly after invasive eye surgery, corneal transplantation, trauma, and so forth. Regarding stem cells, it has recently been reported that corneal endothelial stem cells divide very slowly and migrate toward the center of the cornea, and that cell clusters located in the extreme periphery may be stem cell niches.[14] In addition, we recently reported that human corneal endothelial stem/progenitor cells are regulated by LGR5 via the Hedgehog and Wnt pathways.[15] Consequently, in cases of early-stage corneal endothelial dysfunction, in which stem cells or progenitor cells are still maintained, drug-based therapies might provide a less-invasive pathway to halt the progression of the disease. However, current treatments for endothelial dysfunction to restore visual acuity are limited to corneal transplantation surgeries, such as penetrating or endothelial keratoplasty (Descemet's stripping automated endothelial keratoplasty and Descemet's membrane endothelial keratoplasty). Although pharmaceutical agents, such as EGF, platelet-derived growth factor, FGF-2, and small interfering RNA of Connexin 43 are reportedly effective for enhancing the proliferation of corneal endothelial cells,[33-35] those agents have yet to be introduced into the clinical setting.[2] To date, and to the best of our knowledge, no clinically practical medical therapy has been developed for the treatment of corneal endothelial dysfunction.

HCECs reportedly remain arrested at the G_1 phase of the cell cycle throughout their life span,[36] and regulation of cell-cycle G_1/S progression plays a central role in cell proliferation. Mitogenic stimulation induces entry into the G_1 phase, which prepares cells for DNA duplication in the S phase. Progression of cells from G_1 to S phase is highly regulated, and numerous proteins function as positive or negative regulators. One of the early G_1-phase positive regulatory proteins is cyclin D, which binds Cdk4 or Cdk6, forming an active kinase complex. In the absence of mitogenic stimulation, the cyclin D/Cdk4/Cdk6 complex remains associated with p27. Mitogenic stimulation sequesters p27 from the cyclin D/Cdk4/Cdk6 complex to the cyclin E/Cdk2 complex, which phosphorylates p27 and leads to the subsequent degradation of p27. Activation of cyclin D/Cdk4/Cdk6 in early G_1 and cyclin E/Cdk2 in late G_1 results in hyperphosphorylation of pRb and the release of the E2F transcription factor from the repressed complex pRb/E2F.[37] Thus, p27 plays a key role throughout the G_1 phase of the cell cycle. In both HCECs and nonhuman CECs, the corneal endothelium reportedly employs phosphorylation of p27 as the major mechanism for G_1/S progression.[26,36,38-40] Furthermore, this removal mechanism of p27 is mediated by PI 3-kinase signaling.[24-26] The findings of Joyce[41] revealed that not only p27, but also other cyclin-dependent kinase inhibitors, such as p21Cip1 and p16INK4a, are involved in the negative regulation of the CEC cycle, and that both p21Cip1 and p16INK4a increase with age. In line with the clinical application of ROCK inhibitor, the effect of ROCK inhibitor on p21Cip1 and p16INK4a needs to be further investigated, as corneal endothelial disorder patients are often relatively advanced in age.

In this present study, we determined the molecular mechanism by which ROCK inhibitors Y-27632 and Y-39983 stimulate the proliferation of both MCECs and HCECs. Our findings demonstrated that Y-27632 activates PI 3-kinase signaling, which subsequently regulates the following two respective pathways necessary for G_1/S progression: upregulation of cyclin D, and downregulation of p27. Moreover, our findings that Y-27632 rapidly increased the expression of Cdc25A, which is an essential phosphatase for Cdk2 activation, coincides with the findings of previous reports.[24,25] Upregulated cyclin D and removal of p27 by ROCK inhibitor both enable cyclin D/Cdk4 and cyclin E/Cdk2 complexes to hyperphosphorylate pRb, thus leading to activation of E2F and subsequent G_1/S progression. Although earlier studies have shown that inactivation of Rho by C3 blocks G_1/S progression in Swiss 3T3 fibroblast,[42,43] our findings, which are contrary to the findings of those studies, are explainable by the fact that the effect of ROCK signaling is cell-type dependent.[28,44] Our results provide the first evidence that ROCK is negatively involved in the cell proliferation pathway via PI 3-kinase signaling, at least in corneal endothelium.

The proven safety of Fasudil suggests that ROCK is a genuine and significant drug target.[28] In addition, several pharmaceutical companies have been developing ROCK inhibitors as therapeutic agents for various kinds of diseases, such as cardiovascular disease, cancer, and neurodegenerative disease.[28] We recently performed the first case series in a clinical trial involving eight patients treated with a topical instillation of Y-27632 eye drops, and the findings revealed that it is effective for treating corneal endothelial dysfunction patients with focal edema.[12,13] However, one disadvantage related to developing ROCK inhibitors in eye-drop form is the poor stability of the inhibitor in solution.[18] On the other hand,

Y-39983 exhibits stability in solution and was developed as a more potent inhibitor of ROCK activity. The IC_{50} of Y-39983 and Y-27632 for ROCK are 0.0036 μM and 0.11 μM, respectively, suggesting that the inhibition of ROCK by Y-39983 was 30 times greater than that obtained by Y-27632.[18] Coincidentally, our current findings also show that 0.3 μM of Y-39983 exhibits a proliferative potential on MCECs equal to 10 μM of Y-27632. Because Y-39983 has the same specificity for ROCK as Y-27632, the ratio of IC_{50} for inhibition of ROCK/protein kinase C for Y-39983 was 117, whereas that for Y-27632 was 82,[18] thus indicating that Y-39983 is a potential candidate for treating corneal endothelial dysfunction due to its high potency and a low off-target effect. In fact, Y-39983 has reportedly been developed as an eye drop for the treatment of glaucoma, thus validating it pharmacologically.[17,18] Although sporadic punctate subconjunctival hemorrhage in vascular endothelial cells was observed in a toxicological study, no serious side effects were exhibited in ocular tissues.[18] The finding that Y-39983 eye drops in the concentration of 0.003% to 0.03% showed proliferative ability on corneal endothelium, and that those concentrations are lower than that for glaucoma eye drops, suggests that it is possible to develop Y-39983 as an eye drop that exhibits no severe side effects, although the concentration requires optimization via pharmacokinetic experiments.

In summary, our data demonstrated that ROCK inhibitors employ both cyclin D (positive G_1 regulator) and p27 (negative G_1 regulator) via PI 3-kinase signaling to promote the proliferation of CECs. Furthermore, Y-39983 may be a better pharmacological agent than Y-27632 for facilitating corneal endothelium wound healing due to its effectiveness at lower concentrations, and those findings encourage a further development of ROCK inhibitor eye drops as a novel therapy for corneal endothelial dysfunction.

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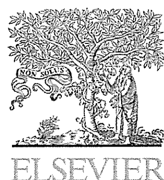

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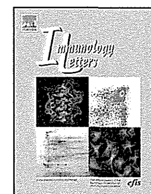

Suppression of polyI:C-inducible gene expression by EP3 in murine conjunctival epithelium

Keywords:
Prostaglandin E receptor 3 (EP3)
Toll-like receptor 3 (TLR3)
GeneChip
Conjunctival epithelium

To the Editor,

We previously reported that EP3, a subtype of prostaglandin E_2 receptors (EP1-EP4), negatively regulates eosinophilic infiltration in murine experimental allergic conjunctivitis (EAC) induced by TLR3, which causes reduced eosinophilic conjunctival inflammation in TLR3/EP3 double knock-out (DKO) mice although in EP3-KO mice eosinophilic conjunctival inflammation is pronounced [1]. We also documented that in human conjunctival epithelial cells, the EP3 agonist suppressed the production of cytokines such as CXCL10, CXCL11, IL6, CCL5, TSLP, and MCP-1 induced by polyI:C, a TLR3 ligand [2]. EP3 was dominantly expressed in conjunctival epithelial cells [3], airway epithelial cells [4], and keratinocytes [5].

To examine the effects of EP3 against polyI:C-inducible gene expression in conjunctival epithelium we performed gene expression analysis of the polyI:C-stimulated conjunctival epithelium in wild-type, EP3-KO-, and EP3/TLR3 DKO mice.

Balb/c mice were purchased from CLEA (Tokyo, Japan). EP3/TLR3 DKO mice were produced by interbreeding EP3-KO- and TLR3-KO mice at Kyoto Prefectural University of Medicine [1]. All experimental procedures were approved by the Committee on Animal Research of Kyoto Prefectural University of Medicine, Kyoto, Japan.

For the *in vivo* analysis of murine conjunctival epithelial cells we prepared a 100 μg/ml polyI:C solution in 50% VISCOAT® (Alcon Laboratories Ltd, Fort Worth, TX)/PBS [6]. The polyI:C solution (each about10 μl) was injected subconjunctivally and dropped into the eyes as described elsewhere [6]. At 6 h after the injection, murine conjunctival tissues were resected and then murine conjunctival epithelium were detached and collected (Supplemental methods). Collected murine conjunctival epithelium almost consisted of epithelial cells (Supplemental Fig. 1). Quantitative RT-PCR was on an ABI-prism 7000 instrument (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The primers for the murine samples are shown in Supplemental Table 1. Microarray analysis was with Affymetrix GeneChip® mouse gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA). Throughout the process we followed Affymetrix instructions (Supplemental Methods).

Using GeneChip® we first examined the comprehensive effects of gene expression in polyI:C-stimulated conjunctival epithelium of wild-type mice. We found that after 6-h stimulation, 31 transcripts were up-regulated more than 10-fold (Supplemental Table 2). Quantitative RT-PCR confirmed that 21 of the 31 transcripts (Cxcl10, Rsad2, Ifi205, Mx1, Cmpk2, Iigp1, Mx2, Iigp2, Ifit3, Gbp5, Cxcl11, H28, Slfn8, Plscr2, Slfn4, Usp18, Sectm1a, Oas2, Dhx58, Ccl5, Isg15) were significantly (>3-fold) up-regulated. Next, to identify the transcripts regulated by EP3 we compared the gene expression of these 21 transcripts in polyI:C stimulated conjunctival epithelium of wild-type and EP3-KO mice by quantitative RT-PCR. We found that all 21 transcripts were expressed significantly stronger in polyI:C stimulated conjunctival epithelium of EP3-KO mice (Fig. 1A). We also confirmed that the mRNA expression of these 21 transcripts was significantly reduced in polyI:C stimulated conjunctival epithelium of EP3/TLR3 DKO- compared to EP3-KO mice (Fig. 1A). *Ptger3* was almost undetectable in EP3-KO and EP3/TLR3-DKO mice as was TLR3 in EP3/TLR3-DKO mice (Fig. 1B).

GeneChip® analysis also showed that the number of 4 transcripts was more than 5 times greater in polyI:C stimulated conjunctival epithelium of EP3-KO- than wild-type mice although in wild-type mice these 4 transcripts were not significantly up-regulated after 6-h polyI:C stimulation (data not shown). Quantitative RT-PCR confirmed that the number of 2 of the 4 transcripts (Oas1g and Oas1a) was more than 100-fold higher in polyI:C stimulated EP3 KO- than wild-type mice (Fig. 1C).

We found that EP3 suppresses polyI:C-inducible genes in murine polyI:C stimulated conjunctival epithelium.

Of the 21 transcripts down-regulated by EP3, 13 (Cxcl10, Rsad2, Ifi205, Mx1, Iigp1, Mx2, Iigp2, Ifit3, Cxcl11, H28, Usp18, Oas2, and Isg15) are IFN-inducible genes. Our observations on EP3-KO mice suggest that Oas1g and Oas1a are markedly suppressed by EP3; they also are IFN-inducible genes and we posit that EP3 regulates the IFN-related response. It is of interest that there was no significant difference between wild-type and EP3/TLR3-DKO mice with respect to many of the 21 transcripts that were significantly up-regulated in EP3-KO mice. This suggests that polyI:C-inducible genes are regulated not only by TLR3 but also by other molecules

Abbreviations: EP3, prostaglandin E receptor 3; TLR3, toll-like receptor 3; EAC, experimental allergic conjunctivitis; DKO, double knock-out; TSLP, thymic stromal lymphopoietin; MCP-1, monocyte chemoattractant protein-1; polyI:C, polyinosinic:polycytidylic acid; Cxcl10, chemokine (C-X-C motif) ligand 10; Rsad2, radical S-adenosyl methionine domain containing 2; Ifi205, interferon activated gene 205; Mx1, myxovirus (influenza virus) resistance 1; Cmpk2, cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial; Iigp1, interferon inducible GTPase 1; Mx2, myxovirus (influenza virus) resistance 2; Iigp2, interferon inducible GTPase 2; Ifit3, interferon-induced protein with tetratricopeptide repeats 3; Gbp5, guanylate binding protein 5; Cxcl11, chemokine (C-X-C motif) ligand 11; H28, histocompatibility 28; Slfn8, schlafen 8; Plscr2, phospholipid scramblase 2; Slfn4, schlafen 4; Usp18, ubiquitin specific peptidase 18; Sectm1a, secreted and transmembrane 1A; Oas2, 2′-5′ oligoadenylatesynthetase 2; Dhx58, DEXH (Asp-Glu-X-His) box polypeptide 58; Ccl5, chemokine (C-C motif) ligand 5; Isg15, ISG15 ubiquitin-like modifier; Oas1g, 2′-5′ oligoadenylatesynthetase 1G; Oas1a, 2′-5′ oligoadenylatesynthetase 1A.

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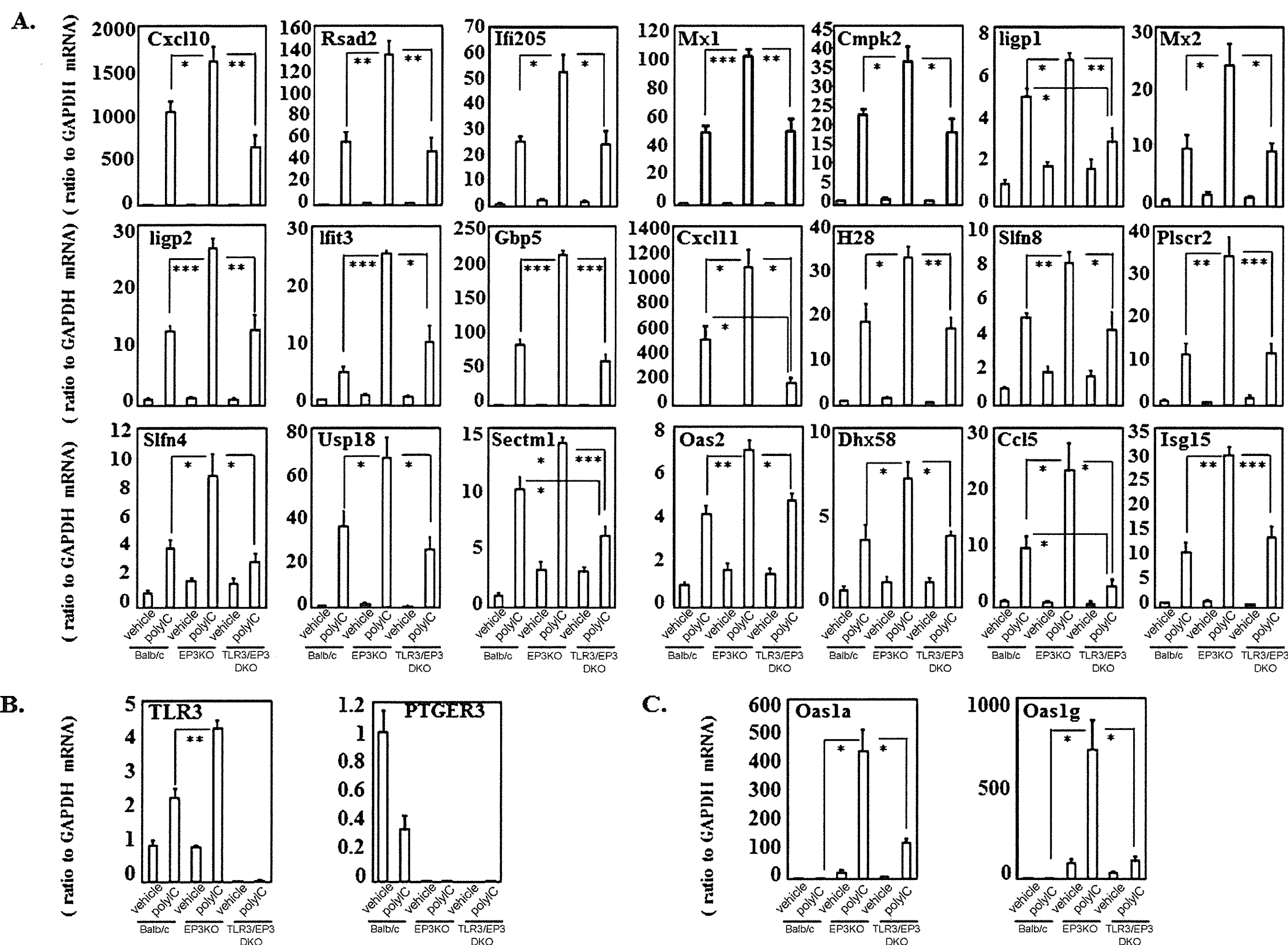

Fig. 1. Expression of transcripts induced by the polyI:C stimulation of conjunctival epithelium of wild-type-, EP3-KO-, and EP3/TLR3-KO mice. Quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y*-axis shows the increase in specific mRNA over unstimulated samples from wild-type mice. Data are representative of 3 separate experiments and show the mean ± SEM from one experiment carried out in 4 mice per group (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.005$).

such as MDA5 and RIG-I. We now know that EP3 suppresses polyI:C-inducible genes in polyI:C, a TLR3 ligand, stimulated conjunctival epithelium.

EP3 negatively regulates the eosinophilic infiltration of TLR3-induced murine EAC [1] and, EP3 and TLR3 were dominantly expressed in conjunctival epithelial cells [3,7]. In conjunctival epithelium EP3 suppresses polyI:C, a TLR3 ligand, inducible genes, suggesting that the conjunctival epithelium plays a critical role in the regulation of allergic conjunctivitis. Okuma et al. [8] recently reported that dysfunction of epithelial cells by the disruption of IκBζ induction elicits ocular surface inflammation via the activation of self-reactive lymphocytes, indicating that epithelial cells have an important role in the regulation of inflammation.

Elsewhere [1,8,9] we suggested that the pathogenesis of ocular surface inflammation such as Stevens–Johnson syndrome with severe ocular surface complications is associated with anomalies in innate immune reactions, especially reactions that involve epistatic interactions between TLR3 and EP3. We think that a lack of balance between TLR3 and EP3 is involved in triggering ocular surface inflammation [9].

In summary, we found that EP3 suppressed polyI:C, a TLR3 ligand, inducible genes in polyI:C stimulated murine conjunctival epithelium. Our findings suggest that EP3 and TLR3 in conjunctival epithelium play a critical role in regulating ocular surface inflammation.

Contributors

Material contributions to the research: Mayumi Ueta, Katsura Mizushima, Yuji Naito, Shuh Narumiya, Katsuhiko Shinomiya, Shigeru Kinoshita.

Writing and review contributions to the manuscript: Mayumi Ueta.

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Financial relationship disclosure

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2013.08.010.

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Author Response: Human Corneal Endothelium Regeneration: Effect of ROCK Inhibitor

We read with interest the Letter to the Editor from Galvis et al.[1] in regard to our recent published article entitled "The ROCK inhibitor eye drop accelerates corneal endothelium wound healing."[2] We greatly appreciate their interest in our study, and wish to respond with some additional information for clarification on various points.

It should be noted that the primary aim of our article was to report the effect of ROCK-inhibitor eye drops to promote corneal endothelial wound healing by stimulating the in vivo proliferation of corneal endothelial cells in a monkey model, followed by a limited clinical study to confirm the safety of ROCK-inhibitor eyedrop treatment with transcorneal freezing. Based on these animal and human study data, we aimed to illustrate the possibility of a pharmacologic treatment for certain types of corneal endothelial dysfunctions, for example, the early phase of Fuchs' corneal dystrophy, via the use of ROCK-inhibitor eyedrops.

Based on our reports, and those from other researchers, showing the existence of corneal endothelial stem/precursors with higher proliferative ability in the peripheral area of the cornea, it should be noted that there is the possibility that the reestablishment of each patient's endothelium was not solely a direct result of the ROCK-inhibitor administration, but could have been the result of denudation of the pathologic endothelial cells. At present, it is too early to make a definitive statement regarding the therapeutic effect of ROCK-inhibitor eyedrops for the patients' corneal endothelial dysfunctions because the exact mechanism by which ROCK-inhibitor accelerates the proliferation of human corneal endothelial cells (HCECs) has yet to be elucidated and the individual response to the ROCK-inhibitor varies greatly per patient in such a small cohort as reported in our article.

In regard to the central corneal thickness (CCT) measurements of our patients, we consistently used anterior segment optical coherence tomography (AS-OCT), which is used widely in the clinical setting. In our study, CCT measurements were obtained by AS-OCT before and after the ROCK-inhibitor treatment in all patients, thus minimizing intraindividual or operator-dependent variability. Of the 4 cases of Fuchs' corneal dystrophy, 1 case (case 2) did not show a remarkable clinical effect of ROCK-inhibitor treatment at 3 months, and that case was excluded from postoperative evaluation of CCT at 6 months due to the fact that the patient underwent Descemet's stripping automated endothelial keratoplasty (DSAEK) 4 months after treatment. In 1 case of laser iridotomy-induced bullous keratopathy (case 5), the patient's corneal edema became less severe in accordance with the CCT measurement; however, examination by specular microscopy could not be performed due to the residual corneal edema, and best-corrected visual acuity failed to improve due to the severe nuclear cataract. Since corneal endothelial damage was quite severe in all of the 8 cases referred to our university hospital for DSAEK surgery, it was impossible to obtain a pretreatment endothelial cell count in most of those patients, especially in the diffuse edema group. We reviewed the specular microscopy images of 4 patients with central edema as reference data, yet unfortunately, and except for the images of case 1, those images were not of suitable quality for publication. More detailed clinical data of case 1 has been published recently in another journal as a case report,[3] which had been accepted for publication before the publication of this present article (this matter was declared at the time of the submission of our article to the present Journal). In that case report, we showed a panoramic image of corneal endothelial cells taken by wide-field contact specular microscopy. We observed the presence of a high density of smaller cells in the central cornea from which endothelial cells had been removed before ROCK-inhibitor administration compared to the peripheral area. Though that finding is indirect evidence, it may suggest that the in vivo proliferation of corneal endothelial cells was stimulated by the ROCK inhibitor. We have not examined karyotype change in corneal endothelium after ROCK-inhibitor treatment. Though it might be useful for confirming the safety of this new concept of therapy, in reality it is impossible to perform due to the large number of mitotic cells that would need to be obtained from the patients' eyes.

In our recent published article, 10 mM of ROCK-inhibitor eyedrops were administered for only 1 week, as a longer period of administration was cost prohibitive and 7 days was all that was needed to investigate the safety of using the drops. A longer period of administration is expected to be more effective, and our current ongoing clinical study of ROCK-inhibitor eye drops for post-DSAEK patients was designed to administer 1 mM of ROCK-inhibitor Y-27632 for 6 months. In addition, based on our series of fundamental research pertaining to the use of ROCK-inhibitor,[4] we currently are conducting a drug library screening in an attempt to elucidate other low molecular weight chemical compounds useful for the treatment of corneal endothelial diseases.

Though several groups have reported methods for the cultivation of HCECs, it still is quite difficult to prevent the fibroblastic change of HCECs in culture and to obtain consistently a successful HCEC culture with endothelial phenotypes that are morphologically correct and functional. We recently established a successful protocol for the cultivation of HCECs by using an inhibitor of TGF-β[5] with human mesenchymal stem cell conditioned medium.[6] It should be noted that the cellular response of cultivated HCECs to ROCK inhibitor is influenced greatly by the culture conditions, and that further investigation is needed to elucidate the mechanism by which ROCK inhibitor promotes the proliferation of corneal endothelial cells.

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