

FIGURE 2. Involvement of ROCK-inhibitor Y-27632 on G₁/S progression. MCECs were serum starved for 24 hours prior to the treatment of cells with growth medium containing 10 μ M 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 μ M 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 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 G₁/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 μ M 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 G₁ 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 G₁/S progression. In the absence of Y-27632, 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

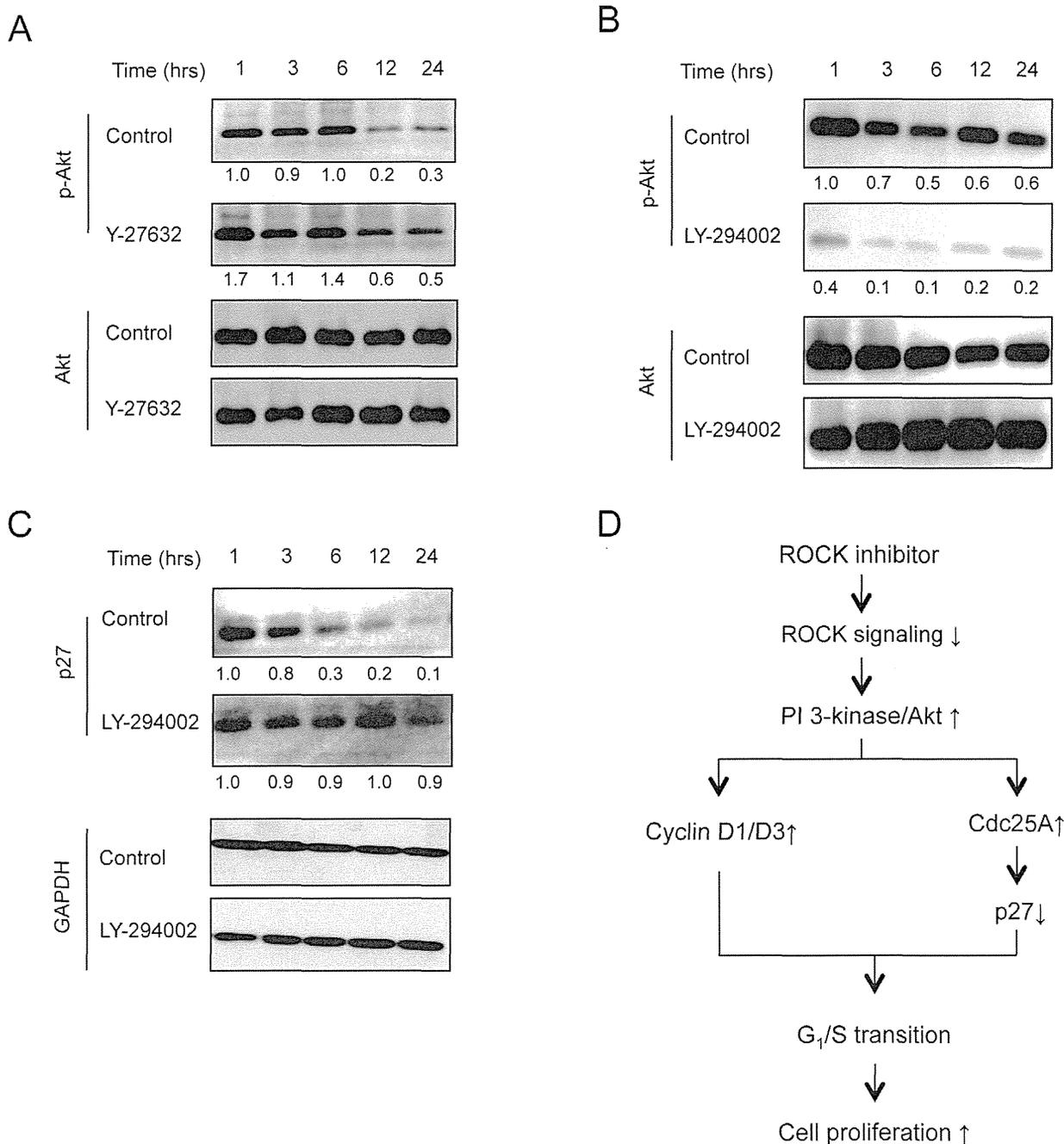


FIGURE 3. Involvement of PI 3-kinase signaling in Y27632-mediated p27 degradation and upregulation of cyclin D. (A–C) Serum-starved MCECs were treated with or without Y27632 for 1, 3, 6, 12, or 24 hours. Phosphorylation of Akt, total Akt, and p27 was evaluated by Western blotting. The phosphorylation of Akt was sustained 1.7-fold higher in the Y27632-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 G₁/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 Y27632 for 1, 3, 6, 12, or 24 hours. Y27632 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 Y27632-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 G₁ phase of the cell cycle allow for G₁/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

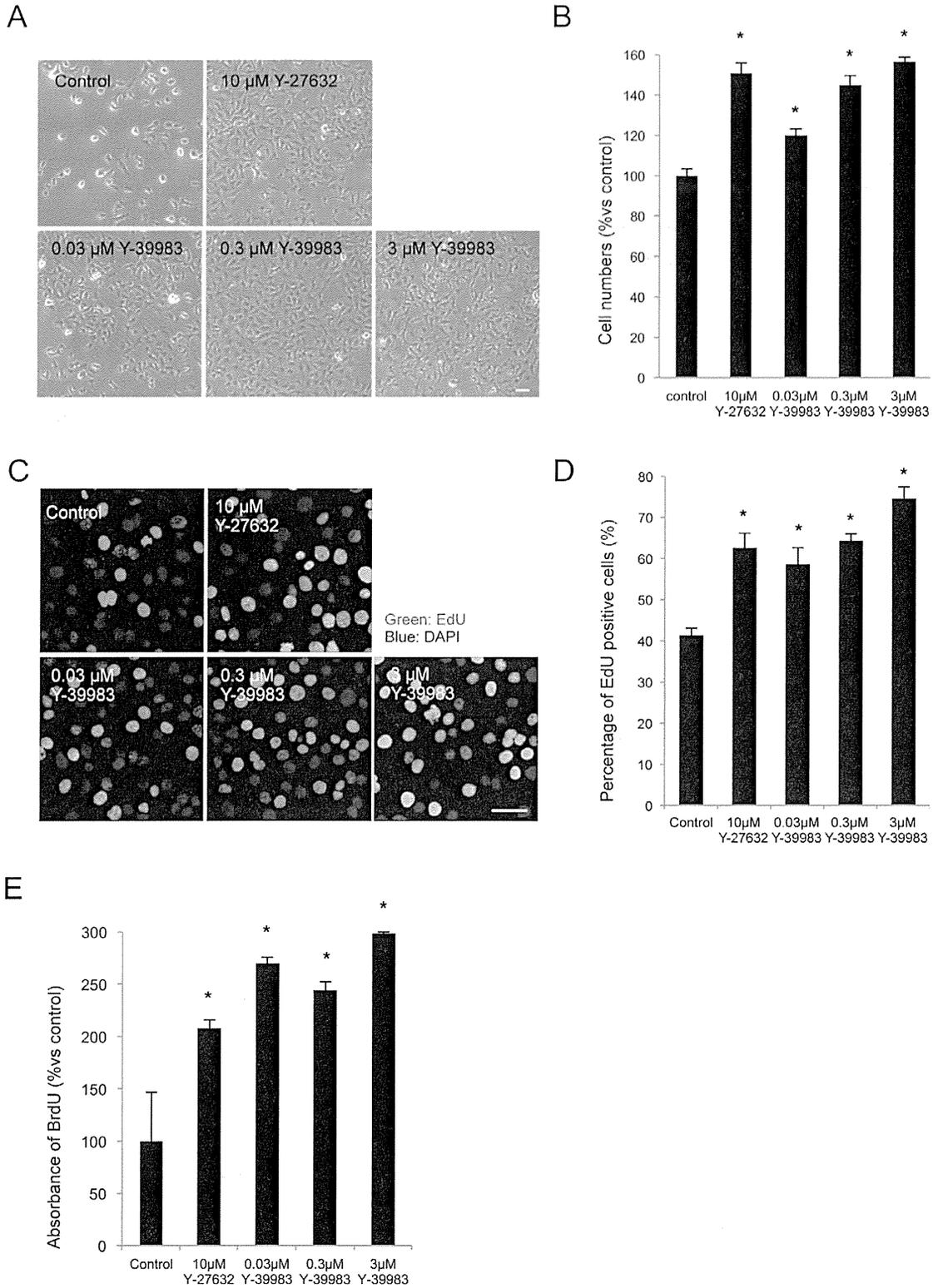


FIGURE 4. Effect of ROCK-inhibitor Y39983 on the proliferation of MCECs. (A, B) MCECs were seeded at a density of 5.0×10^3 cells/cm² for 24 hours and then incubated with serum starvation for an additional 24 hours in the presence or absence of Y39983. The MCECs were inspected by phase-contrast microscopy. The numbers of MCECs increased from 1.2- to 1.5-fold following stimulation with Y39983 for 24 hours. Five samples were prepared for each group, and the experiments were performed in duplicate. *Scale bar:* 200 μ m. (C, D) MCECs seeded at a density of 5.0×10^4 cells/cm² were maintained for 24 hours, followed by serum starvation for an additional 24 hours in the presence or absence of Y39983. 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 Y39983 (0.03–3.00 μ M). *Scale bar:* 200 μ m. (E) The effect of Y39983 on the proliferation of MCECs was evaluated by BrdU incorporation assay. BrdU incorporation was enhanced 240% to 300% by Y39983 (0.03–3.00 μ M), while it was enhanced 200% by 10 μ M Y27632. **P* < 0.01. All experiments were performed in duplicate.

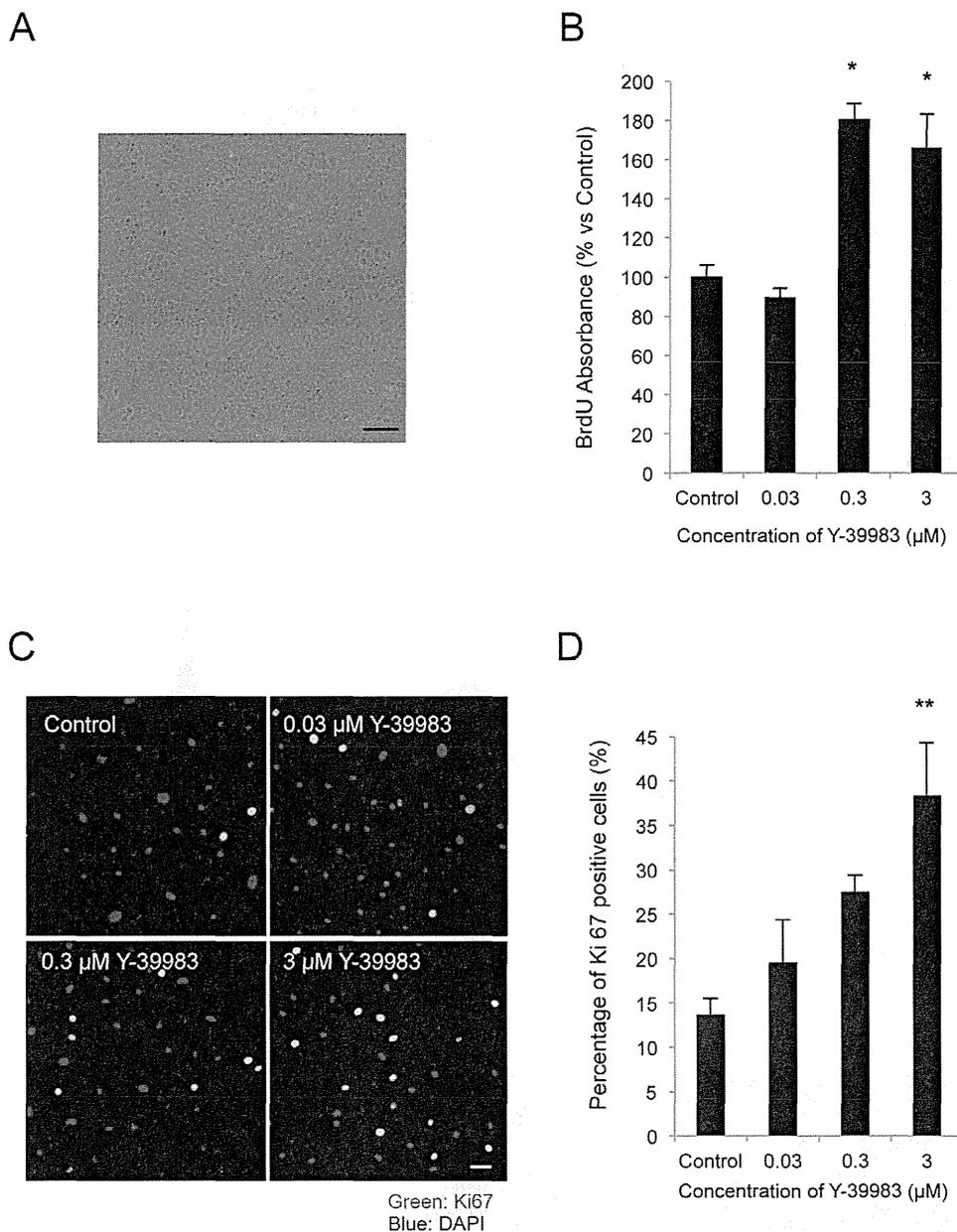


FIGURE 5. Effect of Y-39983 on the proliferation of HCECs. (A) Representative phase-contrast image of cultured HCECs. Scale bar: 50 μm. (B) HCECs were cultured, and the effect of Y-39983 on the proliferation of HCECs was evaluated by BrdU incorporation assay. BrdU incorporation into the newly synthesized 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 (IC₅₀) 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 concentration³⁰ 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

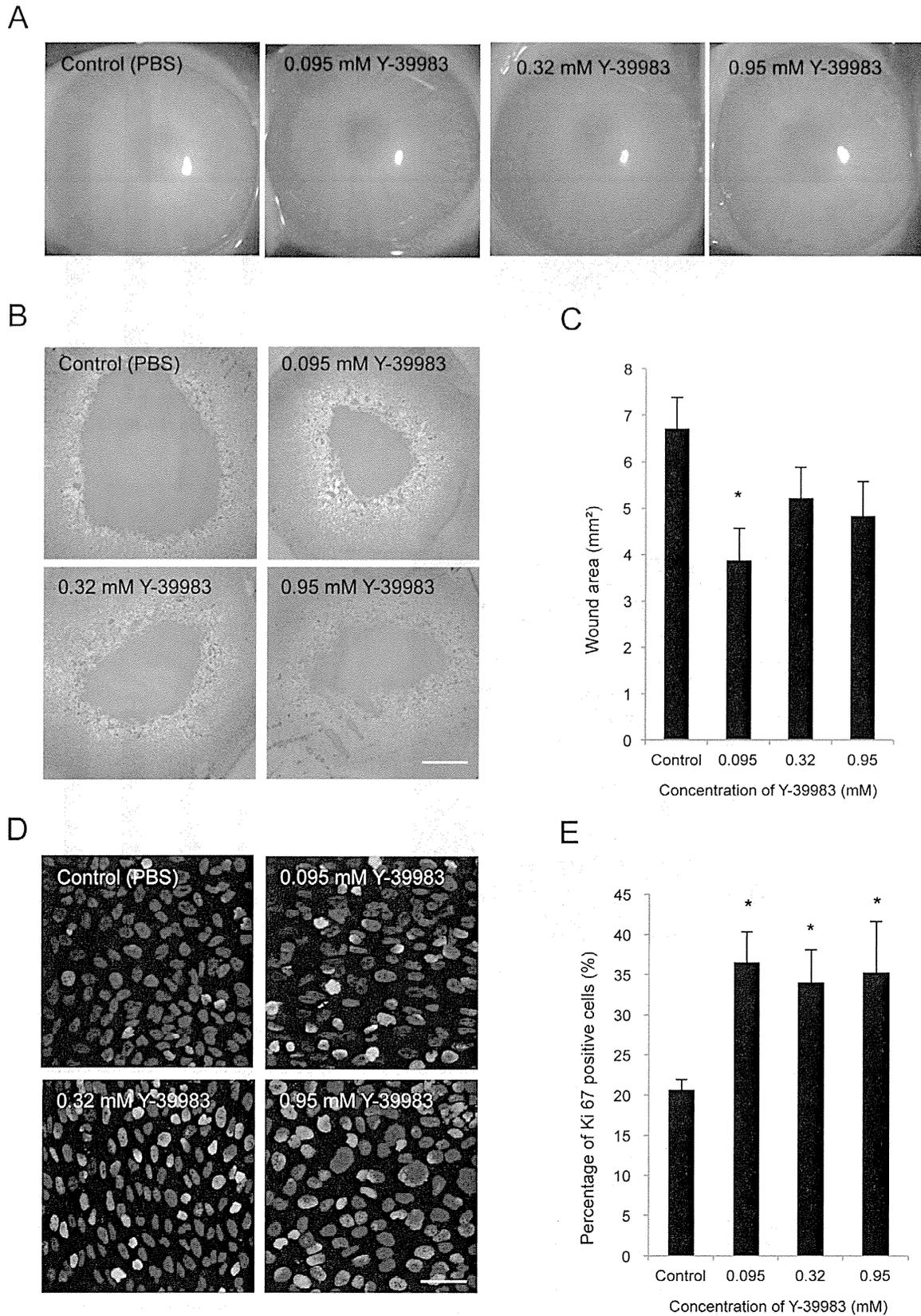


FIGURE 6. The effect of Y39983 eye-drop instillation on wound healing and cell proliferation in an in vivo rabbit model. **(A)** The corneal endothelium of 27 Japanese white rabbits was damaged by transcorneal freezing. Then, 0.095, 0.32, or 0.95 mM of Y-39983 was topically instilled in one eye of each 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 μ m. **(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 Y39983-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, Y39983 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 Y39983 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 Y39983. There was a tendency for the Y39983-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 Y39983 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 Y39983 and 37% in the corneas treated with 0.95 mM Y39983 (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 Y39983 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 G₁ phase of the cell cycle throughout their life span,³⁶ and regulation of cell-

cycle G₁/S progression plays a central role in cell proliferation. Mitogenic stimulation induces entry into the G₁ phase, which prepares cells for DNA duplication in the S phase. Progression of cells from G₁ to S phase is highly regulated, and numerous proteins function as positive or negative regulators. One of the early G₁-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 G₁ and cyclin E/Cdk2 in late G₁ 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 G₁ phase of the cell cycle. In both HCECs and nonhuman CECs, the corneal endothelium reportedly employs phosphorylation of p27 as the major mechanism for G₁/S progression.^{26,36,38-40} Furthermore, this removal mechanism of p27 is mediated by PI 3-kinase signaling.²⁴⁻²⁶ The findings of Joyce⁴¹ 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 Y27632 and Y39983 stimulate the proliferation of both MCECs and HCECs. Our findings demonstrated that Y27632 activates PI 3-kinase signaling, which subsequently regulates the following two respective pathways necessary for G₁/S progression: upregulation of cyclin D, and downregulation of p27. Moreover, our findings that Y27632 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 G₁/S progression. Although earlier studies have shown that inactivation of Rho by C3 blocks G₁/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 Y27632 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 IC₅₀ 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 IC₅₀ 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 G₁ regulator) and p27 (negative G₁ 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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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 E₂ 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 about 10 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, Slnf8, Plscr2, Slnf4, 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; Slnf8, schlafen 8; Plscr2, phospholipid scramblase 2; Slnf4, 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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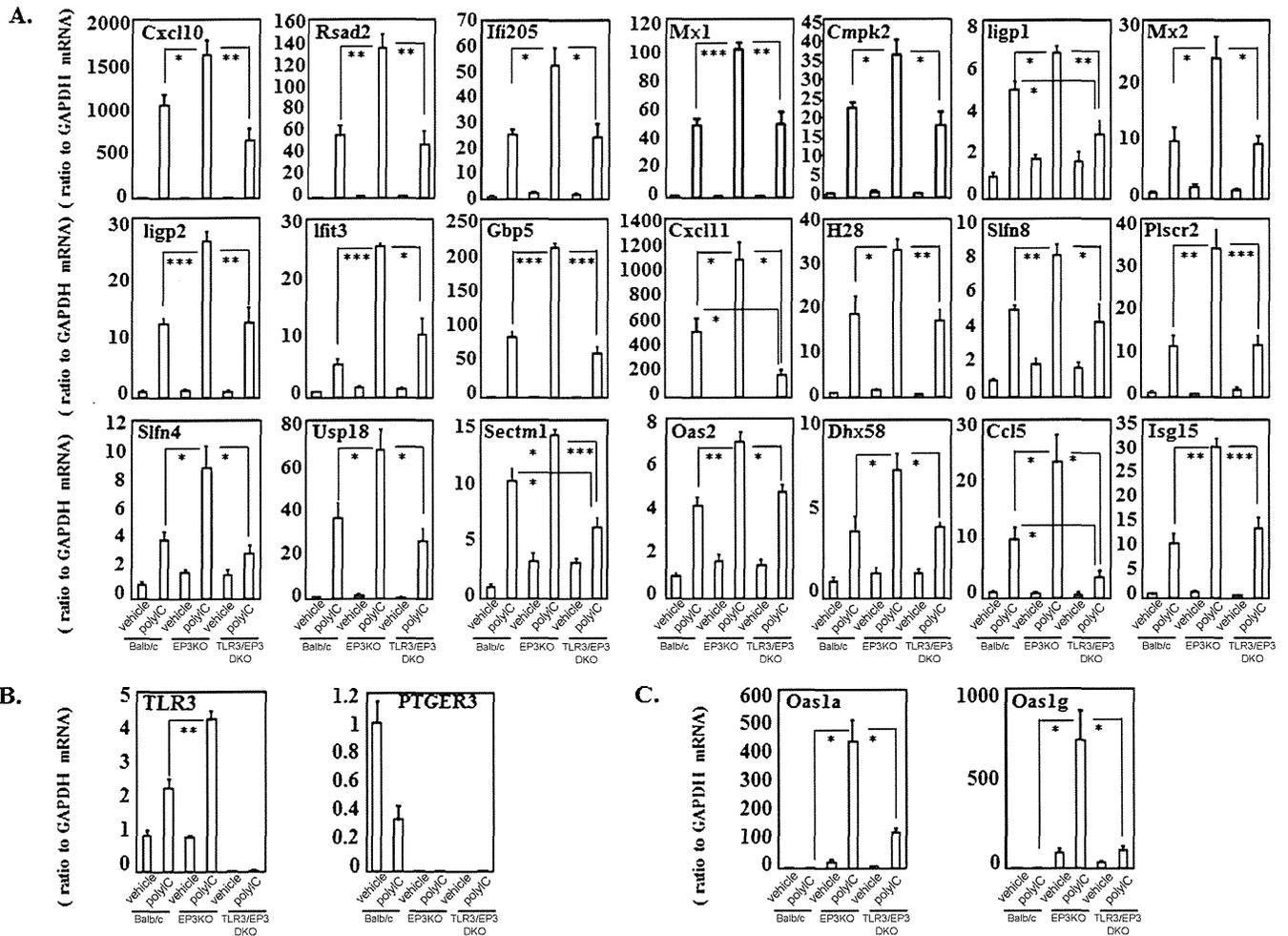


Fig. 1. Expression of transcripts induced by the polyI:C stimulation of conjunctival epithelium of wild-type-, EP3-KO-, and EP3/TLR3-KO mice. Quantification data were normalized to the expression of the housekeeping gene GAPDH. The Y-axis shows the increase in specific mRNA over unstimulated samples from wild-type mice. Data are representative of 3 separate experiments and show the mean \pm SEM from one experiment carried out in 4 mice per group (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

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 $\kappa B\zeta$ 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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Mayumi Ueta^{a,b,*}

^a Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

^b Research Center for Inflammation and Regenerative Medicine, Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Katsura Mizushima
Yuji Naito

Department of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto, Japan

Shuh Narumiya
Department of Pharmacology and Faculty of Medicine, Kyoto University, Kyoto, Japan

Katsuhiko Shinomiya
Shigeru Kinoshita
Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan

* Corresponding author at: Department of Ophthalmology, Kyoto Prefectural University of Medicine, Hirokoji, Kawaramachi, Kamigyo-ku, Kyoto 602-0841, Japan. Tel.: +81 75 251 5578; fax: +81 75 251 5663.

E-mail address: mueta@koto.kpu-m.ac.jp (M. Ueta)

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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.

Noriko Koizumi^{1,2}
Naoki Okumura^{1,2}
Shigeru Kinoshita²

¹Department of Biomedical Engineering, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan; and
²Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

E-mail: nkoizumi@mail.doshisha.ac.jp

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Corneal Endothelial Expansion Promoted by Human Bone Marrow Mesenchymal Stem Cell-Derived Conditioned Medium

Makiko Nakahara¹, Naoki Okumura^{1,2}, EunDuck P. Kay¹, Michio Hagiya^{2,3}, Kiwamu Imagawa³, Yuuki Hosoda^{2,3}, Shigeru Kinoshita², Noriko Koizumi^{1*}

1 Department of Biomedical Engineering, Faculty of Life and Medical Sciences, Doshisha University, Kyotanabe, Japan, **2** Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan, **3** Research Division, JCR Pharmaceuticals Co., Ltd., Kobe, Japan

Abstract

Healthy corneal endothelium is essential for maintaining corneal clarity, as the damage of corneal endothelial cells and loss of cell count causes severe visual impairment. Corneal transplantation is currently the only therapy for severe corneal disorders. The greatly limited proliferative ability of human corneal endothelial cells (HCECs), even *in vitro*, has challenged researchers to establish efficient techniques for the cultivating HCECs, a pivotal issue for clinical applications. The aim of this study was to evaluate conditioned medium (CM) obtained from human bone marrow-derived mesenchymal stem cells (MSCs) (MSC-CM) for use as a consistent expansion protocol of HCECs. When HCECs were maintained in the presence of MSC-CM, cell morphology assumed a hexagonal shape similar to corneal endothelial cells *in vivo*, as opposed to the irregular cell shape observed in control cultures in the absence of MSC-CM. They also maintained the functional protein phenotypes; ZO-1 and Na⁺/K⁺-ATPase were localized at the intercellular adherent junctions and pump proteins of corneal endothelium were accordingly expressed. In comparison to the proliferative potential observed in the control cultures, HCECs maintained in MSC-CM were found to have more than twice as many Ki67-positive cells and a greatly increased incorporation of BrdU into DNA. MSC-CM further facilitated the cell migration of HCECs. Lastly, the mechanism of cell proliferation mediated by MSC-CM was investigated, and phosphorylation of Akt and ERK1/2 was observed in HCECs after exposure to MSC-CM. The inhibitor to PI 3-kinase maintained the level of p27^{Kip1} for up to 24 hours and greatly blocked the expression of cyclin D1 and D3 during the early G1 phase, leading to the reduction of cell density. These findings indicate that MSC-CM not only stimulates the proliferation of HCECs by regulating the G1 proteins of the cell cycle but also maintains the characteristic differentiated phenotypes necessary for the endothelial functions.

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Competing Interests: Please note that Makiko Nakahara, Naoki Okumura, Shigeru Kinoshita, and Noriko Koizumi have financial competing interests (patents currently pending, “culture medium including human bone marrow mesenchymal stem cell-derived conditioned medium for corneal endothelial expansion” patent application in Japan; 2012-196725, application date; September 7, 2012). EunDuck P. Kay has no conflicts of interest. Michio Hagiya, Kiwamu Imagawa, and Yuuki Hosoda are paid employees of JCR Pharmaceuticals Co., Ltd., Ashiya, Japan. Please also note that this statement does not alter the authors’ adherence to all of the PLOS ONE policies on sharing data and materials.

* E-mail: nkoizumi@mail.doshisha.ac.jp

Introduction

Human corneal endothelium is known to play a critical role in maintaining corneal transparency by regulating corneal hydration [1]. The proliferative ability of human corneal endothelial cells (HCECs) is severely limited *in vivo* [2], therefore, cell loss due to the aging process or pathological conditions causes a concurrent compensatory migration of the existing cells and the enlargement of those cells to achieve a contact-inhibited monolayer. Maintenance of such a phenotype is necessary for functional integrity and corneal deturgescence [3], [4]. If the density of corneal endothelial cells (CECs) is below 500 cells/mm², Na⁺/K⁺-ATPase pump and barrier functions are not compensated by residual CECs. The failure of endothelial functions is known to subsequently cause stromal and epithelial edema, as well as loss of corneal clarity and visual acuity. In addition, irreversible corneal haziness is often

observed in corneal endothelial disorders such as Fuchs’ corneal dystrophy, pseudophakic bullous keratopathy, or trauma-related injuries [5], [6].

The treatment of severe corneal disorders requires either full-thickness corneal transplantation or endothelial keratoplasty to restore clear vision. Recently, highly effective surgical techniques for the treatment of corneal disorders, i.e., Descemet’s stripping automated endothelial keratoplasty (DSAEK) and Descemet’s membrane endothelial keratoplasty (DMEK), have been developed, with these procedures being aimed at replacing penetrating keratoplasty [7–10]. However, the worldwide shortage of transplantable donor corneas, the continual cell damage after transplantation, and primary graft failure are issues that remain to be resolved [11], [12]. To overcome these problems, our group, as well as several other groups, have explored new treatment

protocols for corneal endothelial dysfunctions through the use of tissue engineering techniques [13–17]. Among researchers worldwide, the common goal is to establish optimum experimental protocols for the *in vitro* expansion of HCECs for clinical application.

HCECs are arrested at the G1 phase of the cell cycle [2], [18], and this characteristic property of HCECs indicates that HCECs have the potential to proliferate in response to growth stimulation factors. Recently, we reported that Y-27632, a specific inhibitor of the Rho-associated coiled-coil forming kinases (ROCKs), promoted the adhesion and proliferation of monkey CECs [17], [19]. It has also been reported that FGF-2 stimulates the cell proliferation of HCECs through degradation of p27^{Kip1} (p27) [20]. The findings from these studies confirm that the proliferative potential of HCECs can be resumed and that such cells can be cultivated for clinical transplantation into the eye, thus replacing the endothelial keratoplasty. Although a variety of methods to expand HCECs *in vitro* have been explored, protocols for the expansion of HCECs for tissue engineering therapy have yet to be established [21], [22]. We recently reported that the use of conditioned medium (CM) obtained from NIH-3T3 (NIH-3T3-CM) resulted in efficient cultures of HCECs [23]. However, the use of NIH-3T3-CM faces the obstacle that CM derived from mouse cells contains a xenoantigen for human cells [24], [25]. To overcome this difficult obstacle, CM obtained from human bone marrow (BM)-derived mesenchymal stem cells (MCSs) (MSC-CM) was investigated in this present study, as BM-derived stem cells reportedly promote tissue repair by the secretion of cytokines and growth factors that enhance regeneration of injured cells, thus stimulating the proliferation and differentiation of endogenous stem-like progenitors found in most tissues [26–28].

In the present study, we provide evidence suggesting that CM obtained from BM-MSCs stimulates cell proliferation and motility of HCECs, while maintaining the contact-inhibited monolayer with functional adherent junctions and pump functions. Our findings show that the proliferative action of MSC-CM is facilitated via the downregulation of p27 and the upregulation of cyclin D through phosphatidylinositol 3-kinase (PI 3-kinase) and extracellular signal-regulated kinase 1/2 (ERK1/2) pathways. These results indicate that MSC-CM provides a feasible means by which to expand proliferative and functional HCECs for use as a subsequent clinical intervention for corneal endothelial dysfunction.

Materials and Methods

Ethics statement

The human tissue used in this study was handled in accordance with the tenets set forth in the Declaration of Helsinki. Informed written consent was obtained from the next of kin of all deceased donors in regard to eye donation for research. Human donor corneas were obtained from SightLife™ (<http://www.sightlife.org/>, Seattle, WA). All tissue was recovered under the tenants of the Uniform Anatomical Gift Act (UAGA) of the particular state in which the donor consent was obtained and the tissue was recovered.

Cell cultures

All human corneas had been stored at 4°C in storage medium (Optisol; Chiron Vision, Irvine, CA) for less than 14 days prior to the use of the associated HCECs in the culture. Donor age ranged from 51 to 68 years. The culture medium was prepared according to published protocols, but with some modifications [23], [29]. The Descemet's membrane/corneal endothelium complex was

stripped and digested with 1 mg/mL collagenase A (Roche Applied Science, Penzberg, Germany) at 37°C for 2 hours, followed by washing with OptiMEM-I (Life Technologies, Carlsbad, CA). HCECs obtained from the individual donor corneas were resuspended in basal growth medium (OptiMEM-I, 8% fetal bovine serum (FBS), 5 ng/mL epidermal growth factor (EGF), 20 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MO), 200 mg/L calcium chloride, 0.08% chondroitin sulfate (Sigma-Aldrich), and 50 µg/mL gentamicin (Life Technologies)) and plated into 2 wells of a 12-well plate coated with FNC Coating Mix® (Athena Environmental Sciences, Inc., Baltimore, MD). The HCECs were maintained in a humidified atmosphere at 37°C in 5% CO₂, and the culture medium was replaced with fresh media every 2 days. When the cells reached confluency in 14 to 28 days, they were rinsed in Ca²⁺ and Mg²⁺-free phosphate buffered saline (PBS), trypsinized with 0.05% Trypsin-EDTA (Life Technologies) for 5 minutes at 37°C, and passaged at a 1:2 ratio. U0126 (10 µM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and LY294002 (10 µM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used to inhibit MEK and PI 3-kinase, respectively.

Preparation of NIH-3T3-CM

Inactivation of the 3T3 fibroblasts was performed as described previously [30], [31]. Briefly, confluent 3T3 fibroblasts were incubated with 4 µg/mL mitomycin C (MMC) (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) for 2 hours, and then seeded onto plastic dishes at a cell density of 2×10^4 cells/cm². Next, the attached cells were washed 3 times with PBS, and the medium was replaced with basal culture medium containing OptiMEM-I, 8% FBS, 5 ng/mL EGF, 20 µg/mL ascorbic acid, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, and 50 µg/mL of gentamicin. The NIH-3T3 was maintained for an additional 24 hours. The medium was collected and centrifuged at 2000 xg for 10 minutes, and the supernatant was filtered through a 0.22-µm filtration unit (EMD Millipore Corporation, Billerica, MA) and used as NIH-3T3-CM.

Preparation of MSC-CM

BM-MSCs were obtained from JCR Pharmaceuticals Co., Ltd. (Kobe, Japan). BM-MSCs passaged 3 times were used for the experiments. The BM-MSCs were plated at a cell density of 1.3×10^4 cells/cm² and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, and were then maintained for 1 day. The attached cells were washed 3 times with PBS, and the medium was replaced with basal growth medium. The BM-MSCs were then maintained for an additional 24 hours. The medium was collected and centrifuged at 2000 xg for 10 minutes, and the supernatant was filtered through a 0.22-µm filtration unit (EMD Millipore Corporation) and used as MSC-CM.

Total RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

HCECs after 5 passages were seeded at a cell density of 1.6×10^4 cells/cm² and maintained for 1 day, and the medium was replaced with either MSC-CM or NIH-3T3-CM. The cultures were maintained for 8 days. Total RNA was isolated by use of the RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The quality of the RNA preparations was measured by use of the NanoDrop® (Thermo Fisher Scientific Inc., Waltham, MA) spectrophotometer. First-strand cDNA was synthesized with 1 µg of total RNA by use of the ReverTra Ace® (Toyobo Corporation, Osaka, Japan) reverse transcriptase kit. The

cDNA samples were subjected to PCR with specific primers as listed in Table 1; genes involved in the transport of the corneal endothelium were analyzed in comparison with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. PCR reactions were then performed with Extaq DNA polymerase (Takara Bio Inc., Otsu, Japan) as follows: denaturation at 94°C for 30 seconds, 33 cycles of annealing at 54°C for 30 seconds, and elongation at 72°C for 30 seconds. The PCR products were separated by electrophoresis on 1.5% agarose gels, followed by ethidium bromide staining and detection under ultraviolet illumination.

Cell proliferation assay

HCECs were cultured at the density of 5000 cells/well in a 96-well plate in the presence or absence of CM derived from NIH-3T3 or BM-MSC. DNA synthesis was detected as incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA by use of the Cell Proliferation Biotrak ELISA system, version 2 (GE Healthcare Life Sciences, Buckinghamshire, UK) according to the manufacturer's instructions. Briefly, HCECs were incubated with 10 μ M BrdU for 24 hours at 37°C and 5% CO₂ in a humidified atmosphere. Cultured cells were incubated with fixation solution for 2 hours and incubated with 100 μ l of monoclonal antibody against BrdU for 30 minutes. The BrdU absorbance was measured directly using a spectrophotometric microplate reader at a test wavelength of 450 nm.

Immunofluorescent staining

Cultured HCECs on a 48-well cell culture plate were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then incubated for 30 minutes with 1% bovine serum albumin (BSA). Immunocytochemical analyses of ZO-1 (Zymed Laboratories, South San Francisco, CA) and Na⁺/K⁺-ATPase (Upstate Biotech, Lake Placid, NY) were respectively performed with a 1:200 dilution of ZO-1 polyclonal antibody and a 1:200 dilution of Na⁺/K⁺-ATPase monoclonal antibody. Either Alexa Fluor[®] 488-conjugated goat anti-mouse (Life Technologies) or Alexa Fluor[®] 594-conjugated goat anti-rabbit IgG (Life Technologies) was used for the secondary antibody with a 1:1000 dilution. Nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA). The cells were then examined by fluorescence microscopy (BZ-9000; Keyence, Osaka, Japan).

Scratch-induced directional migration assay

HCECs were cultured in 6-well plates in basal growth media. When the cells reached confluence, they were maintained in either

control basal growth medium, NIH-3T3-CM, or MSC-CM for an additional 7 days. Scrape-wounding of the cells was performed using a plastic pipette tip. Following scraping, the medium containing detached cells was removed and replaced with basal growth medium, NIH-3T3-CM, or MSC-CM; cells were further maintained for 20 hours until the monolayer was restored. Cell migration and the recovery to a cell monolayer were determined by phase contrast microscopy. The width of the wound area was measured using ImageJ software (U.S. National Institutes of Health, Bethesda, MD), and three fields from each well were measured.

Western blot analysis

The HCECs were washed with ice-cold PBS and then lysed with ice-cold RIPA buffer (Bio-Rad Laboratories, Hercules, CA) containing Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich) and Protease Inhibitor Cocktail (Nacalai Tesque, Kyoto, Japan). The lysates were centrifuged at 15,000 rpm for 10 minutes at 4°C to sediment 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). An equal amount of protein was fractionated by SDS-PAGE; proteins were transferred to PVDF membranes. The membranes were then blocked with 3% non-fat dry milk (Cell Signaling Technology, Inc., Danvers, MA) in TBS-T buffer (50 mM Tris, pH 7.5, 150 mM NaCl₂, and 0.1% Tween20) for 1 hour at room temperature, followed by overnight incubation at 4°C with the following primary antibodies: Na⁺/K⁺-ATPase (1:1000; Merck Millipore), ZO-1 (1:1000; Zymed Laboratories), GAPDH (1:3000; Abcam, Cambridge, UK), Akt1 (1:2000; Cell Signaling Technology), phosphorylated Akt (1:2000; Cell Signaling Technology), ERK1/2 (BD, Franklin Lakes, NJ), and phosphorylated ERK1/2 (BD). The blots were washed, and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000: anti-rabbit IgG, anti-mouse IgG; Cell Signaling Technology). The blots were then developed with luminal for enhanced chemiluminescence (ECL) using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Piscataway, NJ), documented by LAS4000S (Fuji Film, Tokyo, Japan), and analyzed with Image Gauge (Fuji Film).

Concentration of MSC-CM

Supernatants of MSC cultured in OptiMEM-I supplemented with gentamicin were collected after 24 hours. After centrifugation at 1000 rpm for 10 minutes to remove cell debris, cell-free supernatant was concentrated 17-fold by centrifugation at 2500 xg

Table 1. Oligonucleotide sequences for RT-PCR.

Gene	Sense primer	Anti-sense primer	Size (bp)
<i>keratin 12</i>	5'-GGCCTACATGAAGAAGAACAC-3'	5'-CTCGATCTCCAGGTTCTGAAAG-3'	295
<i>CLCN3</i>	5'-GAGTTTGGCTTTCTTGCGAGTT-3'	5'-GAAAAGATATTTCCGACGAAC-3'	203
<i>VDAC3</i>	5'-ATAAGTTGGCTGAAGGGTTGAA-3'	5'-TTCTGTGACAGTTTGATTTGG-3'	235
<i>SLC4A4</i>	5'-GCTTGCAGATTACTACCCCATC-3'	5'-TTGAACACTCCTCTTCGACAA-3'	209
<i>p-120</i>	5'-AGGATCCAGCAAACGATACAGT-3'	5'-AGGTCAGCTATGGCAGAAAGAG-3'	244
<i>ZO-1</i>	5'-TTCTGAGGCTGTAACCATTTT-3'	5'-AATTGGATACCACTGGGCATAG-3'	245
<i>Na⁺/K⁺-ATPase</i>	5'-ACGGCAGTGATCTAAAGGACAT-3'	5'-GAAGAATCATGTGACAGCCTTG-3'	255
<i>GAPDH</i>	5'-GAGATCCCTCCAAATCAAGTG-3'	5'-GAGTCCTCCACGATACCAAAG-3'	245

doi:10.1371/journal.pone.0069009.t001

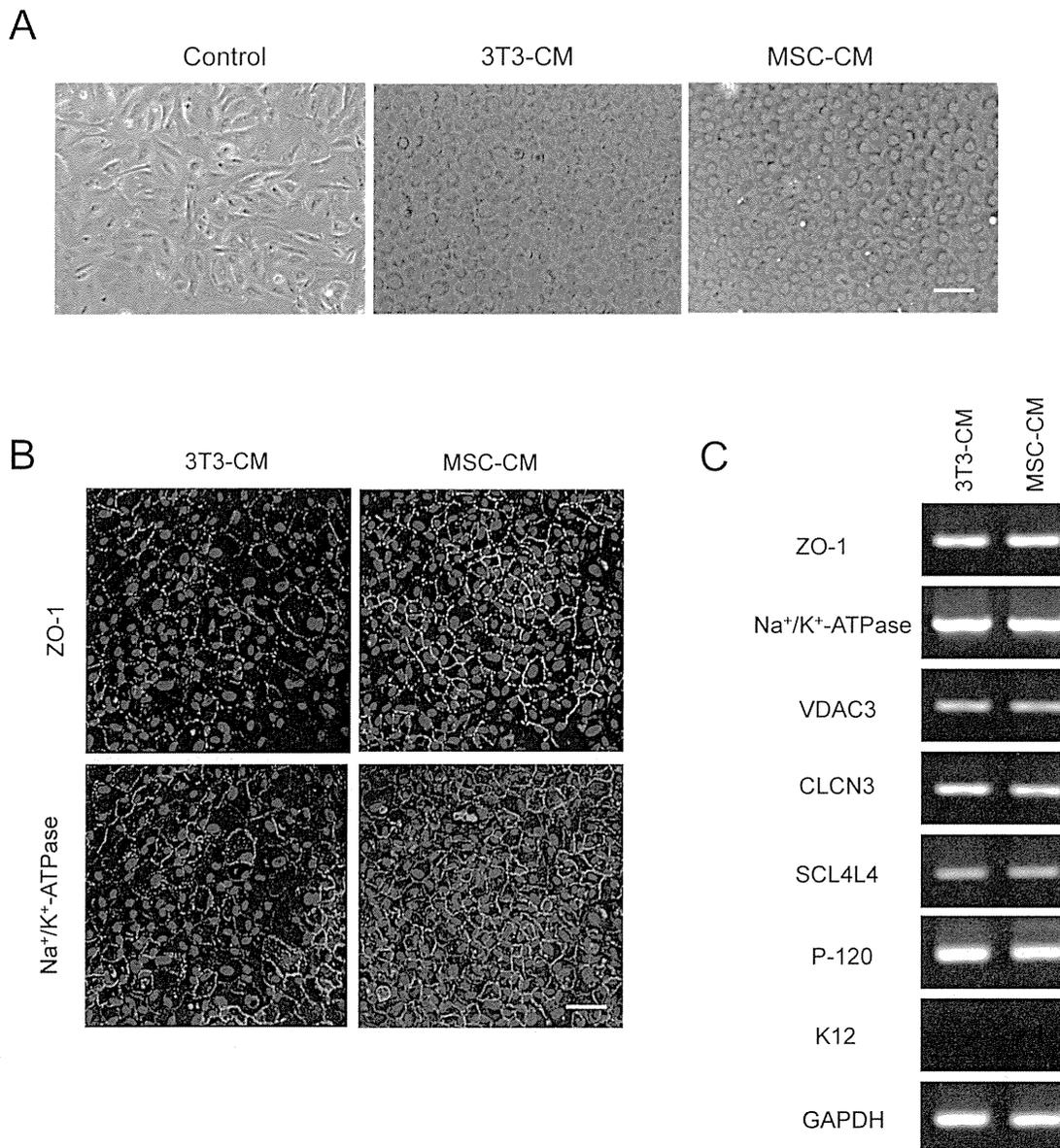


Figure 1. MSC-CM and NIH3T3-CM maintain corneal endothelial phenotype *in vitro* expansion. (A) Effect of MSC-CM on morphology of primary cultures of HCECs. Representative phase-contrast images of primary culture from different CMs. Cultured HCECs were maintained in basal growth medium, MSC-CM, or NIH3T3-CM for 30 days. Scale bar: 200 μ m. (B) HCECs cultured in either MSC-CM or NIH3T3-CM for 14 days expressed ZO-1 and Na⁺/K⁺-ATPase. The pictures are representative of 2 independent experiments. (C) Expression of genes involved in the active transmembrane transporter activity in HCECs cultured with both NIH3T3-CM and MSC-CM was assessed by RT-PCR. The experiments were performed in duplicate.

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for 3 hours using Ultra-PL 3 ultrafiltration units (Amicon; EMD Millipore) with a 3-kDa molecular weight cutoff.

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 with Dunnett's multiple-comparisons test. Results were expressed as mean \pm SEM.

Results

MSC-CM and NIH-3T3-CM maintain corneal endothelial phenotype *in vitro* expansion

Current isolation and cultivation methods to establish HCECs *in vitro* face an unexpected obstacle due to spontaneous morphological fibroblastic change and severely limited proliferative ability. Therefore, we tested the CM obtained from human BM-MSCs in this study. HCECs were maintained in basal growth medium, NIH-3T3-CM, or MSC-CM for 30 days. The control cells maintained in basal growth medium showed loss of the

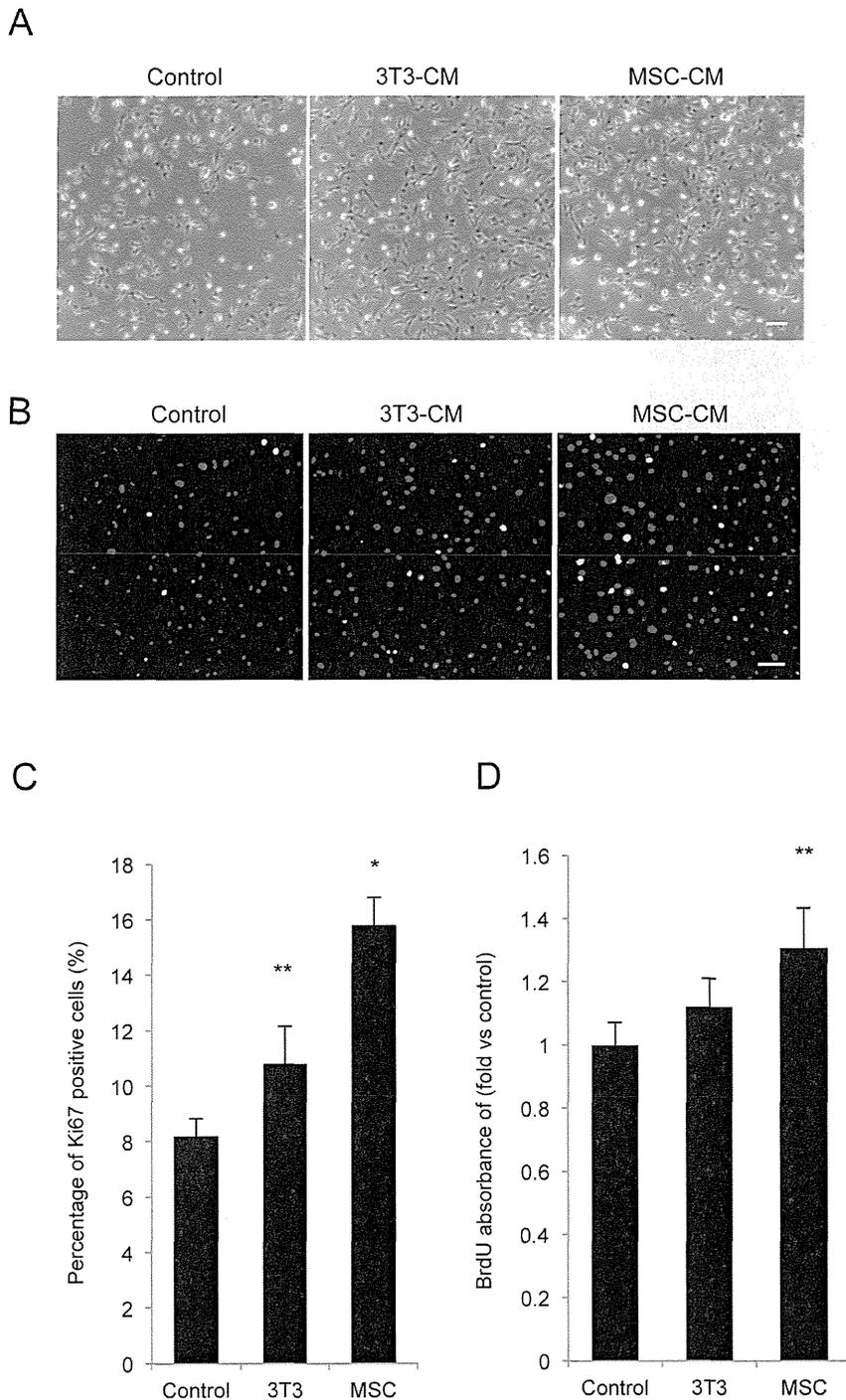


Figure 2. MSC-CM enhances the proliferation of HCECs. (A) Phase-contrast images of HCECs cultured with MSC-CM. HCECs were seeded and cultured with MSC-CM, NIH3T3-CM, or basal growth medium (control) for 5 days. Scale bar: 200 μ m. (B+C) To test proliferative potential, HCECs maintained for 5 days under the experimental conditions were immunostained with the cell-cycle-progression population marker Ki67, and the percentages of Ki-67 positive cells were then evaluated. The experiment was performed in duplicate. Scale bar: 200 μ m. (D) HCECs were cultured in basal growth medium (control), NIH3T3-CM, or MSC-CM. Proliferation of HCECs was evaluated by BrdU incorporation assay after 5 days of incubation. The experiment was performed in triplicate. * $p < 0.01$, ** $p < 0.05$. doi:10.1371/journal.pone.0069009.g002

characteristic polygonal cell morphology, whereas HCECs maintained in either CM demonstrated a contact-inhibited monolayer of hexagonal cells (Figure 1A). Immunostaining of ZO-1 and Na⁺/K⁺-ATPase was clearly outlined at the intercellular adherent junction in HCECs maintained with either MSC-CM or NIH-

3T3-CM (Figure 1B), similar to the previous findings [32]. Expression of genes involved in the active transmembrane transporter activity was assessed by RT-PCR (Figure 1C). The transcripts of ZO-1, Na⁺/K⁺-ATPase, voltage dependent anion channel3 (VDAC3), chloride channel protein 3 (CLCN3), sodium

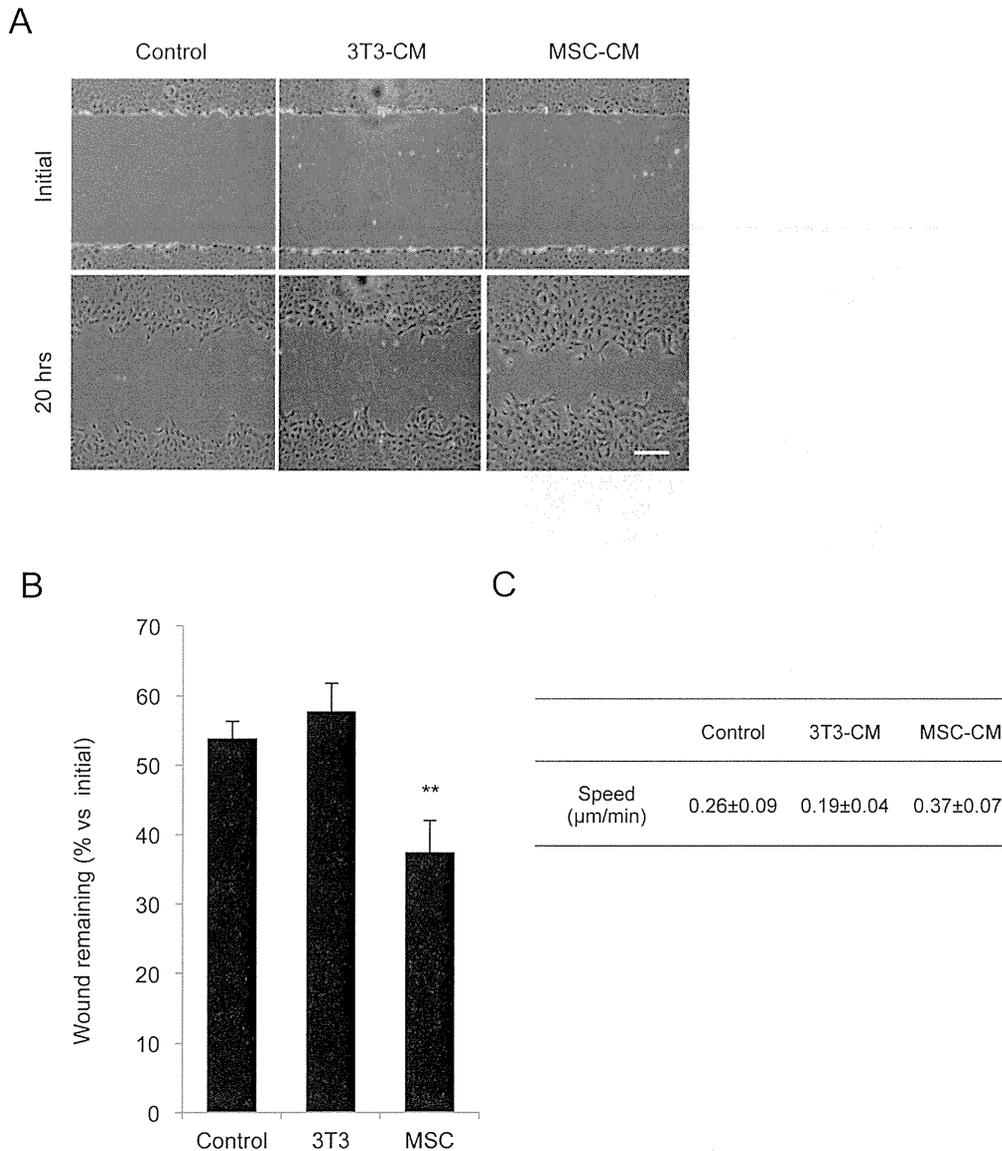


Figure 3. MSC-CM promotes cell motility in an *in vitro* wound model. (A+B) HCECs were cultured with basal growth medium (control), NIH3T3-CM, or MSC-CM for 40 days, and the monolayer cells were then wounded by scratching. After 20 hours, the remaining wound area was quantified by Image J software. ** $p < 0.05$. Scale bar: 200 μm . (C) The speed of cell motility was measured from the image. The experiments were performed in triplicate.

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bicarbonate co-transporter member4 (SLC4A4), and p-120 were expressed in HCECs, while keratin 12 (K12) was not expressed.

Effect of MSC-CM on the proliferation of HCECs

HCECs were cultured in basal growth medium, MSC-CM, or NIH-3T3-CM (Figure 2A), and the proliferative potential was then assessed using two respective methods: Ki67 staining and BrdU incorporation into the newly synthesized DNA. HCECs maintained for 5 days under the experimental conditions were immunostained with the cell cycle progression population marker Ki67 (Figure 2B). The control cells showed 8.2% Ki67-positive cells, whereas HCECs treated with MSC-CM showed 15.8% Ki67-positive cells (Figure 2C). When incorporation of BrdU into the newly synthesized DNA was measured, HCECs maintained in MSC-CM showed a much higher incorporation of BrdU into

DNA than did the control cells (Figure 2D). Of interest, HCECs maintained in NIH-3T3-CM demonstrated lower proliferative potential when compared to HCECs maintained in MSC-CM.

Effect of MSC-CM on the wound closure of HCECs

Scratch-induced directional migration assay was employed to compare the wound closure in HCECs maintained in MSC-CM to those of control cells and the NIH-3T3-CM-treated cells (Figure 3A, B). The wound was introduced to the confluent cultures and wound closure was measured 20 hours after the initial wounding. Cells maintained in MSC-CM demonstrated the fastest healing rate; 63% of the initial wound area was covered with cells. On the other hand, both the control cells and the cells maintained in NIH-3T3-CM showed that much less area of the initial wound was recovered by cells. When wound healing over a 20-hour

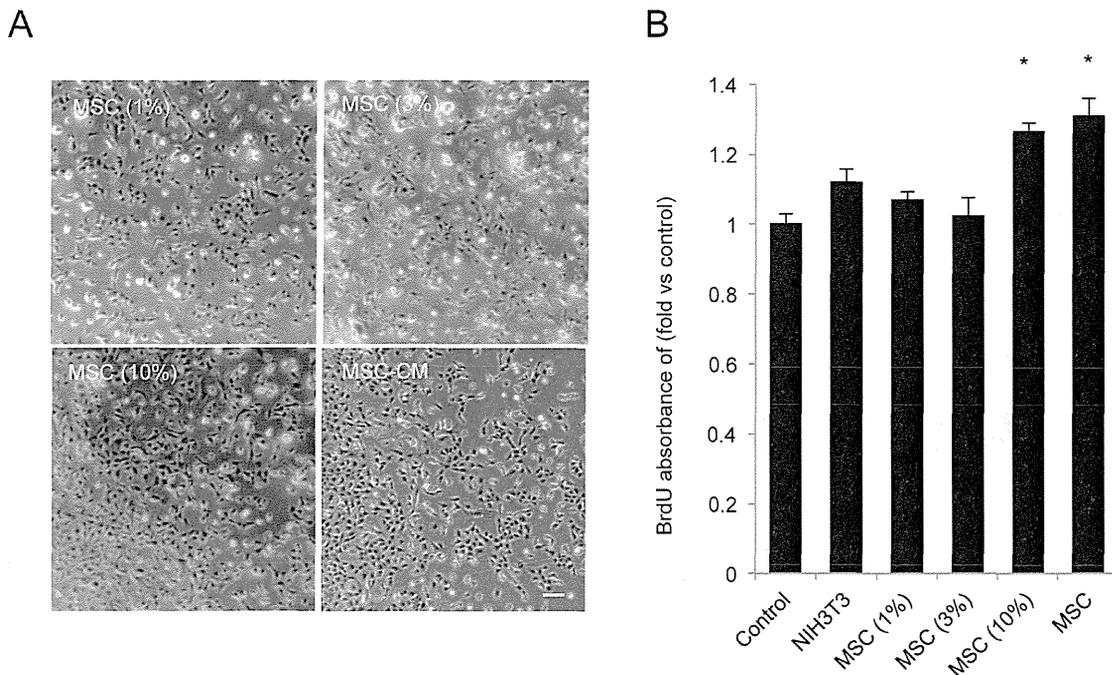


Figure 4. MSC-CM-derived factors enhance HCEC proliferation. (A) HCECs were maintained with basal growth medium supplemented with 1, 3, or 10% of concentrated MSC-CM or full strength MSC-CM. (B) The effect of soluble factors from MSC-CM on the proliferation of HCECs was evaluated by BrdU incorporation assay after 4 days of incubation. * $p < 0.01$. The experiments were performed in duplicate. Scale bar: 200 μm . doi:10.1371/journal.pone.0069009.g004

period was converted into the migration rate of HCECs, the MSC-CM-treated HCECs demonstrated 0.37 $\mu\text{m}/\text{min}$, the control cells showed 0.26 $\mu\text{m}/\text{min}$, and the NIH-3T3-CM-treated cells showed 0.19 $\mu\text{m}/\text{min}$ (Figure 3C), similar to the earlier findings [33].

Effect of MSC-CM-derived factors on CEC proliferation

The fact that the full strength of MSC-CM exerted proliferative activity led us to examine whether or not there is a dilution-dependent activity of MSC-CM on the proliferation of HCECs. To test the dilution effect, MSC-CM was concentrated and added to basal growth medium at the final concentration of 1%, 3%, or 10%. Then, the proliferative activity of the concentrated MSC-CM was compared to that of the full-strength CM (Figure 4A, B). Cells maintained in 10%-strength MSC-CM showed BrdU incorporation into DNA similar to the level achieved with the full-strength CM. On the other hand, MSC-CM at the strength of 1% and 3% produced no proliferative activity. These findings indicated that the soluble factors derived from MSC promote proliferation of HCECs, and also that the effect is dose-dependent.

Involvement of PI 3-kinase and ERK1/2 in the proliferation of HCECs in response to MSC-CM stimulation

It has been known that CECs, regardless of the species, utilize PI 3-kinase and ERK1/2 pathways for cell proliferation mediated by FGF-2 [20], [34]. Therefore, we tested whether or not MSC-CM activated the PI 3-kinase and ERK1/2 pathways. When serum-starved cells were treated with MSC-CM for 15, 30, 60, or 180 minutes, phosphorylation of Akt was greatly induced from 15 minutes following treatment of the cells with CM. Such enhancement on the phosphorylation of Akt sustained for 60

minutes, after which the phosphorylation of Akt was greatly reduced (Figure 5A). The control cells showed faint levels of phosphorylated Akt. Phosphorylation of ERK1/2 was also enhanced 15 minutes following treating the cells with MSC-CM, and such phosphorylation attenuated up to 180 minutes (Figure 5B). To test whether or not the cell proliferation was induced by either PI 3-kinase or MEK, cell density was measured in the presence of the respective inhibitors to PI 3-kinase and ERK1/2; both LY294002 (PI 3-kinase inhibitor) and U0126 (MEK inhibitor) were found to block cell proliferation (Figure 5C). The cells treated with either inhibitor showed an enlarged cell shape due to the lesser cell numbers (Figure 5D). Cell proliferation observed in CECs was linked to the degradation of p27, the potent inhibitor of the G1 phase of the cell cycle [20], [34]. Therefore, we examined the amount of p27 in the absence or presence of LY294002 at the early G1 phase (8 hours) or the late G1 phase (24 hours). p27 appeared to be maintained at a low level regardless of the G1 stage in the presence of MSC-CM (Figure 5E), whereas p27 level was greatly increased in the presence of LY294002 during the late G1 phase of the cell cycle. On the other hand, cyclin D1 and cyclin D3 expressed in the presence of MSC-CM were greatly reduced by the action of LY294002 during the early G1 phase (8 hours) of the cell cycle (Figure 5E). These findings indicated that MSC-CM may employ PI 3-kinase signaling to regulate cell cycle progression through the action on p27 and cyclin D.

Discussion

Human corneal endothelium is a physiologically important monolayer of the cornea, as the simple but crucial role of the endothelium is to maintain cornea clarity. In order to keep the entire cornea transparent, it is essential for corneal endothelium to