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Inhibition of Matrix Metalloproteinase Activity Reverses Corneal Endothelial-Mesenchymal Transition

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Ex vivo culture or regeneration of corneal endothelial cells often is subjected to gradual endothelial-mesenchymal transition and loss of function. Here, we found that during *ex vivo* culture, bovine corneal endothelial cells underwent endothelial-mesenchymal transition and had an up-regulated expression and activity of matrix metalloproteinases. Inhibition of matrix metalloproteinase activity in confluent bovine corneal endothelial cells decreased the level of endothelial-mesenchymal transition regulators: snail and slug. The phosphorylation and degradation of the key Wnt signaling pathway modulator active β-catenin also were accelerated with the broad-spectrum matrix metalloproteinase inhibitor Marimastat, which may result from decreased N-cadherin shedding and increased intact N-cadherin molecules on the cell membrane. Intracameral injection of Marimastat also suppressed basic fibroblast growth factor—induced endothelial-mesenchymal transition in a rat corneal endothelium cryo-injury model and significantly diminished the corneal edema. Our study indicated that inhibition of matrix metalloproteinase activity can reverse endothelial-mesenchymal transition and preserve the function of corneal endothelial cells both during *ex vivo* culture and *in vivo*. This may offer a potential therapeutic target in regenerative medicine for the treatment of corneal endothelial dysfunctions. *(Am J Pathol 2015, 185: 2158—2167; http://dx.doi.org/10.1016/j.ajpath.2015.04.005)*

Corneal endothelial cells (CECs), a monolayer of cells located in the posterior part of the cornea, play an important role in maintaining corneal clarity by active pumping.[1] Because of limited proliferative capacity, the cell number decreases with age, trauma, intraocular surgery, or corneal endothelial dystrophy. Low corneal endothelial cell count may lead to corneal edema and decreased visual acuity.[2,3] The options of surgical treatment include penetrating keratoplasty and endothelial keratoplasty. Nevertheless, issues such as organ shortage or allograft rejection have yet to be resolved. Accordingly, it is necessary to develop methods to promote *in vivo* regeneration or *ex vivo* culture of human CECs.

To overcome the limited proliferative capability of human CECs, many growth factors or supplements have been used, including basic fibroblast growth factor (bFGF), epidermal growth factor, and endothelial cell growth supplements.[4–6] However, CECs tend to undergo endothelial-mesenchymal transition (EnMT), resembling epithelial-mesenchymal transition (EMT), on these proliferation-stimulating agents, and then lose their polarized characteristic, which are essential for their physiological function.[7–10] As a result, EnMT has become an important issue in cultivating corneal endothelial cells, especially during *ex vivo* expansion of CECs in the field of tissue engineering.[11]

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Disclosures: The authors have submitted a patent on using matrix metalloproteinase inhibitor to maintain the phenotype of corneal endothelial cells during *ex vivo* culture.

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Table 1 List of Antibodies Used in the Experiments and their Concentrations

Protein	Assay	Company	Catalog number	Concentration (ng/μL)
ABC	IF	Millipore	05-665	5
ABC	WB	Millipore	05-665	2
Snail	IF	Santa Cruz Biotechnology	sc-28199	1
Snail	WB	Cell Signaling Technology	3895	4.2
Slug	IF	Santa Cruz Biotechnology	sc-15391	1
Slug	WB	Santa Cruz Biotechnology	sc-15391	0.4
MMP-2	WB	Santa Cruz Biotechnology	sc-13595	0.4
MMP-3	WB	GeneTex	GTX103647	2
MMP-7	WB	GeneTex	GTX104658	2
MMP-9	WB	GeneTex	GTX100458	2
GAPDH	WB	Santa Cruz Biotechnology	sc-25778	0.2
α-SMA	WB	Abcam	ab32575	0.048
α-SMA	IF	Abcam	ab32575	0.12
β-catenin	WB	Thermo Fisher Scientific	PA5-16192	0.2
Phospho-β-catenin	WB	Cell Signaling Technology	9561	0.142
N-cadherin	WB	BD Biosciences	610921	0.5
N-cadherin	WB	Santa Cruz Biotechnology	sc-7939	0.4
Calnexin	WB	Origene	TA305929	1

ABC, active β-catenin; α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IF, immunofluorescence; MMP, matrix metalloproteinase; WB, Western blot.

EMT is characterized by the loss of cell—cell adhesion and cell polarity, which enables epithelial cells to migrate and invade, and is pivotal in embryonic development as well as many diseases.[12] Among the signaling pathways leading to EMT, the Wnt/β-catenin pathway plays a key role.[13] In addition to participating in the formation of the adherens junction complex, β-catenin, especially the active form that is unphosphorylated at Ser33, Ser37, and Thr41, has been found to serve as a coactivator of T-cell and lymphoid enhancer factors.[14] The occupancy of T-cell and lymphoid enhancer factors/β-catenin transcriptional activation complex at the target promoter regions can activate the downstream genes, including matrix metalloproteinase (MMP), which may degrade extracellular matrix, process growth receptors, cleave junctional proteins, and then facilitate cellular migration and invasion.[15—22] However, MMPs are not only the effectors of EMT, but also directly can turn on the EMT process in many cell types, including the lens epithelium.[23—26]

As in EMT, the Wnt/β-catenin pathway is involved in the EnMT; it has been shown that the Wnt/β-catenin pathway is activated during the *ex vivo* culture of human CECs by adding EDTA and bFGF.[27] However, the role of MMPs as a trigger of EnMT has not been explored in CECs. Moreover, if MMPs indeed are involved in modulating the EnMT, reasonable speculation ensures that targeting MMPs may change, or even reverse, the EnMT process. Herein, we used a bovine CEC (BCEC) *ex vivo* culture model together with a rat corneal endothelium cryo-injury model to investigate the role of MMPs in EnMT. Our results indicate that MMP activity is involved in EnMT, and reversing the EnMT process by MMP inhibition provides a new avenue for treating corneal endothelial dysfunction.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium, Ham's/F12 medium, HEPES buffer, phosphate-buffered saline (PBS), gentamicin, amphotericin B, fetal bovine serum, bFGF, trypsin, selenium, and DAPI were purchased from Invitrogen Corp. (Carlsbad, CA). Dimethyl sulfoxide, human epidermal growth factor, insulin, transferrin, cholera toxin, bovine serum albumin, Triton X-100, Ponceau, Marimastat, MG132, and BIO were purchased from Sigma-Aldrich (St Louis, MO). The antibodies used were active β-catenin (ABC; Millipore Corp., Billerica, MA); snail, slug, N-terminal N-cadherin (clone H63), MMP-2, and glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA); total β-catenin (Thermo Fisher Scientific, Inc., Waltham, MA); α-smooth muscle actin (α-SMA; Abcam, Inc., Cambridge, MA); phospho-β-catenin, snail (Cell Signaling Technology, Danvers, MA); C-terminal N-cadherin (BD Biosciences, San Jose, CA); MMP-3, MMP-7, and MMP-9 (GeneTex, Irvine, CA); calnexin (Origene, Rockville, MD); and Alexa Fluor—conjugated goat IgGs (Invitrogen Corp.). The catalog numbers and the concentrations of antibodies used in the experiment are summarized in Table 1.

BCEC Cultures

All procedures followed in this study were in accordance with the principles of the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the National

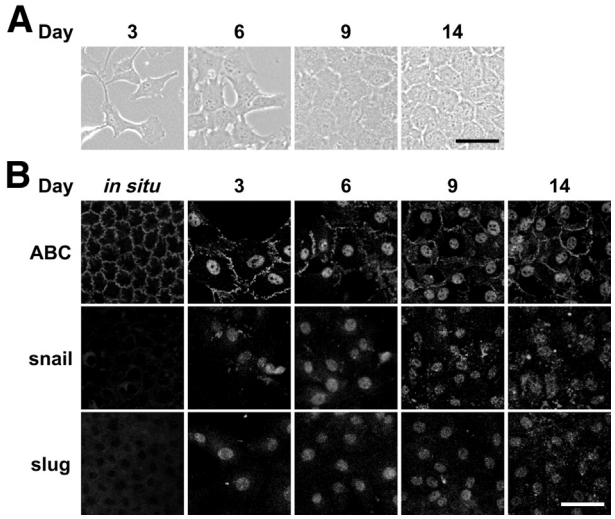

Figure 1 Phenotypic change of bovine corneal endothelial cells (BCECs) during *ex vivo* culture indicates endothelial-mesenchymal transition. **A:** Isolated P1 BCECs were plated, and phase-contrast images were captured on days 3 to 14. **B:** Corneal button (*in situ*) and BCECs cultured for the indicated periods were immunostained for active β-catenin (ABC), snail, and slug. Scale bars: 50 μm.

Taiwan University Hospital. The BCECs were cultivated as described previously with modifications.[28] Briefly, fresh bovine eyes were acquired from the local abattoir, disinfected by iodine solution for 3 minutes, and then washed with PBS. The corneal buttons were harvested, and the CECs were peeled with the Descemet's membrane under the dissecting microscope. After digestion with trypsin at 37°C for 30 minutes, the BCECs were collected by centrifugation, seeded into a dish, cultured in supplemented hormonal epithelial medium composed of equal volumes of HEPES-buffered Dulbecco's modified Eagle's medium and Ham F12, supplemented with 5% fetal bovine serum, 0.5% dimethyl sulfoxide, 2 ng/mL human epidermal growth factor, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 1 nmol/L cholera toxin, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. The dish was incubated at 37°C in an atmosphere of 95% air/5% CO_2, and the culture medium was changed every 3 days. When the cells reached confluence, they were trypsinized and passaged at a ratio of 1:3. Cultivated BCECs at passage 1, and a seeding density of 1×10^4 per well in a 24-well plate were used for all experiments. For inhibiting MMP activity, 10 μmol/L of Marimastat was added to the supplemented hormonal epithelial medium through the cell culture or at the indicated time point.

Immunostaining

Cultured BCECs on cover slides were fixed at the indicated time points in 4% paraformaldehyde (pH 7.4) for 30 minutes at room temperature, permeabilized with 0.5% Triton X-100 for 5 minutes, and blocked with 10% bovine serum albumin for 30 minutes. The cells then were incubated with the indicated primary antibodies overnight at 4°C. After washing twice with PBS for 15 minutes, samples were incubated with Alexa Fluor—conjugated secondary antibody (1:100) at room temperature for 1 hour. All samples were counterstained with DAPI at room temperature for 5 minutes. After several washes, all samples were mounted in fluorescent mounting solution (VectA Mount; Vector Laboratories, Burlingame, CA). Immunofluorescent images were obtained using laser scanning confocal microscope (LSM-510; Zeiss, Oberkochen, Germany).

MMP Activity Assay

To determine the MMP activity in the culture media, we used the SensoLyte 520 generic MMP assay kit (Anaspec,

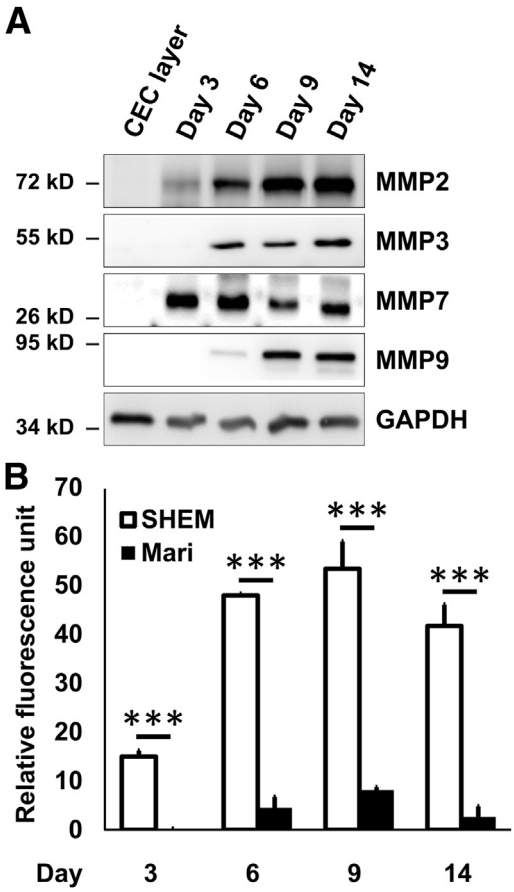

Figure 2 Increased expression and activity of matrix metalloproteinases (MMPs) are noted during *ex vivo* culture of bovine corneal endothelial cells (BCECs). **A:** Total lysates collected from BCECs scratched from the corneal button (CEC layer) and *ex vivo* cultured P1 BCECs were subjected to SDS-PAGE followed by Western blot analysis to assess the expression of MMPs at the indicated time points. **B:** Media collected from *ex vivo* cultured P1 BCECs possess MMP enzymatic activity to cleave fluorogenic substrate that is reduced significantly by the addition of 10 μmol/L Marimastat in the media. The media were collected before changing the culture medium, which was replenished routinely every 3 days. The samples collected at 14 days were 2-day-old culture medium, which thereby showed a slightly lower MMP enzymatic activity compared with the samples from day 9 culture, which contained 3-day-old culture media. The graph represents the means ± SD from three replicates. ***$P < 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Mari, Marimastat; SHEM, supplemented hormonal epithelial medium.

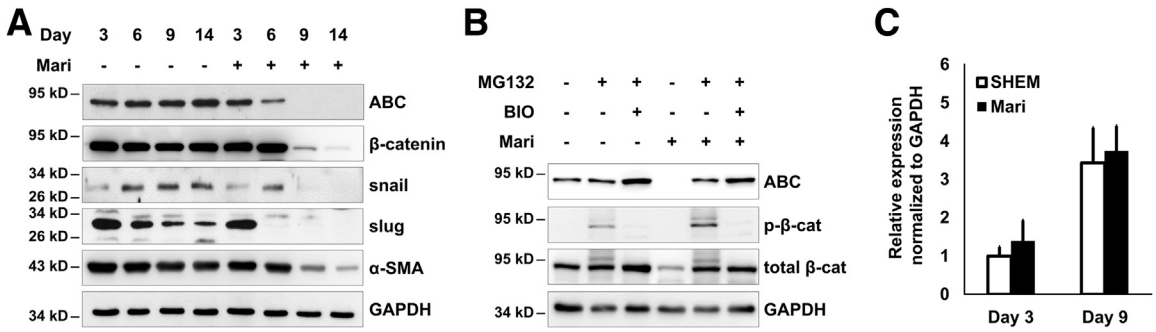

Figure 3 Marimastat induces degradation of active β-catenin (ABC) through phosphorylation in bovine corneal endothelial cells (BCECs). **A:** Total lysates of P1 BCECs with or without 10 μmol/L of Marimastat (Mari) were collected at the indicated time points, and were subjected to SDS-PAGE, followed by Western blot analysis using the indicated antibodies. **B:** BCECs were cultured in the medium with or without 10 μmol/L of Marimastat, and were harvested on day 9. Twenty-four hours before the cell harvest, BCECs were treated with or without 10 μmol/L of MG132, and 5 μmol/L of BIO. The cell lysates were subjected to SDS-PAGE followed by Western blot analysis using the indicated antibodies. **C:** The β-catenin mRNA expression of BCECs cultured with or without Marimastat was examined by quantitative real-time PCR at the indicated time points. The data are presented as relative β-catenin mRNA expression normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in triplicate. Error bars represent SD. p-β-cat, phospho-β-catenin; SHEM, supplemented hormonal epithelial medium; total-β-cat, total β-catenin.

Fremont, CA) according to the manufacturer's instructions. Briefly, the media were collected at the indicated time point and were incubated with the 5-FAM/QXL520 fluorescence resonance energy transfer substrate for 1 hour in a 96-well plate at room temperature. On cleavage by MMPs, the fluorescence of 5-FAM was measured with the excitation at 490 nm and emission at 520 nm.

Protein Extraction and Western Blot Analysis

BCECs were lysed at the indicated time points with RIPA lysis buffer (Pierce Biotechnology, Rockford, IL) containing protease inhibitor cocktail (Roche, Mannheim, Germany) and 0.1% SDS. For membrane protein extraction, the MEM-PER Plus kit was used (Thermo Fisher Scientific, Inc., Waltham, MA). The proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), blocked with blocking solution overnight, and detected by the indicated primary antibodies. After washing and incubation with appropriate secondary antibodies conjugated with horseradish peroxidase, immunoreactive bands were observed by chemiluminescence.

RNA Extraction and Real-Time PCR

For real-time PCR, total RNAs were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and were reverse-transcribed to cDNA by the RevertAid cDNA synthesis kit (Thermo Fisher Scientific, Inc.). Real-time PCR was performed by using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The reactions were performed in a 15 μL volume containing 10 pmol/L of each oligonucleotide primer, SYBR Green Real-Time PCR Master Mixes (Applied Biosystems), and 50 ng of cDNA. The PCR program included initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes. The specificity of the reaction was analyzed by melting curve analysis, and the PCR product was confirmed by size using 2% agarose gels followed by ethidium bromide staining. The sequences of the primer pairs used were as follows: glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-AGGGTCATCATCTCTGCACCTT-3′ and reverse, 5′-TGGTCATAAGTCCCTCCACGATG-3′); β-catenin (forward, 5′-AGAGTGAAGAAGGAGCGGAAGTG-3′ and reverse, 5′-TTTCATCGTTGTCCTGGAGCA-3′).

Rat Corneal Endothelium Damage Model

The rat corneal endothelium damage procedure was performed as described previously with modifications.[29] Twelve-week-old male Sprague-Dawley rats (Lasco, Taiwan) were anesthetized with an intramuscular injection of xylazine (Ropum 2%, 5.6 mg/kg; Bayer Suisse, Lyssach, Suisse) and tiletamine plus zolazepam (Zoletil 50, 18 mg/kg; Virbac, São Paulo, Brazil). Alcaine Ophthalmic Solution (0.5%; Alcon, Fort Worth, TX) was instilled into the right eye of each rat to minimize the blink reflex. A 3-mm−diameter stainless steel probe, cooled with liquid nitrogen, was applied to the central cornea of the right eye for 30 seconds for 3 consecutive days. Both 0.1% atropine and 0.3% gentamicin sulfate were instilled after the procedure. Rats were divided into three groups ($n = 9$ in each group), and received two rounds of intracameral injection. The first injection was performed immediately after the last endothelial damage, and the second injection was performed 3 days later. In the first injection, 0.02 mL of PBS was injected intracamerally into the rats in group 1, whereas intracameral injection of 0.02 mL of 25 ng/mL bFGF was performed in groups 2 and 3. In the second injection, PBS was injected into the rats in groups 1 and 2, whereas 0.02 mL of 10 μmol/L Marimastat was injected into the rats in group 3. During the follow-up evaluation, the corneas were observed and photographed with a slit-lamp microscope, and the corneal thickness was measured by ultrasound biomicroscopy (S-Sharp Corp., New Taipei City, Taiwan). The

animals were euthanized 3 days after the last injection with an overdose of intramuscular thiamylal sodium injection (Shinlin Sinseng Pharmaceutical, Lungtan, Taiwan), and the corneas were harvested for further analysis, including hematoxylin and eosin staining and immunofluorescence staining. Another six rats served as uninjured controls, and also were subjected to the examinations and analysis described earlier.

Statistical Analysis

The data are expressed as means ± SD. Differences between the groups were compared using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

EnMT Occurs during *ex Vivo* Culture of BCECs

After being seeded on the culture plate, the BCECs initially appeared fibroblast-like on days 3 and 6. They then became more hexagonal on reaching complete confluence after day 9 (Figure 1A). To confirm whether the morphologic change reflected the process of EnMT, immunofluorescence staining was performed to detect the subcellular localization of EMT regulators, snail and slug,[30] as well as the unphosphorylated and transcriptionally active ABC. In the excised bovine corneal button, none of the ABC, snail, or slug was detected in the nucleus (Figure 1B). However, during *ex vivo* culture of BCECs, ABC, snail, and slug all were translocated into the nuclei, and were detected there through day 14 (Figure 1B). These results implied that EnMT indeed occurred and that the Wnt/β-catenin pathway was activated during *ex vivo* culture of BCECs.

MMP Activity Is Required for EnMT

Many previous studies have shown that MMPs can trigger the process of EMT.[15–19] To determine the role of MMP in EnMT, we examined the expression of MMPs in BCECs. There was no MMP expression in the BCECs scratched from the excised corneal button (Figure 2A). When cultured *ex vivo*, MMP-2, MMP-3, MMP-7, and MMP-9 were expressed gradually from days 3 to 14 (Figure 2A), which were active enzymatically as assessed in the conditioned media of the cultured BCECs (Figure 2B). The increased MMP activity can be suppressed significantly by incubating the BCECs with Marimastat, a broad-spectrum MMP inhibitor. These results indicated that MMPs were secreted and remained active during *ex vivo* culture of BCECs.

To further investigate the role of MMP activity in the process of EnMT, EnMT key regulators, β-catenin, snail, and slug, as well as EnMT marker α-SMA, were analyzed in BCECs maintained in the presence of Marimastat. Although Western blot analysis showed that the level of ABC, total β-catenin, snail, slug, and α-SMA were overly expressed in BCECs as

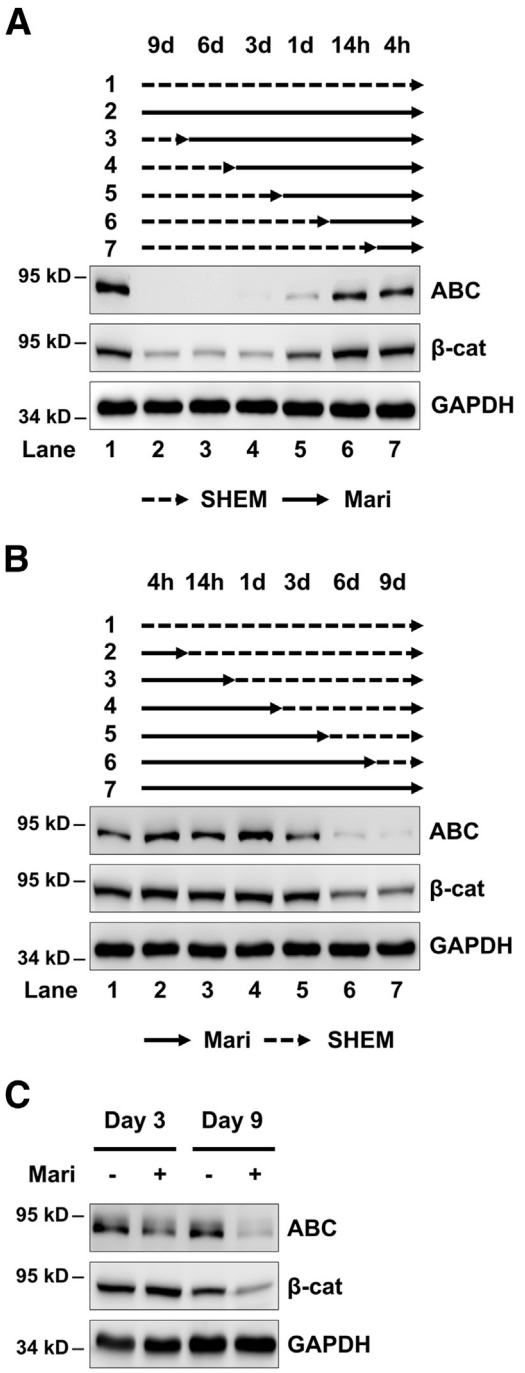

Figure 4 The β-catenin degrading effect of Marimastat depends on the stage of cell culture. **A:** Cells were first cultured in supplemented hormonal epithelial medium (SHEM; **dashed arrow**), and then 10 μmol/L of Marimastat was added to the medium for the indicated duration (**solid arrow**) before the cells were harvested on day 9. Cell lysates were subjected to SDS-PAGE followed by Western blot analysis using the indicated antibodies. **B:** Bovine corneal endothelial cells (BCECs) were prepared similarly by the procedures described in panel **A**, except 10 μmol/L Marimastat was added to the medium for the indicated period (**solid arrow**) before the culture medium was changed to SHEM (**dashed arrow**). **C:** P1 BCECs were harvested on days 3 and 9. One day before cell harvest, 10 μmol/L of Marimastat was added to the medium in the experimental group. Cell lysates were subjected to SDS-PAGE followed by Western blot analysis using the indicated antibodies. ABC, active β-catenin; β-cat, β-catenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

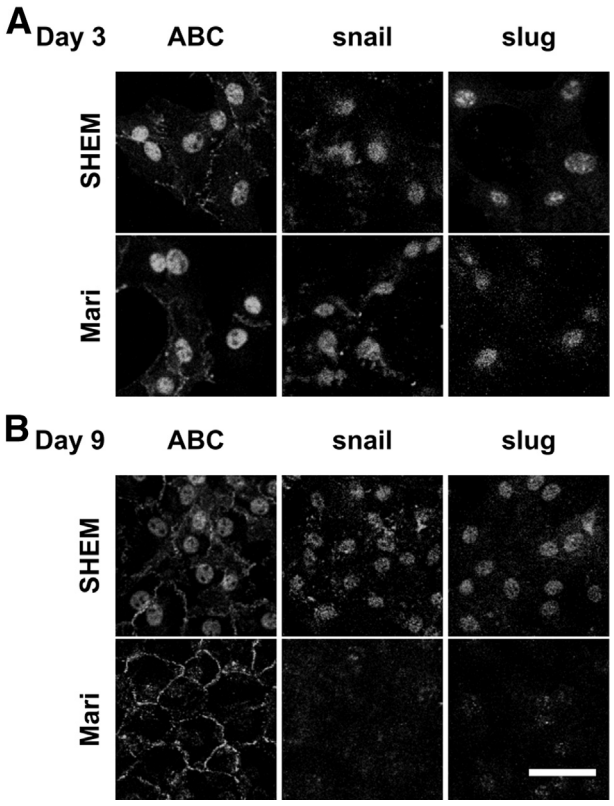

Figure 5 Marimastat reduces nuclear accumulation of active β-catenin (ABC) and epithelial-mesenchymal transition regulators in the late stage of bovine corneal endothelial cell (BCEC) culture. BCECs were cultured for either 3 (**A**) or 9 (**B**) days before being processed for ABC, snail, and slug immunostaining. Marimastat (10 μmol/L) was added to the culture medium 24 hours before harvest in the indicated samples whereas supplemented hormonal epithelial medium (SHEM) served as the negative control. Scale bar = 50 μm.

shown in Figure 1B, the expression of all these EnMT regulators markers was suppressed by Marimastat addition during *ex vivo* culture (Figure 3A). This result signifies the involvement of MMPs in the EnMT process occurring during the *ex vivo* culture of BCECs. According to previous studies, the degradation of ABC mainly is through sequential phosphorylation by casein kinase-1α and glycogen synthase kinase-3β.[31,32] To determine the mechanism that leads to diminished ABC and total β-catenin levels, we harvested *ex vivo* cultured BCECs pretreated with or without Marimastat on day 9, and analyzed the level of phospho-Ser33/Ser37/Thr41 β-catenin. Figure 3B shows that the level of ABC was reduced after incubating BCECs with Marimastat. However, the phospho-β-catenin could not be detected whether Marimastat was added or not. Because phospho-β-catenin is known to undergo subsequent ubiquitination by β-transducin repeat-containing protein, a ubiquitin E3 ligase, and then degradation by proteasome,[33] we incubated BCECs with the proteasome inhibitor MG132 for 24 hours, and indeed found that the level of phosphorylated β-catenin significantly increased in BCECs by Marimastat. To validate that β-catenin was phosphorylated by GSK-3β, BCECs were pretreated with the GSK-3β inhibitor BIO for 24 hours. Phospho-β-catenin could not be detected in the presence of BIO (Figure 3B). The decreased β-catenin expression by MMP inhibition does not seem to result from a secondary effect on down-regulated transcription because quantitative real-time PCR analysis showed no significant difference in β-catenin gene expression with or without Marimastat (Figure 3C). Collectively, these results indicated that inhibition of MMP activity can reverse the process of EnMT in *ex vivo* cultured BCECs, which results from activation of the Wnt/β-catenin signaling pathway.

The Effect of Marimastat Is Related to Cellular Confluence

As mentioned previously, Marimastat inhibited the phenomenon of EnMT in BCECs through facilitating β-catenin degradation. Interestingly, both ABC and β-catenin levels were highly maintained despite Marimastat treatment on day 3 (approximately 50% confluence), whereas it became apparent on day 6 (approximately 80% confluence), and significant on day 9 when the cells became completely confluent (Figure 3A). To investigate the time point and the incubation duration required for Marimastat to exert its effect, we incubated BCECs with Marimastat for different durations before cells were harvested for further analysis on day 9. When BCECs were incubated with Marimastat for a short duration of either 4 or 14 hours immediately before harvest, both ABC and total β-catenin levels remained high (Figure 4A). However, ABC diminished significantly when BCECs were incubated with Marimastat for more than 1 day (Figure 4A). In contrast, when Marimastat was added from the beginning of cell culture, ABC degradation could be noted only when Marimastat was added for more than 6 days (Figure 4B). These results suggest that the cellular confluence might modulate the effect of Marimastat on the ABC degradation and EnMT. To define the role of cellular confluence for the effect of Marimastat definitively, we harvested the BCECs on days 3 and 9 when cells reached 50% and 100% confluence, respectively, and BCECs were incubated with Marimastat for 24 hours before cell harvest. The levels of ABC with or without Marimastat pretreatment did not differ significantly on day 3 (Figure 4C). In contrast, total β-catenin and ABC on day 9 decreased significantly with Marimastat treatment. These results imply that MMP regulation of the EnMT process depends on cellular confluence.

Imaging studies further showed that ABC, snail, and slug still were evident in the nucleus of BCECs even when Marimastat was added on day 3 (Figure 5). However, Marimastat significantly decreased the nuclear staining of ABC, snail, and slug on day 9 when the BCECs became fully confluent (Figure 5). Taken together, these results indicate that Marimastat facilitates ABC degradation and thereby inhibits EnMT when cellular confluence is reached.

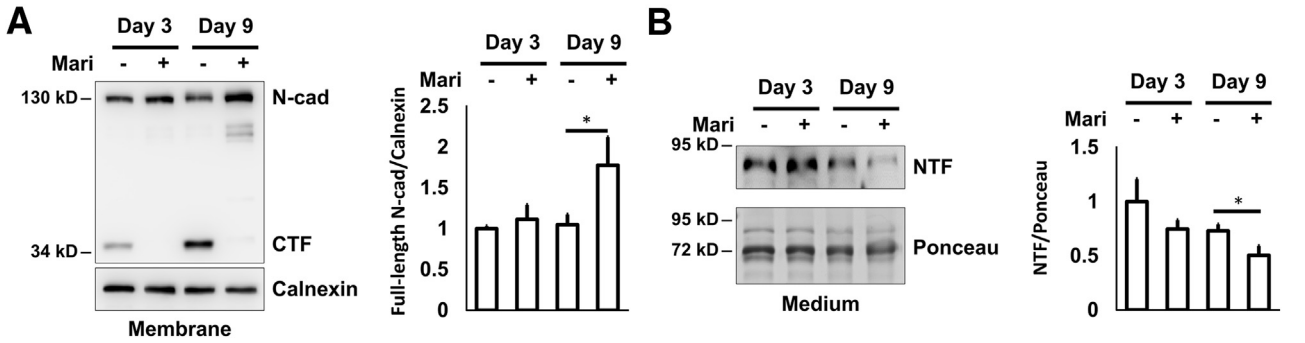

Figure 6 Marimastat inhibits the cleavage of N-cadherin. **A:** Marimastat (10 μmol/L) was added 24 hours before bovine corneal endothelial cells (BCECs) were harvested at the indicated time points, and membranous proteins were extracted and subjected to SDS-PAGE followed by Western blot analysis using the antibody against C-terminus of N-cadherin. The relative expression levels of full-length N-cadherin were normalized to Calnexin, a membrane protein control, and then set the value of control BCECs on day 3 as one. The graph represents the means ± SD from three replicates. **B:** Culture media were changed to serum-free media with or without 10 μmol/L Marimastat 24 hours before harvesting the supernatants. Then the supernatants were harvested at the indicated time points, and were subjected to SDS-PAGE followed by Western blot analysis using the antibody against N-terminus of N-cadherin and nonspecific protein staining by Ponceau. The differences of N-cadherin expression in the medium were assessed by normalization of the signal from the Western blot analysis to the Ponceau staining intensity, and then set the value of control BCECs on day 3 as one. The graph represents the means ± SD from three replicates. $*P < 0.05$. CTF, C-terminal fragment of N-cadherin; N-cad, N-cadherin; NTF, N-terminal fragment of N-cadherin.

Marimastat Inhibits EnMT through Inhibition of N-Cadherin Cleavage

Because of the obvious involvement of cellular density in the effect of Marimastat on accelerating ABC degradation and reversing EnMT, we suspect that cadherin family proteins might be the molecular target for Marimastat. Previous studies have reported that the molecules of cellular contacts might be active regulators of EMT.[34] For example, cadherin-based cellular adhesion is known to be able to promote the phosphorylation and degradation of β-catenin.[35,36] Among the different cadherin family members, N-cadherin is known to be regulated through dynamic post-translational proteolysis.[19] Therefore, we hypothesized that Marimastat may regulate the EnMT of BCECs through modulating the expression pattern of N-cadherin, the major cadherin of corneal endothelial cells.[37] To this end, we performed membrane protein extraction followed by Western blot analysis for N-cadherin. The result showed that Marimastat increased the level of full-length N-cadherin on the membrane, especially on day 9 BCEC culture (Figure 6A). Correspondingly, the 40-kDa C-terminal fragment, a degradation product of full-length N-cadherin, was found only in BECEs cultured with supplemented hormonal epithelial medium, but became undetectable in the presence of Marimastat. Furthermore, by using antibody against the N-terminal epitope of N-cadherin, we detected the 90-kDa N-terminal fragment, the proteolytically released ectodomain of N-cadherin, in the medium, which was decreased significantly by Marimastat on day 9 culture of BCECs (Figure 6B). Intriguingly, the effect of Marimastat on diminishing N-terminal fragment was not apparent on day 3, as compared with that on day 9, corresponding to a time point when the EnMT process of BCECs was shown previously to be relatively refractory to MMP inhibition (Figures 4 and 5). These results indicated that Marimastat can inhibit N-cadherin cleavage during *ex vivo* culture of BCECs, thereby exerting its EnMT-reversing effect.

Marimastat Suppresses bFGF-Induced EnMT *in Vivo*

A significant number of studies have established that bFGF can stimulate the proliferation of CECs, but the accompanying EnMT process through the Wnt/β-catenin pathway jeopardizes further therapeutic implication of bFGF in expanding CECs for clinical application.[27,38,39] To evaluate the EnMT reversing effect of Marimastat *in vivo* and explore its potential in the clinical setting, we performed intracameral injection of bFGF followed by Marimastat in a rat corneal endothelium damage model. The size of the central corneal bullae and the area of microcystic edema significantly decreased after intracameral injection of bFGF followed by Marimastat compared with bFGF or PBS alone (Figure 7A). H&E staining also showed an intact corneal endothelial layer with less corneal edema with Marimastat (Figure 7B). Ultrasound biomicroscopy showed that the corneal thickness was decreased in the bFGF/Marimastat-treated group compared with the bFGF or PBS group, presumably resulting from the water pumped out by healthy corneal endothelia (Figure 7C). Microscopically, Marimastat injection greatly decreased the intranuclear staining of ABC and the cytoplasmic staining of α-SMA induced by bFGF, and restored the hexagonal morphology of CECs (Figure 7D). These results indicate that Marimastat reversed EnMT and restored the phenotype and functionality of corneal endothelium *in vivo*.

Discussion

CECs are noted for their EnMT propensity during cell proliferation, which severely compromises the possibility

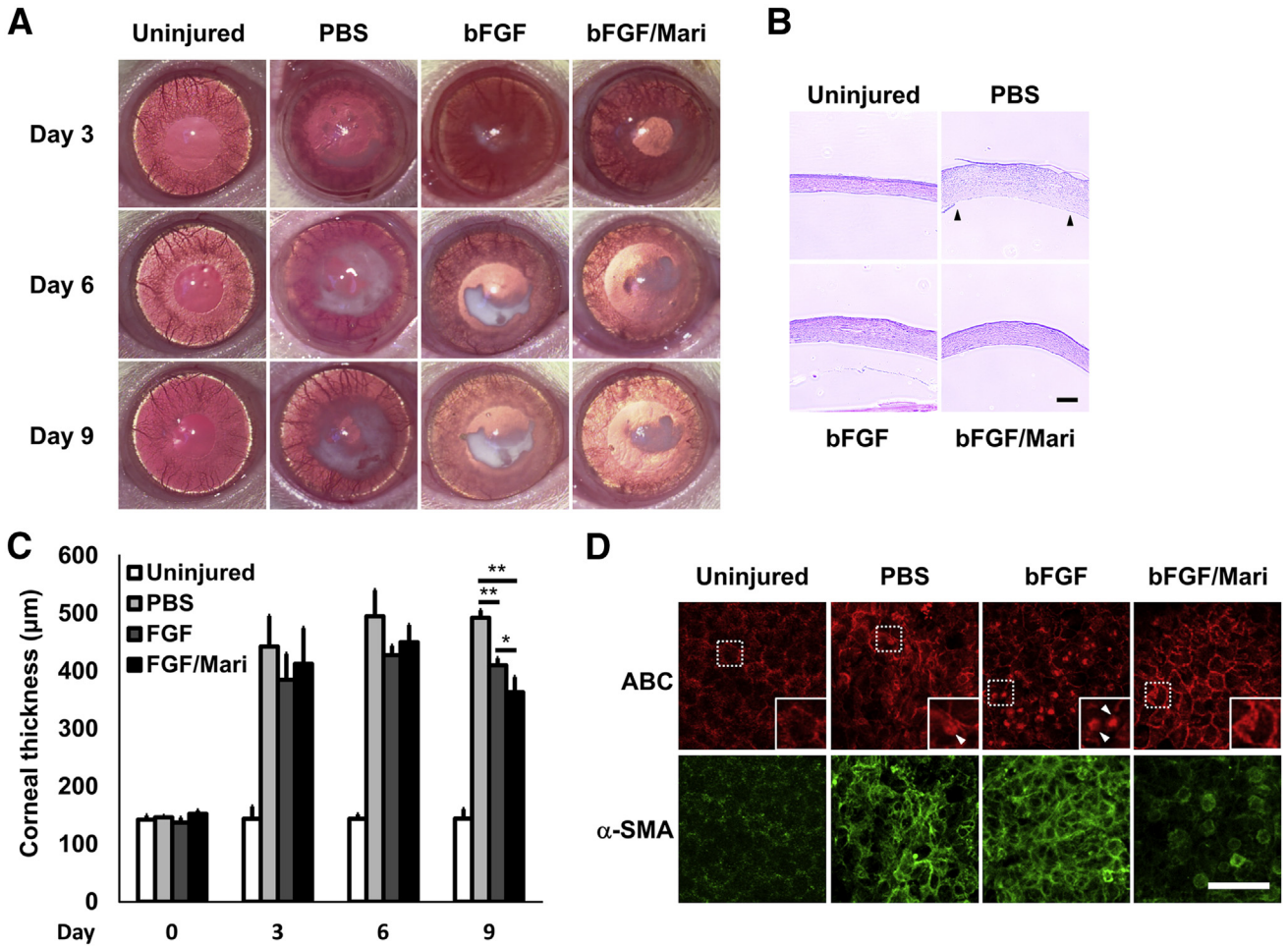

Figure 7 Marimastat suppresses bFGF-induced endothelial-mesenchymal transition *in vivo*. The rat corneas were subjected to cryo-injury for 3 consecutive days (days 0 to 3). The phosphate-buffered saline (PBS) group ($n = 9$) received two rounds of 0.02 mL PBS injection on days 3 and 6, separately. The basic fibroblast growth factor (bFGF) group ($n = 9$) received 0.02 mL of 50 ng/mL bFGF injection on day 3 followed by PBS injection on day 6. The bFGF/Mari group ($n = 9$) received 0.02 mL of 50 ng/mL bFGF injection on day 3 followed by 0.02 mL of 10 μmol/L Marimastat injection on day 6. Uninjured corneas ($n = 6$) served as controls. **A:** Representative external eye photographs from each group at the indicated time points. Day 3: immediately after 3 consecutive days of cryo-injury. Day 6: 3 days after the first intracameral injection. Day 9: 3 days after the second intracameral injection. **B:** Hematoxylin and eosin staining from the day 9 corneas shows the corneal endothelium defect in the PBS group (**arrowheads**) and the intact endothelial layer in the bFGF and bFGF/Mari groups. **C:** Rat corneal thickness was measured by an ultrasound biomicroscope before cryo-injury (day 0) at the indicated time points. The graph represents the means ± SD. **D:** Immunofluorescence staining of the rat corneal buttons harvested at day 9 using the indicated antibodies. **Insets: Arrowheads** point to nuclear staining of active β-catenin (ABC). $*P < 0.05$, $**P < 0.01$. Scale bars: 200 μm (**B**); 50 μm (**D**). α-SMA, α-smooth muscle actin.

of clonal expansion of CECs for therapeutic purposes.[11] As a result, reversing EnMT is mandatory to preserve the normal phenotype and polarized property for CECs to perform essential cellular functions. In this study, we found that BCECs prepared by trypsinization underwent EnMT during *ex vivo* culture. Consistent with the previous study, we observed that ABC also showed nuclear translocation during *ex vivo* culture of CECs, indicating the involvement of the Wnt/β-catenin signaling pathway.[27] Interestingly, we found that the expression and activity of MMPs increased during *ex vivo* expansion of CECs, and Marimastat, a broad-spectrum MMP inhibitor, not only suppressed the activity of MMPs secreted by BCECs, but also the expression levels of ABC, snail, slug, and α-SMA. Furthermore, we found that the phosphorylation of β-catenin by GSK-3β and subsequent degradation was increased with Marimastat, which lead to blockade of the Wnt/β-catenin signaling pathway and reversal of EnMT in CECs.

It has been shown that MMPs can initiate EMT through cleavage of cell—cell junction protein.[40,41] For example, the ectodomain of N-cadherin, the major junctional protein on CECs, is reported to be cleaved by many MMPs, including MMP-2, MMP-7, MMP-9, MMP-12, MT1-MMP, MT5-MMP, and ADAM-10,[18,42–46] and the biological consequence is multifold. After the proteolytic cleavage, the cell adhesion is lost, and the soluble ectodomain fragments released from the cell surface then interfere with the homophilic interaction of the adjacent cadherin molecules, or even trigger a new signaling pathway.[47] Moreover, Maher et al[35] showed that E-cadherin—based adhesion can enhance the sequestration

of β-catenin and its phosphodestruction. Similarly, Shoval et al[46] showed that overexpression of full-length N-cadherin can antagonize the EMT properties of neural crest cells. In this study, our data showed that Marimastat inhibited the cleavage of N-cadherin, decreased the level of C-terminal and N-terminal fragments, and increased the level of full-length N-cadherin on the cell membrane, which may enhance the cell adhesion and further contribute to reverse EnMT through β-catenin degradation and inhibit any further signaling arising from the proteolytic by-products of N-cadherin.

Surprisingly, Marimastat could facilitate only β-catenin degradation when BCECs reached confluence, but showed little effect in inhibiting N-cadherin cleavage in the subconfluent cellular state. This result strongly implies the existence of a MMP-independent mechanism that governs the N-cadherin shedding in the subconfluent BCECs. Indeed, Jang et al[48] showed that in cortical neuron cells, N-cadherin cleavage is regulated by calpain, a calcium-dependent cysteine protease, instead of MMPs. Furthermore, the behavior of EMT also may be regulated by cell confluence because previous studies have shown that the integrity of cell−cell contacts determines the response to transforming growth factor-β1−induced EMT through Rho/Rho kinase-mediated myosin light-chain phosphorylation.[34,49] Apparently, more studies are pending to elucidate the signaling pathway leading to EnMT in subconfluent BCECs.

To validate the EnMT reversing effect *in vivo*, we generated cryo damage on rat corneal endothelium and performed an intracameral injection of Marimastat. As mentioned previously, Marimastat only reverses EnMT when cells reach confluence. Therefore, we injected bFGF first to accelerate the healing of corneal endothelium by stimulating the EnMT process, and then injected Marimastat to reverse EnMT. In the bFGF only group, although the corneal endothelial defect could be filled by regenerated CECs, it was associated with significant corneal edema, indicating poor pumping function of CECs. On the contrary, Marimastat injection subsequent to bFGF treatment greatly dissipated the corneal edema, and the final corneal thickness was decreased further compared with bFGF injection alone. These results suggest that the combination of growth-stimulating agents and an EnMT-reversing agent such as Marimastat offers a promising therapeutic option to combat corneal endothelial dysfunction.

Given the importance of regulating the extent of EnMT in repairing corneal injury, a number of strategies have been used to reverse EnMT. Li et al[50] showed that rat CECs undergo EnMT after serial passages *in vitro*, which is dominated by the Notch signaling pathway, and can be reversed by the Notch inhibitor. Okumura et al[51] found that the fibroblastic phenotype of *ex vivo* cultured primate CECs shows augmented transforming growth factor-β signaling, which can be counteracted by a selective inhibitor. In our study, we have shown that EnMT in BCECs can be reversed by Marimastat, a broad-spectrum MMP inhibitor. Our data not only provide further insight regarding the mechanistic role of MMPs in EnMT, but also offer a new therapeutic target to regenerate phenotypically and functionally normal corneal endothelium.

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