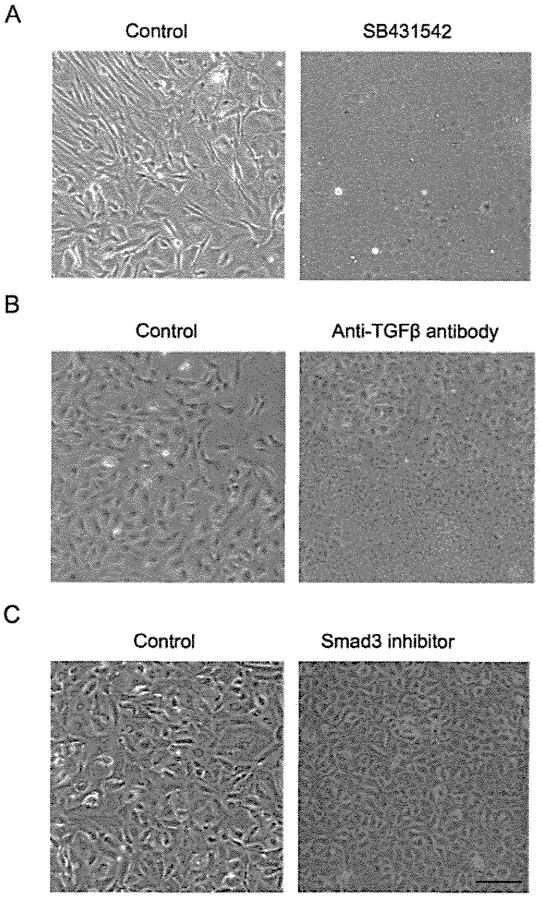

Figure 6. Inhibition of the TGFβ pathway suppressed the fibroblastic change of HCECs. (A) HCECs cultured with SB431542 (1 μM) exhibited the hexagonal cell shape and contact-inhibited monolayer, while the control CECs exhibited the fibroblastic morphology. (B+C) Both neutralizing antibody to TGF-β (500 ng/ml) and Smad3 inhibitor (3 mM) blocked cells from acquiring fibroblastic phenotypes. Scale bar: 50 μm. The experiment was performed in duplicate.
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and at the cytoplasm. Of interest, the cytoplasmic location of type I collagen appeared to be at the Golgi complex, the intracellular localization of which is essential for secretion, and these findings are similar to the published data [28]. On the other hand, type I collagen staining in the normal phenotypes was not clearly observed (Fig. 2A). RT-PCR analysis was used to determine the expression of major ECM proteins. The type I collagen transcript [α1(I) mRNA] was found to be abundantly expressed in the fibroblastic phenotypes, while the expression of α1(I) mRNA was negligible in the normal phenotypes (Fig. 2C). Unlike the type I collagen transcript, the basement membrane collagen phenotype α1(IV) mRNA was expressed in both the normal and fibroblastic phenotypes, yet to a lesser degree in the normal phenotype. Collagen phenotype α1(VIII) mRNA was expressed in both phenotypes at similar levels. Expression of fibronectin and integrin α5 was observed in the fibroblastic phenotypes, as opposed to the normal phenotypes in which the two transcripts were not expressed (Fig. 2C). On the other hand, β1 integrin mRNA was expressed in both phenotypes at similar levels (Fig. 2C).

Next, signaling pathways were determined to elucidate what might cause fibroblastic phenotypes of CECs. Since Smad2, p38, ERK1/2, and JNK are reportedly all involved in the EMT pathway [18–20,29,30], we therefore tested whether Smad2 and the MAPKs were involved in an endothelial-mesenchymal transformation similar to the EMT observed in epithelial cells (Fig. 3). Phosphorylation of Smad2 was found to be greatly promoted in the fibroblastic phenotypes when compared to that in the normal phenotypes. Phosphorylation of p38 and ERK1/2 was greatly enhanced in the fibroblastic phenotypes, while activation of JNK was negligible. These findings suggested that TGF-β signaling may exert the key role for the fibroblastic transformation of CECs.

TGF-β-mediated endothelial-mesenchymal transformation and use of TGF-β receptor inhibitor to block it in primate CECs

The findings shown in Fig. 3 led us to directly test whether SB431542, the TGF-β receptor inhibitor, was able to block the EMT process observed in the fibroblastic phenotypes. Phase contrast imaging demonstrated that primate CECs cultured in the presence of SB431542 exhibited the authentic polygonal cell shape and contact-inhibited monolayer, while the control CECs exhibited the fibroblastic morphology (Fig. 4A). Moreover, the SB431542-treated CECs showed the characteristic plasma membrane staining of Na^+/K^+-ATPase and ZO-1, while the control CECs lost their staining, suggesting that endothelial functions were maintained in the SB431542-treated cells (Fig. 4B). Furthermore, the expression of Na^+/K^+-ATPase and ZO-1 was strongly upregulated in the SB431542-treated fibroblastic phenotypes at both the protein (Fig. 4C) and mRNA levels (Fig. 4D). These data further confirmed that TGF-β might be the direct mediator of the endothelial to mesenchymal transformation observed in primate CEC cultures. Therefore, we tested whether the normal phenotypes were transformed to fibroblastic cells when exposed to the exogenous TGF-β, as in the findings shown in Fig. 5A. Of interest, the staining pattern of Na^+/K^+-ATPase and ZO-1 at the plasma membrane of the normal phenotypes was greatly reduced upon exposure of polygonal cells to TGF-β (Fig. 5B). The growth factor also markedly reduced the expression of the two proteins at protein levels in a concentration-dependent manner (Fig. 5C), while phosphorylation of Smad2 was greatly increased in a concentration-dependent manner (Fig. 5D). These data suggest that even the normal phenotypes of primate CECs are prone to acquire fibroblastic phenotypes in response to TGF-β-stimulation.

Two distinct phenotypes of HCEC cultures and the use of TGF-β receptor inhibitor to block endothelial-mesenchymal transformation

The interesting findings observed in primate CECs led us to further study whether HCECs were subjected to the similar undesirable prerequisite cellular changes leading to endothelial-mesenchymal transformation. Of great interest, cultivated HCECs lost the characteristic contact-inhibited monolayer and polygonal phenotypes, and acquired fibroblastic cell morphology like primate CECs (Fig. 6A). However, consistent with the primate CECs when the CECs were cultivated with the specific inhibitor to the TGF-β receptor (SB431542), the inhibitor was able to block alteration of the cell shape to fibroblastic phenotypes. Similar to the inhibitory effect of SB431542 on fibroblastic phenotypes, both neutralizing antibody to TGF-β (Fig. 6B) and Smad3 inhibitor (Fig. 6C) also blocked cells from acquiring fibroblastic phenotypes. We then tested whether SB431542 was able to maintain endothelial function. The findings shown in Fig. 7A and Fig. 7B demonstrated that blocking the TGF-β receptor signaling enabled the subcellular localization of Na^+/K^+-ATPase and ZO-1 at the plasma membrane and their protein expression to be maintained. Of

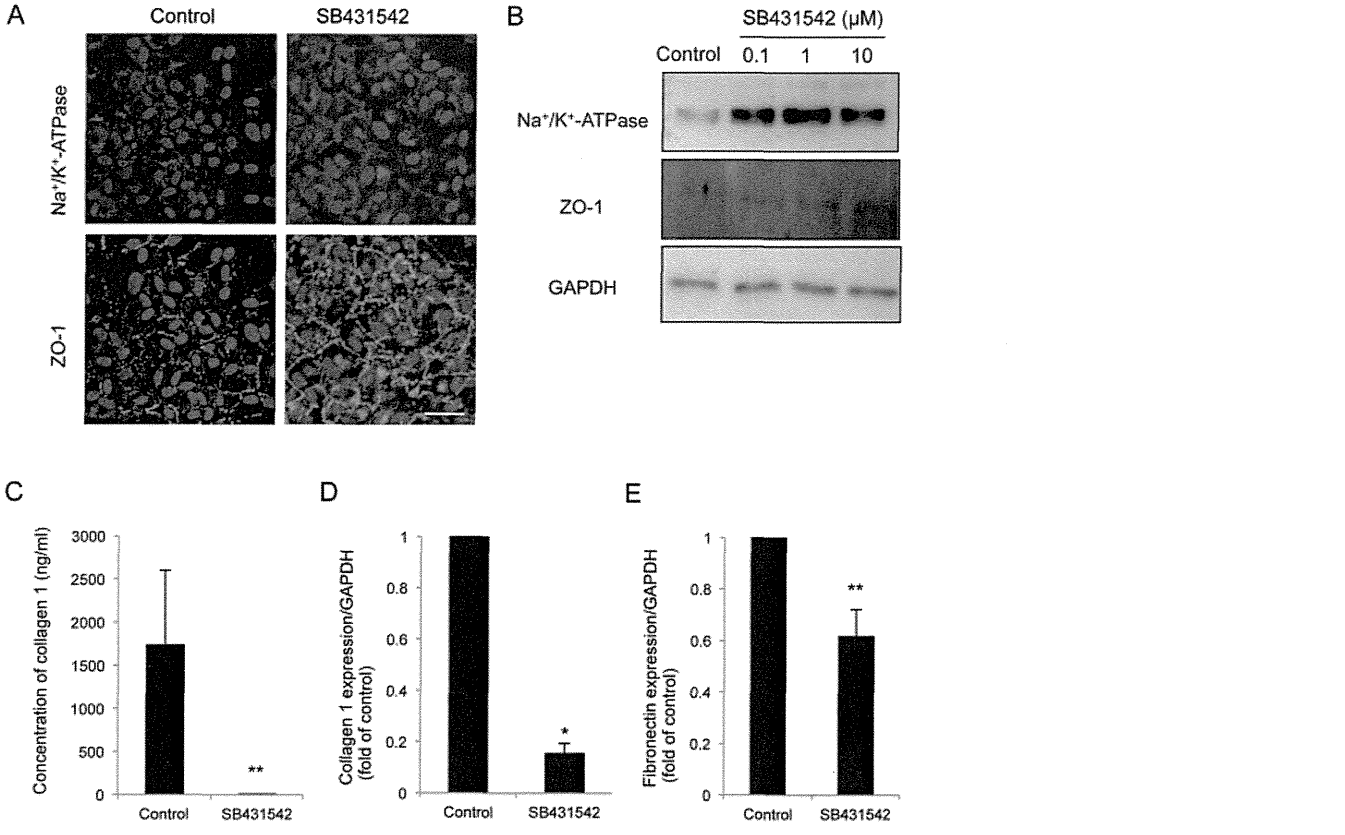

Figure 7. SB431542 maintained the functions and suppressed the fibroblastic change of HCECs. (A+B) Blocking the TGF-receptor signaling by SB431542 (A: 1 μM, B: 0.1, 1, and 10 μM) enabled the subcellular localization of Na^+/K^+-ATPase and ZO-1 at the plasma membrane and their protein expression to be maintained. Scale bar: 100 μm. (C) ELISA assay revealed that SB431542 significantly downregulated the secretion of type I collagen to the culture supernatant. **P<0.05. (D+E) Quantitative PCR showed that SB431542 significantly reduced the expression of type I collagen and fibronectin at the mRNA level. *$p<0.01$, ** $p<0.05$. Samples were prepared in duplicate. Immunoblotting, ELISA, and quantitative PCR were performed in duplicate.
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great importance, ELISA assay revealed that SB431542 markedly downregulated the secretion of type I collagen to the culture supernatant (Fig. 7C). Coincidentally, SB431542 markedly reduced the expression of type I collagen and fibronectin at the mRNA level (Fig. 7D, E).

Use of BMP-7 to suppress fibroblastic changes and maintain endothelial functions

Bone morphogenetic protein-7 (BMP-7) promotes MET and specifically inhibits the TGF-β-mediated epithelial-to-mesenchymal transition. Thus, that molecule has been used to antagonize the EMT process [31–34]. We therefore tested whether BMP-7 was able to antagonize the prerequisite changes of HCECs. The fibroblastic HCECs were treated with BMP-7 in a concentration ranging from 10 to 1000 ng/ml. Of important note, the elongated cell shapes of the fibroblastic phenotypes were reversed to the polygonal cell morphology in response to the presence of BMP-7 in a concentration-dependent manner (Fig. 8A). BMP-7 enabled the hexagonal cell morphology and actin cytoskeleton distribution at the cortex to be maintained (Fig. 8B), similar to that observed in normal CECs [35], and it also maintained the subcellular localization of Na^+/K^+-ATPase (Fig. 8C) and ZO-1 (Fig. 8D) at the plasma membrane. Thus, BMP-7 at the concentration of 1000 ng/ml was able to maintain CECs in polygonal and contact-inhibited phenotypes with a positive expression of function-related markers (Fig. 8E, F).

Discussion

Corneal endothelial dysfunction accompanied by visual disturbance is a major indication for corneal transplantation surgery [36,37]. Though corneal transplantation is widely performed for corneal endothelial dysfunction, researchers are currently seeking alternative methods to restore healthy corneal endothelium. The fact that corneal endothelium is cultured and stocked as 'master cells' from young donors allows for the transplantation of CECs with high functional ability and for an extended period of time. In addition, an HLA-matching transplantation to reduce the risk of rejection [38,39] and overcoming the shortage of donor corneas might be possible. Tissue bioengineering is a new approach to develop treatments for patients who have lost visual acuity [40]. To date, there are two methods that utilize bioengineering approaches: 1) use of cultured donor HCECs adhered on bioengineered constructs [4,5,7,9], and 2) transplantation of cultivated HCECs into the anterior chamber [11,41–43]. Regardless of which of the two methods is applied to clinical settings, establishment of an efficient cultivation technique for HCECs is essential and inevitable [44]. Many researchers have noticed that establishing a consistent long-term culture of HCECs is challenging [40]. Although the successful cultivation of HCECs has been reported by several groups, the procedures involved in the isolation and subsequent cultivation protocols varied greatly between laboratories [44]. One of the most difficult problems is

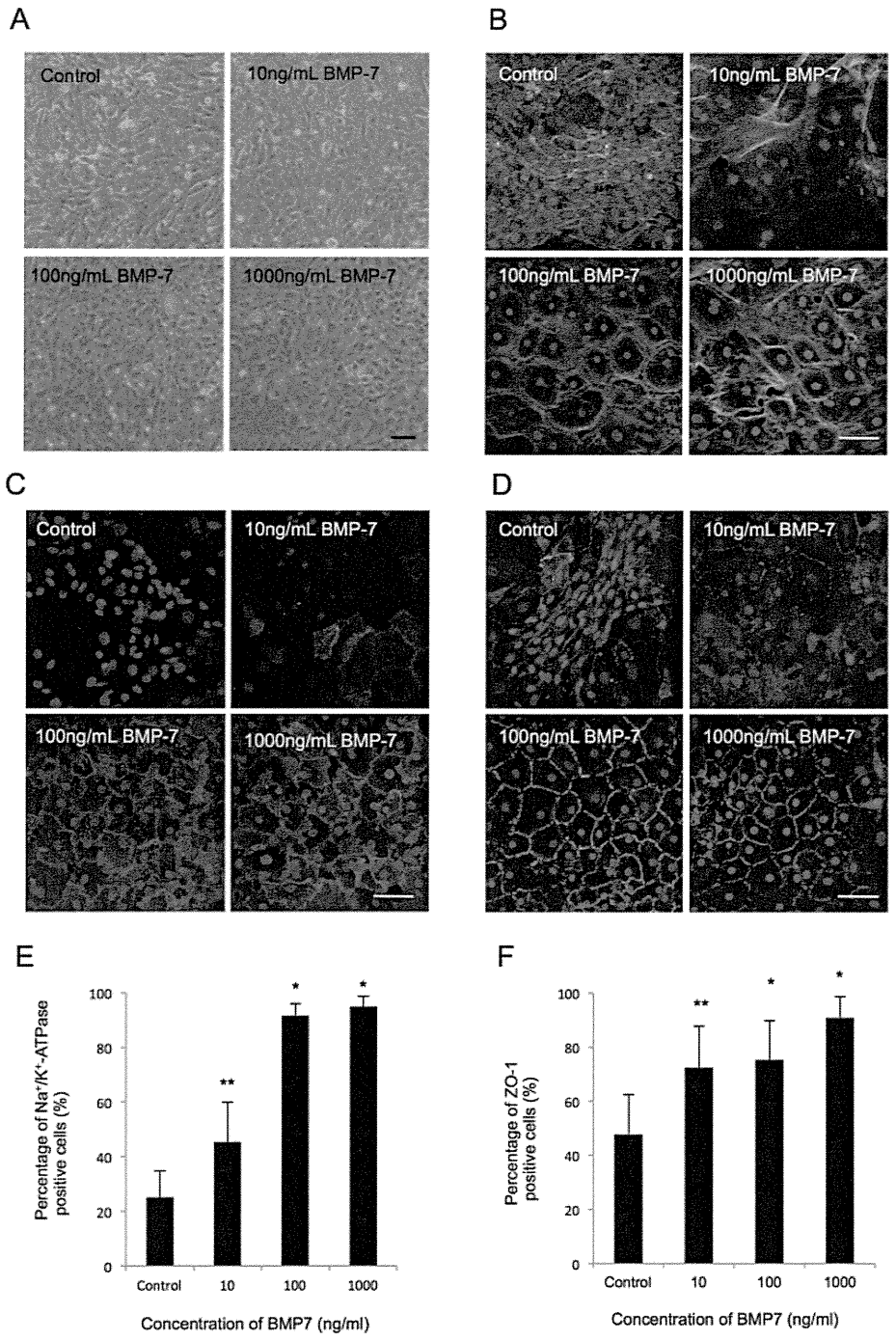

Figure 8. BMP7 suppressed fibroblastic change and maintained the functions of HCECs. (A) The elongated cell shapes of the fibroblastic phenotypes were reversed to a polygonal cell morphology in response to the presence of BMP-7 in a concentration-dependent manner. Scale bar: 50 μm. (B) BMP-7 enabled normal hexagonal cell morphology and actin cytoskeleton distribution at the cortex to be maintained. Scale bar: 100 μm. (C+D) BMP-7 maintained the subcellular localization of Na^+/K^+-ATPase and ZO-1at the plasma membrane. Scale bar: 100 μm. (E+F) The percentages of both Na^+/K^+-ATPase and ZO-1 positive cells treated with BMP-7 were significantly higher than in the control. * $p<0.01$, ** $p<0.05$. The experiment was performed in duplicate.
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that HCECs are vulnerable to undergoing massive fibroblastic change over each passage [40]. Therefore, it is essential to find means to circumvent the spontaneous transformation of the CECs in order to maintain the physiological phenotypes for the subsequent use for transplantation.

Transformation of endothelial cells to fibroblastic cells is designated as endothelial- mesenchymal transformation. Such transformation is triggered by TGF-β via the Smad2/3 pathway [16]. Endothelial-mesenchymal transformation causes the loss of the characteristic endothelial phenotypes, such as loss of the contact-inhibited monolayer and loss of the apical junctional

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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Rho-associated kinase (ROCK) inhibitor eye drop treatment as a possible medical treatment for Fuchs corneal dystrophy

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ABSTRACT

Purpose: To report a case of Fuchs corneal dystrophy that was successfully treated by Rho-associated kinase (ROCK) inhibitor eye drops, subsequent to transcorneal freezing of damaged corneal endothelial cells.

Case report: A 52-yr-old Japanese male with a diagnosis of late-onset Fuchs corneal dystrophy was referred to our hospital as a candidate for keratoplasty. Best corrected vision was 20/20 in the right eye and 20/63 in the left. Multiple guttae were observed in both eyes. The right cornea was clear, but the left showed severe central oedema, with a central corneal thickness (CCT) of 703μm. We were unable to perform specular microscopy in the central cornea, but endothelial cells were observed in the mid-periphery at a density of 757 cells/mm^2. The patient was treated by a corneal endothelial denudation in the pre-pupillary region followed by the topical administration of a selective ROCK inhibitor, Y-27632, as eye drops for one week. Follow-up of 24 months is reported.

Results: Corneal clarity recovered and vision improved to 20/20 two weeks after treatment. At six months vision had improved to 20/16 and CCT measured 568μm, significantly lower than its pre-treatment value. Endothelial function and vision have been well maintained up to the most recent observation, 24 months post-treatment. The

average corneal endothelial density in the central and peripheral cornea was 1549.3±89.7 and 705.0±61.1 cells/mm^2, respectively.

Conclusions: The case highlights the possibility of medical treatments involving the use of ROCK inhibitor eye drops as an alternative to graft surgery for certain forms of corneal endothelial disease.

Keywords: corneal endothelium; medical treatment; Rho kinase inhibitor; Fuchs corneal dystrophy

INTRODUCTION

The proliferative ability of human corneal endothelial cells is severely limited *in vivo*. As a consequence corneal endothelial damage caused by trauma, intraocular surgery, or disease such as Fuchs corneal dystrophy often results in severe visual disturbance. Corneal transplantation, including corneal endothelial transplantation surgeries such as Descemet's stripping automated endothelial keratoplasty (DSAEK) and Descemet's membrane endothelial keratoplasty (DMEK), is a beneficial and realistic treatment for patients with endothelial dysfunction, however, patients will not be totally free from the risk of graft rejection. Moreover, corneal endothelial cell loss is a potential problem in the long term[1]. Despite the value and potential of endothelial graft surgery, however, a purely pharmacological approach to endothelial recovery remains an attractive proposition.

Previously, we reported that a selective Rho-associated kinase (ROCK) inhibitor, Y-27632, promoted the proliferation of primate corneal endothelial cells *in vitro*[2], as well as the healing of the corneal endothelium *in vivo*[3]. Here, we present a case of Fuchs corneal dystrophy scheduled for DSAEK surgery, but successfully treated by ROCK inhibitor eye drop treatment subsequent to transcorneal freezing.

CASE REPORT

A 52-yr-old Japanese male with blurred vision due to corneal endothelial dysfunction was referred to the Kyoto Prefectural University of Medicine in May 2008. Visual acuity was 20/20 in the right eye and 20/63 in the left. Multiple guttae , typical of Fuchs corneal dystrophy, were observed in both eyes by slit lamp examination as well as by non-contact specular microscopy (EM-3000^{TM}, TOMEY Corporation, Nagoya, Japan) (Figures 1A, B). The right cornea was clear, although the corneal endothelial density was 632 cells/mm^2. The left cornea showed severe central oedema accompanied by epithelial bullae (Figures 2A, B). The central corneal thickness was 703μm in the patient's affected left eye. We were unable to perform specular microscopy in the central cornea owing to the oedema, but endothelial cells were observed in the mid-periphery at a density of 757 cells/mm^2 (Figure 1B). The patient was diagnosed as late-onset Fuchs corneal dystrophy[4]. He was scheduled to have a DSAEK surgery, but in April 2010 volunteered for an investigative clinical study of a ROCK inhibitor eye drop treatment.

Treatment was initiated on 18 May 2010 according to a protocol approved by the Institutional Review Board of Kyoto Prefectural University of Medicine. First, diseased corneal endothelium in the pre-pupillary region was removed by transcorneal freezing[3] by gently pressing a 2mm-diameter stainless steel rod which had been cooled

in liquid nitrogen onto the corneal surface for 15 sec. In our previous study using a rabbit model, we confirmed that this transcorneal freezing procedure could make a endothelial defect of approximately the same size as the rod-diameter in a reproducible fashion.[3] After the rod was removed and after the cornea had thawed, 50μl of 10mM ROCK inhibitor, Y-27632 (Wako, Osaka, Japan), was applied topically as eye drops, repeated six times daily for seven days (18 to 24 May 2010). To prevent corneal infection, 0.3% Gatifloxacin hydrate eye drops were also applied four times daily. Epithelial erosion was detected after transcorneal freezing, but had healed by post-treatment day 3 (Figures 2C, D). No side effects, such as persistent epithelial defects or corneal stromal scars, were observed.

The patient's cornea recovered complete clarity two weeks after treatment and vision had improved to 20/20. Six months after treatment central corneal thickness was 568μm, significantly lower than its pre-treatment value. At this time vision had improved to 20/16 (Figures2E, F). Wide-field endothelial examinations 18 months after treatment using contact specular microscopy (Konan Medical, Inc. Nishinomiya, Japan; Figure 3A) showed that the average corneal endothelial densities in the central and peripheral cornea were 1549.3±89.7 and 705±61.1 cells/mm^2, respectively (mean ± SEM; Figure 3B). Although Fuchs corneal dystrophy is a progressive disease, in our

patient corneal clarity and good vision (20/16) have been maintained up to the most recent observation, 2-yrs after treatment (Figures 1G, H).

DISCUSSION

Rho-associated kinases (ROCKs) are protein serine/threonine kinases, which are the first identified and best characterized Rho downstream effectors. The Rho/ROCK pathway is involved in regulating the cytoskeleton, and has an influence on cell migration, apoptosis, and proliferation.[5-8]

We previously reported that a selective ROCK inhibitor, Y-27632, promoted the proliferation of primate corneal endothelial cells *in vitro.*[2] In our previous experiments, Y-27632 promoted cell proliferation up to the time when cells became pre-confluent, but did not promote proliferation in confluent cells whose proliferation had been stopped by contact inhibition. Based on this, and on the results of experiments in rabbits,[3] we hypothesized that the topical application of Y-27632 as an eye drop, combined with the prior partial denudation of diseased corneal endothelial cells, might be useful to promote the proliferation *in situ* of the corneal endothelium which is in the early diseased phase. We, thus, came up with the protocol reported here, which shows some potential for the new approach to treat of certain types of corneal endothelial

dysfunction.

In the post-treatment observation of the presented case, contact-specular microscopy revealed relatively small corneal endothelial cells, present at a high cell density, in the central part of cornea from where corneal endothelial cells had been removed by transcorneal freezing. The potential of topical application of ROCK inhibitor suggested by the current report clearly requires a larger comparative study to prove the effect of this new treatment, and plans are underway to conduct this. Regarding the mechanism of action of the procedure, we should also point out that spontaneous remodeling of the human corneal endothelial cells after Descement's stripping has been reported.[9, 10] Based on these reports, and also bearing in mind the existence of corneal endothelial precursors with higher proliferative ability in the peripheral cornea,[11, 12] we cannot rule out the possibility that re-establishment of patient's endothelium was not a direct result of ROCK inhibitor administration, but was the consequence of denudation of the pathologic endothelial cells. Notwithstanding the preliminary nature of the current observation, this case report suggests the possibility of a medical treatment for the early phase of diseases such as Fuchs corneal dystrophy, via the stimulation of non-affected peripheral cells with ROCK inhibitor following the destruction of diseased cells in the central endothelium by transcorneal freezing.

To the best of our knowledge, it is the first report suggesting that the *in vivo* proliferation of a patient's corneal endothelium can be stimulated by interventional medical/pharmaceutical treatment following the destruction of diseased endothelium. We believe our new findings will contribute to the opening up of a new approach to the treatment of corneal endothelial dysfunction.

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FIGURE LEGENDS

Figure 1.

The corneal endothelium observed by non-contact-specular microscopy before treatment. (A) Multiple guttae were present (*), and corneal endothelial cells at a density of 632 cells/mm^2 were observed in the center of the right cornea. (B) We were unable to perform specular microscopy in the center of the left cornea owing to the oedema, however, endothelial cells were observed in the mid-periphery at a density of 757 cells/mm^2. Guttae were also observed (*).

Figure 2.

Slit-lamp photographs of our Fuchs' corneal dystrophy patient before and after transcorneal freezing and ROCK inhibitor treatment. Before treatment, central corneal oedema (A) accompanied by a lesion of epithelial bullae (B) was detected. Three days after treatment the corneal erosion created by the transcorneal freezing had already healed and mild bullae were detected (C, D). It should be noted that less corneal oedema was observed at two days compared to the pre-treatment photograph. Six months after treatment corneal oedema was significantly reduced and cornea had recovered its clarity (E). No epithelial damage was observed by fluorescein staining (F). 2-yrs after

treatment, the patient's cornea remains clear with good (20/16) vision (G, H).

Figure 3.

(A) Wide-field observation of the corneal endothelium by contact-specular microscopy 18 months after treatment. Guttae were detected mainly in the paracentral area. (B) Representative, magnified photographs from nasal peripheral, central and temporal peripheral area. Smaller cells, present at high density, were observed in the central cornea. (Scale bar =100μm)

Figure1