Transmembrane Mucin 1 Blocks Fluorescein Ingress to Corneal Epithelium

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PURPOSE. To determine the role of transmembrane mucins in blocking fluorescein ingress to the corneal epithelium and its deficiency in contributing to corneal fluorescein punctate staining.

METHODS. A dry eye model was established by extirpating lacrimal and Harderian glands in rabbits to correlate the expression of mucins with fluorescein-stained areas on the corneal button using immunofluorescence. Expression of transmembrane mucins was promoted in human corneal epithelial cells (HCECs) by culturing with the mucin-promoting medium (MPM) or diquafosol treatment. Conversely, the expression of mucins was downregulated by knockdown with short hairpin RNA. The role of mucin1 extracellular domain in fluorescein ingress was further verified by overexpression of N-terminally truncated mucin1 in HCECs.

RESULTS. In the rabbit dry eye model, the expression level of mucin1 was significantly decreased in superficial corneal epithelial cells where fluorescein punctate staining was observed. Upregulation of mucin1 and mucin16 in HCECs promoted by MPM or by diquafosol treatment impeded intracellular fluorescein ingress. Downregulation of mucin1 and mucin16 enhanced fluorescence ingress in HCECs after fluorescein staining. Overexpression of truncated mucin1 did not alter the fluorescein intensity of fluorescein-stained HCECs, supporting the notion that the ability of mucin1 to block fluorescein ingress was primarily mediated by its extracellular domain. Minimal inherent expression of mucin16 in the rabbit cornea limited the validation of its role in blocking fluorescein ingress in vivo.

CONCLUSION. Transmembrane mucin1 blocks fluorescein ingress in the corneal epithelium, explaining how fluorescein staining is positive when the level of transmembrane mucins is disturbed in dry eyes.

Keywords: corneal epithelium, fluorescein, MUC1, transmembrane mucin

The ocular surface, comprising corneal, limbal, and conjunctival epithelia and a tear film, is vital to maintaining optical clarity of the eye. Among them, the tear film is essential for a healthy ocular surface.[1] The tear film consists of different layers, of which the outermost lipid layer—secreted by Meibomian glands—prevents the aqueous-mucin layer from evaporation, and the inner mucin layer contribute to stabilization of tear film on the corneal epithelium.[2,3] The mucins in the tear film are of two major forms, including the secreted forms that are released by conjunctival goblet cells and the transmembrane form that are expressed along the apical surface of corneal and conjunctival epithelial cells.

The corneal transmembrane mucins, predominantly the MUC1, MUC4, and MUC16, are large glycoproteins transported through the endoplasmic reticulum and Golgi apparatus to the cell membrane for exocytosis. These glycoproteins carry a variable number tandem repeat region and a sea-urchin sperm protein, enterokinase and agrin domain in the extracellular domain; a transmembrane-spanning domain; and a short cytoplasmic domain.[4] Like all other transmembrane mucins, the extracellular domain of MUC1 constitutes the glycocalyx that hydrates, lubricates, and protects the apical epithelial surface, whereas its cytoplasmic tail additionally participates in cell signaling. Consequently, transmembrane mucins are required to maintain the tear film stability, which is essential for the junctional integrity

of corneal epithelial cells. This notion explains why mucin deficiency owing to the loss of transmembrane mucins contributes to ocular surface deficits in such diseases as dry eye disease,[5–7] corneal erosion,[8] Sjogren's syndrome,[9,10] bacterial conjunctivitis,[11] Stevens-Johnson syndrome,[12] and ocular cicatricial pemphigoid.[13]

Clinically, the corneal epithelial integrity is routinely assessed by fluorescein staining, which is traditionally believed to arise from disruption of tight junctions of the superficial corneal epithelial cells, allowing trapping of fluorescein dye in the intercellular gap.[14–16] Nonetheless, our previous study has shown that fluorescein ingress into the corneal epithelial cells can also occur through the monocarboxylate transporter.[17] Herein, we used both in vivo and in vitro approaches to examine whether the expression of transmembrane MUC1 is causally linked to blocking of fluorescein ingress through corneal epithelial cells as another working mechanism to explain how fluorescein punctate staining might develop in dry eye diseases.

METHODS

Rabbit Dry Eye Model

The experimental procedures to establish the rabbit dry eye model were approved by the Committee for Animal Research of the National Taiwan University Hospital (protocol code no. 20190265) and Taipei Tzu Chi General Hospital (108-IACUC-031) and followed the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This model involved surgical excision of both lacrimal glands and Harderian gland of the right eye of New Zealand albino rabbits (3.0–3.5 kg, six months old) under general anesthesia induced by intramuscular injections of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). A sham surgery was performed on the left eye. After the above survival surgery, the status of the corneal epithelium was assessed by fluorescein staining using fluorescein ophthalmic strips (CH3098; Haag Streit, Köniz, Switzerland) after topical 0.5% proparacaine hydrochloride (Alcaine; Alcon Laboratories, Ft. Worth, TX, USA). Afterward, the corneal button was instantly excised and submitted for cryosection and immunofluorescent staining.

Cryosection and Immunofluorescence Staining

Rabbit corneas were embedded in FSC 22 Frozen Section Media (no. 3801481; Leica, Wetzlar, Germany) for cryosection at 5 μm. The cryosections were fixed with acetone for 10 minutes at 4°C, washed in running tap water for 20 minutes and blocked in phosphate buffered saline solution (PBS) with a 2% normal goat serum for 30 minutes at room temperature. Notably, antigen retrieval was not performed as this procedure may quench the fluorescence of fluorescein staining. The cryosections were then stained with a biotin-conjugated anti-rabbit MUC1 rabbit antibody (no. NBP1-60046B; Novus Biologicals, Littleton, CO, USA), anti-ZO-1 (no. 33-9100; ThermoFisher Scientific, Waltham, MA, USA), or anti-galectin-3 (GAL3) mouse antibody (no. A3A12, Novus Biologicals) for one hour followed by incubation in a Texas Red conjugate of NeutrAvidin biotin-binding protein (no. A2665; Invitrogen, Carlsbad, CA, USA), Alexa Fluor 594 goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (no. A-11012; Invitrogen), or Alexa Fluor

564-conjugated goat anti-mouse IgG antibody (no. A-11003) for one hour in the darkness. Nuclear counterstaining was performed with Hoechst 33342 (no. 62249; ThermoFisher Scientific). Image acquisition was performed using a Zeiss LSM 510-META Confocal laser scanning microscope.

Immunohistochemistry

The expression of MUC1 was examined by immunohistochemistry using the aforementioned 5-µm cryosections. After antigens were retrieved by a Tris-Based antigen unmasking solution (no. H-3301; Vector Laboratories, Burlingame, CA, USA) for one minute, a BLOXALL Endogenous Blocking Solution (no. SP-6000; Vector Laboratories) was used to inactivate endogenous peroxidase, pseudoperoxidase, and alkaline phosphatase. A Streptavidin/Biotin Blocking Kit (no. SP-2002; Vector Laboratories) was used to block all endogenous biotin, biotin receptors, and streptavidin binding sites. Then, sections were blocked in PBS with a 2% rabbit serum for 30 minutes at room temperature and stained with a biotin-conjugated anti-rabbit MUC1 rabbit antibody (no. NBP1-60046B; Novus Biologicals) at 4°C overnight. Detections were done with a Vectastain Elite ABC-HRP Kit (no. PK-6100; Vector Laboratories) for 30 minutes at room temperature. Sections were developed using the ImmPACT DAB Substrate (no. SK-4105, Vector Laboratories) and counter-staining with hematoxylin.

Cell Culture and Treatment

HCECs (ATCC CRL-11515) were cultured in keratocyte serum-free medium (KSFM; no. 17005-042; ThermoFisher Scientific) supplemented with 5 µg/mL human recombinant insulin (no. 12585-014; ThermoFisher Scientific), 500 ng/mL hydrocortisone (no. H0135; Sigma-Aldrich Corp., St. Louis, MO, USA) and 1 × anti-biotic/anti-mycotic (no. 15240-062; ThermoFisher Scientific) and passaged every four days when the cells reached 80% confluence. To induce transmembrane mucin expression, HCECs were cultured in a mucin-promoting medium (MPM), that is, Dulbecco's modified Eagle medium/nutrient mixture F-12 (no. 11330-032; ThermoFisher Scientific), 1 mM $CaCl_2$ (no. C7902; Sigma-Aldrich Corp.), 10% fetal bovine serum (no. 10438-028, ThermoFisher Scientific), 10 ng/mL EGF (no. PHG0311, ThermoFisher Scientific) and 1× anti-biotic/anti-mycotic (no. 15240-062, ThermoFisher Scientific) with the medium changed every two days. For cells cultured in KSFM or MPM, expression of transmembrane mucins was further promoted by addition of 1 mM sodium diquafosol (3% ophthalmic solution, Diquas; Santen, Osaka, Japan) for four or six days with the medium changed every two days.

Short Hairpin Knockdown and Overexpression

Short hairpin (shRNA) to knockdown the MUC1 or MUC16 was performed using MUC1 or MUC16 shRNA cloning lentivectors, which were obtained from the National C6 RNAi Core Facility at Academia Sinica, Taiwan. The target sequences were as follows: shMUC1-1, CCACCAATTTCTCG-GACACTT; shMUC1-2, GACACAGTTCAATCAGTATAA; shMUC16-1, AGCCACCTCATCTATTACCTTCAAGAGAG-GTAATAGATGAGGTGGCT; shMUC16-2, CTGCATGTACTC-CCATCTCTTCAAGAGAGAGATGGGAGTAGATGCAG[18]; and shLuc, GCGGTTGCCAAGAGGTTCCAT. The expression of the truncated MUC1 was performed using MUC1 cDNA ORF

Clone, human (HG12123-UT, SinoBiology) and Lipofectamine 3000 Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

Flow Cytometry

To simultaneously detect the fluorescein ingress and the expression of transmembrane MUC1, HCECs that had been maintained in KSFM or MPM were shifted to culture condition with 2 mM fluorescein-containing KSFM or MPM, respectively, for subsequent cell incubation at 37°C for 15 minutes. Cells were then washed with warm PBS twice, harvested with TrypLE Express Enzyme (1×), and resuspended in PBS containing 1% bovine serum albumin (BSA) in a 1.5 mL Eppendorf tube. The cell suspension was stained with anti-MUC1 antibody [EP1024Y] (ab45166; Abcam, Cambridge, MA, USA) for one hour at room temperature and fixed in 2% paraformaldehyde for 15 minutes at room temperature. To detect the total mucin 1 (including intracellular and membrane bound mucin1), the cell suspension was stained with anti-MUC1 antibody [HMFG2] (ab245693; Abcam) for one hour at room temperature and fixed in 2% paraformaldehyde for 15 min at room temperature. After being washed with 1% BSA/PBS, the cell suspension was incubated with a goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 647 (A21244; Invitrogen) for 30 minutes at room temperature. To detect the expression of K3, K12 or vimentin, cell suspensions were fixed in 2% paraformaldehyde for 15 minutes at room temperature before being stained with an anti-cytokeratin 3/CK-3 antibody [AE5] (ab77869; Abcam), anti-keratin 12/K12 antibody [EPR17882] (ab185627, Abcam), or an anti-vimentin (D21H3) antibody (CS no. 5741, cell signaling) for one hour at room temperature. To detect the expression of GAL3, cell suspensions were fixed and incubated with anti-galectin-3 (GAL3) mouse antibody (no. A3A12; Novus Biologicals). Then, the cell suspensions were incubated with a goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, and an Alexa Fluor 647 (A21244, Invitrogen) and goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (A11001, Invitrogen) for 30 minutes at room temperature. Flow cytometry was performed with BD LSRFortessa Flow Cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Data were analyzed using the FlowJo software program.

Quantitative RT-PCR and PCR

The expression of MUC1, MUC4 and MUC16 in HCECs was determined by quantitative RT-PCR and PCR. The total RNA extraction was performed by using a TRI reagent (no. TR118; Molecular Research Center, Cincinnati, OH, USA), and the cDNA was synthesized by using a High-Capacity cDNA Reverse Transcription Kit (no. 4368814, Applied Biosystems) according to the manufacturer's protocol. Quantitative RT-PCR was performed on the QuantStudio 3 System (Applied Biosystems) using the Maxima SYBR Green/ROX qPCR Master Mix (2X) (no. K0221; ThermoFisher Scientific). Relative quantities of messenger RNA (mRNA) expression of the respective genes were normalized with a GAPDH housekeeping gene. PCRs were performed by using a GoTaq Green Master Mix (no. M712B; Promega Corporation, Madison, WI, USA). The primers used in the quantitative RT-PCR and PCR are as follows: MUC1 forward-5′ TCCTTTCTCTGCCCAGTCTG 3′ and reverse-5′ CAGCTGCCCGTAGTTCTTTC 3′; MUC4 forward-5′ CCTCTCTCCGTGATTCCCTC 3′ and reverse-5′ AAGTCAGCATCGTCCCAGAA 3′; MUC16 forward-5′ GCCTCTACCTTAACGGTTACAATGAA 3′ and reverse-5′ GGTACCCCATGGCTGTTGTG 3′; GAPDH forward-5′ GAGAAGTATGACAACAGCCT 3′ and reverse-5′ ATACCAAAGTTGTCATGGAT 3′; beta-actin forward-5′ CACCATTGGCAATGAGCGGTTC 3′ and reverse-5′ AGGTCTTTGCGGATGTCCACGT 3′.

Western Blot

HCECs were lysed in Pierce RIPA Buffer (no. 89901; ThermoFisher Scientific) containing a 1× Halt Protease and Phosphatase Inhibitor Cocktail (no. 78440; ThermoFisher Scientific). The protein concentration was measured by a Pierce Rapid Gold BCA Protein Assay Kit (no. A53226; ThermoFisher Scientific). An equal amount of protein was boiled in a T-Pro Laemmli (SDS sample) Reagent (no. JB06-F002; T-Pro Biotechnology, Zhonghe, Taiwan), separated by 10% SDS/PAGE, and transferred onto Amersham Hybond P Western blotting membranes, PVDF (no. 10600023; GE Healthcare Life Sciences, Chicago, IL, USA). The membranes were incubated for 1 h in a blocking buffer (Tris-buffered saline solution [TBS] and 5% BSA) and then probed by overnight incubation at 4°C with either anti-MUC16 (no. SC-365002; Santa Cruz Biotechnology, Dallas, TX, USA) or anti-beta-actin (no. ab8226; Abcam) antibody. After being washed in Tris-buffered saline solution–T, the blot was incubated with horseradish peroxidase-conjugated secondary antibodies and detected by the ECL Select Western Blotting Detection Reagent (no. RPN2235; GE Healthcare Life Sciences).

RESULTS

Punctate Fluorescein Staining Correlates With Decreased MUC1 Expression in Superficial Corneal Epithelial Cells in a Rabbit Dry Eye Model

To simulate corneal fluorescein staining in dry eye patients, we created a rabbit dry eye model by surgical excision of both lacrimal glands and Harderian glands.[19] As shown in Figure 1A, rabbit corneas of the experimental group exhibited dry corneal surface (under bright light), multiple fluorescein punctate staining (under cobalt blue light), and superficial corneal epithelial erosion (under a slit beam) three to four weeks after surgery. The immunohistochemistry and immunofluorescence of the frozen sections prepared from their corneal buttons showed that removal of lacrimal glands decreased the MUC1 expression in the superficial epithelial layer (Fig. 1B, left and middle), where positive fluorescein staining was observed in the experimental group but not in the sham-operated group by immunofluorescence (Fig. 1B, right). Notably, the punctate staining was made up of fluorescein-incorporated cells, as evidenced by the colocalization of DAPI nuclear staining within most, if not all, fluorescent spots. These findings were consistently observed in other experimental replicates (Supplementary Fig. S1). To exclude the possibility that corneal fluorescein punctate staining is caused by the loss of tight junction integrity that permits penetration and trapping of fluorescein in the paracellular (intercellular) space, we performed immunostaining of ZO-1, a tight junction-associated protein, to examine the cellular junction of fluorescein-stained cornea of the experimental group. Our results showed that the integrity of tight junction was intact
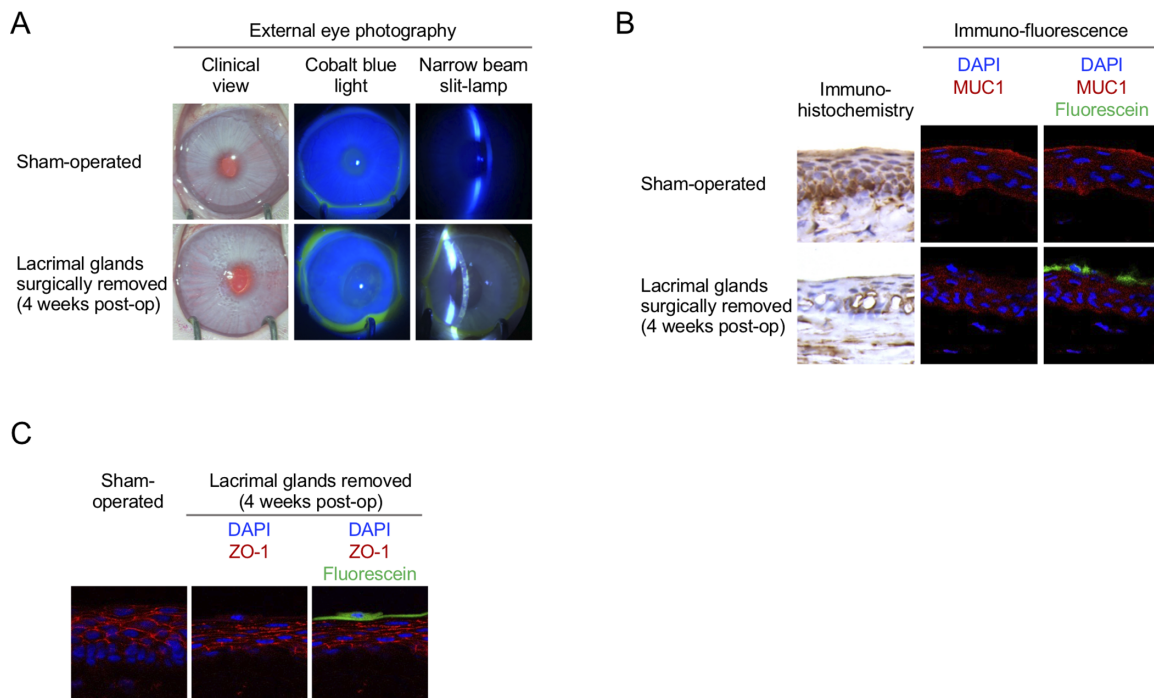
Figure 1. Fluorescein punctate staining are the fluorescein-incorporated corneal epithelial cells of the superficial layer featured with decreased MUC1 expression. **(A)** The clinical or slit lamp view under cobalt blue light of the eyes of sham-operated rabbit model (lower panel) or dry eye rabbit model (*upper panel*) following fluorescein staining. **(B)** The fluorescein-stained corneal epithelia prepared as the frozen tissue sections were stained with MUC1 for immunohistochemistry (*brown*, *left panel*) and immunofluorescence (*middle* and *right panel*) analyses. For immunofluorescence, dual channels (*red* and *blue*, *middle panel*) or triple channels (*red*, *blue*, and *green*, *right panel*) were used. The cell nuclei were counterstained with hematoxylin (*purple*, immunohistochemistry) or DAPI (*blue*, immunofluorescence). **(C)** The frozen corneal epithelia section prepared from sham-operated and lacrimal gland-removed rabbits were immunostained with ZO-1 (*red*), nuclear counterstained with DAPI (*blue*), and the fluorescein ingress (*green*) were analyzed through a confocal microscopy. (*red* and *blue* dual channels, *middle panel*; *red*, *blue*, and *green* triple channels, *right panel*).

(Fig. 1C), suggesting that the positive staining might not be caused solely by disruption of tight junctions.

Upregulation of MUC1 Expression by Superficial Corneal Cells Decreases Fluorescein Ingress

The transmembrane mucins (i.e., MUC1, MUC4, and MUC16) are high molecular weight glycoproteins (200 kDa to 200 MDa).[20] To explore the role of transmembrane mucins in regulating fluorescein ingress into corneal epithelial cells, we modulated the expression of these transmembrane mucins by switching from KSFM to a Dulbecco's modified Eagle medium–based MPM, which is known to promote the expression of transmembrane mucins of human corneal epithelial cell line.[21–23] Specifically, HCECs were plated in MPM for four or seven days until harvest. Alternatively, HCECs were seeded in KSFM for two days and subsequently in MPM for two or five days before harvest. We found that the levels of MUC1, MUC4, and MUC16 transcripts were upregulated in MPM-cultured HCECs (Fig. 2A) and the protein expression of MUC1 was significantly upregulated as determined by immunostaining intensity in flow cytometry (Fig. 2B). To examine whether the increased MUC1 expression would interrupt fluorescein ingress, HCECs were incubated with the anti-MUC1 antibody before cell fixation for flow cytometry analysis; therefore antibodies cannot bind to intracellular molecules, and the antibody signals may indicate the amount of MUC1

expressed on the cell membrane. We showed that the majority of HCECs expressing increased membrane-associated MUC1 exhibited reduced fluorescence. We also used fluorescent microscopy analysis to reveal that the intensity of fluorescein was decreased in HCECs that expressed increased MUC1 under MPM cell culture condition (Fig. 2D).

Another way to upregulate the expression of transmembrane mucins is via the treatment of diquafosol, which is a uridine nucleotide analog functioning as an agonist of the P2Y2 receptor.[24] Our results showed that the transcript level of MUC1 in HCECs was dose-dependently increased whereas that of MUC4 or MUC16 was less significant by the diquafosol treatment (Fig. 3A). Similarly, the protein expression of MUC1 determined by immunostaining intensity in flow cytometry was also increased by diquafosol, while that of MUC4 and MUC16 was not (Fig. 3B). Using the anti-MUC1 antibody to detect extracellular MUC1, we found that HCECs expressing increased membrane-associated MUC1 promoted by diquafosol treatment exhibited reduced fluorescence (Fig. 3C). Collectively, our results suggested that upregulation of MUC1 impeded intracellular fluorescein ingress in HCECs.

Downregulation of MUC1 Increases Fluorescein Ingress

To further support a role of MUC1 in fluorescein ingress, we downregulated the expression of transmembrane mucins via transduction of two different lentiviral-based shRNA
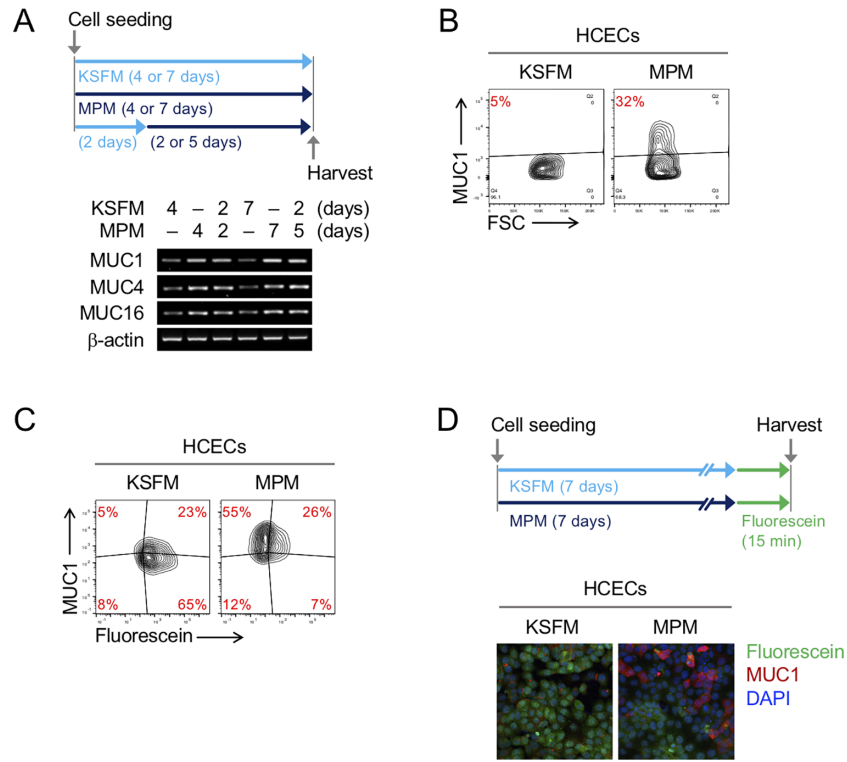
FIGURE 2. The fluorescein intensity is reversely correlated with the level of MUC1 in HCECs cultured in MPM. **(A)** The HCECs that have been grown in KSFM were cultured in MPM for two or five days, and the levels of MUC1, MUC4, and MUC16 mRNA were examined. **(B)** Representative flow cytometric analyses of MUC1 expressions on cell membranes of HCECs cultured in KSFM (*left*) or MPM (*right*) were presented in contour plots. **(C)** Representative flow cytometric analyses of fluorescence intensity and MUC1 expression in HCECs cultured in KSFM (*left*) or MPM (*right*) followed by fluorescein staining were presented in contour plots. **(D)** The HCECs cultured in KSFM or MPM were stained with fluorescein, and the levels of fluorescence (*green*) and MUC1 (*red*) were examined using an immunofluorescence microscopy. Nuclei were stained with DAPI (*blue*). The images were obtained via a confocal microscopy.
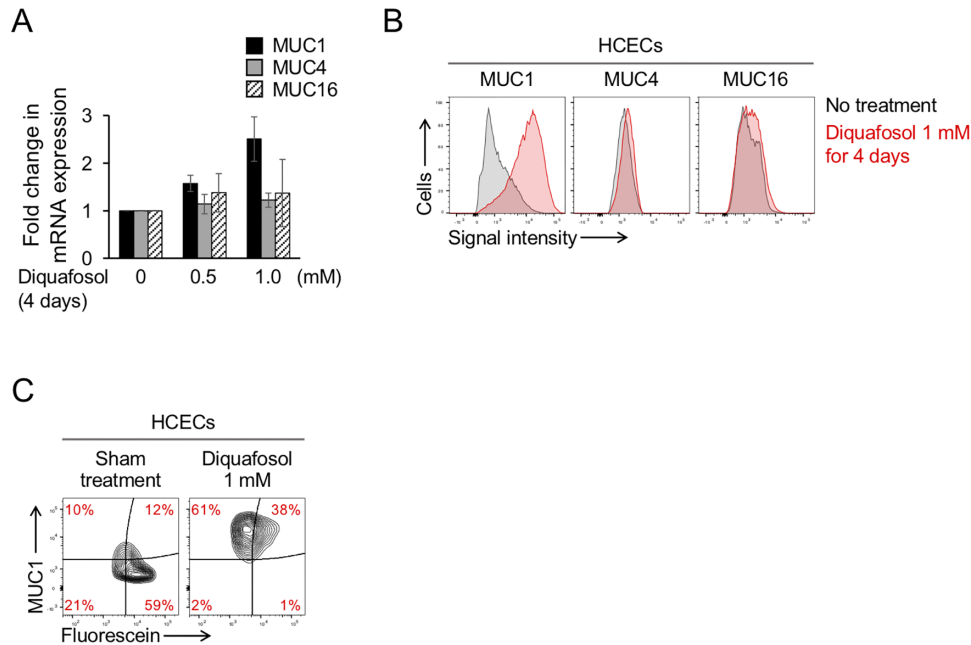

FIGURE 3. Diquafosol treatments preferentially promotes the expression of MUC1 on cell membranes. **(A)** The HCECs were grown in a culture media containing 0, 0.5, or 1.0 mM diquafosol for four days, and the levels of MUC1, MUC4, and MUC16 mRNA of treated cells were examined using qRT-PCR. **(B)** Representative flow cytometric analyses of MUC1, MUC4, and MUC16 expression on cell membranes of HCECs treated with 1 mM diquafosol (*red*) or control (*gray*) were presented in overlaid histograms of signal intensity. **(C)** Representative flow cytometric analyses of fluorescence intensity and MUC1 expression in HCECs treated with sham (*left*) or 1 mM diquafosol (*right*) followed by fluorescein staining were presented in contour plots.
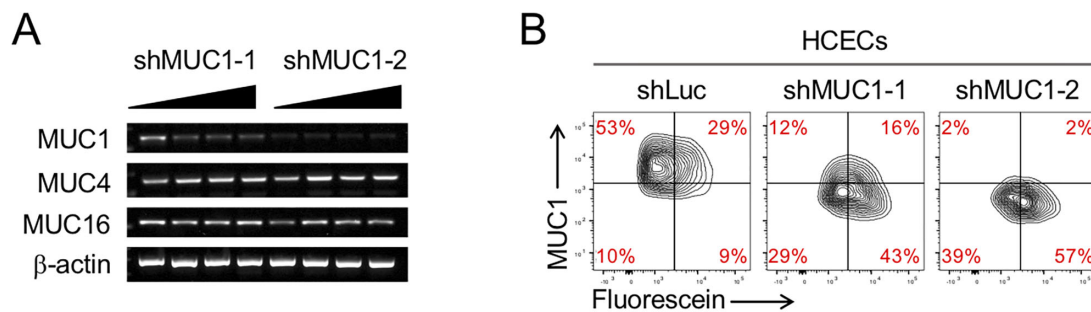
FIGURE 4. Knockdown of MUC1 leads to an increase in fluorescein ingress. **(A)** The mRNA levels of MUC1, MUC4, and MUC16 in HCECs separately transduced with two lentiviral-based shRNA vectors (shMUC1-1 or shMUC1-2) were examined using RT-PCR. The *β*-actin gene was used as an internal control. **(B)** Representative flow cytometric analyses of fluorescence intensity and MUC1 expressions in transduced HCECs followed by fluorescein staining were shown in contour plots.
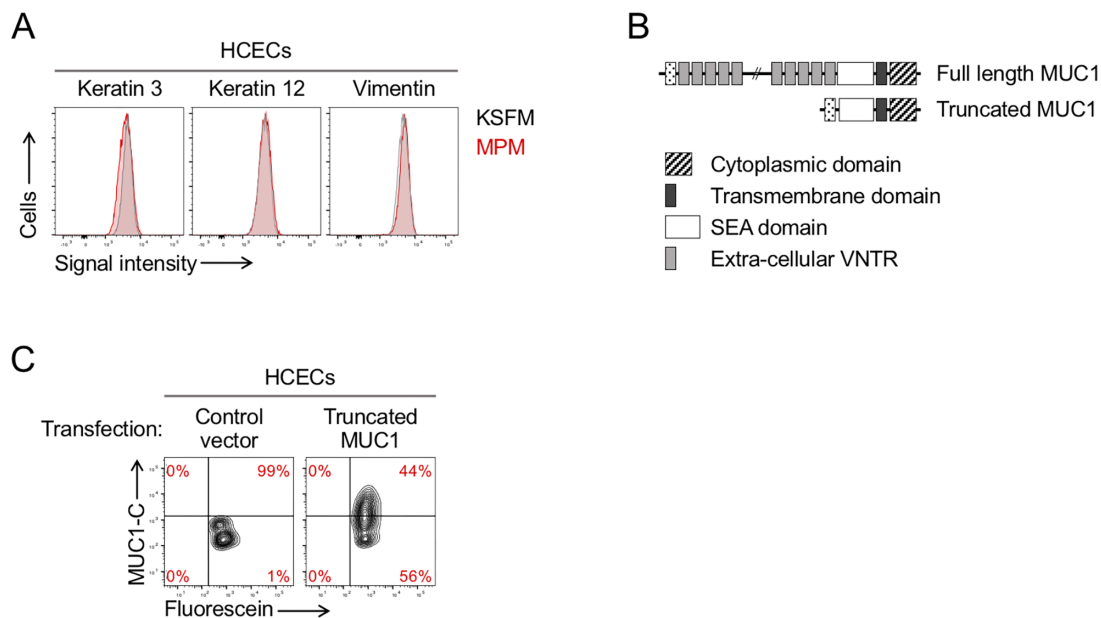

FIGURE 5. The overexpression of MUC1 lacking the extracellular domain is unable to block fluorescein ingress. **(A)** Flow cytometric analyses of keratin 3, keratin 12, and vimentin expressions in HCECs cultured in KSFM (*gray*) or MPM (*red*) were presented in overlaid histograms of signal intensity. **(B)** Schematic diagrams of the wild-type and N-terminally truncated MUC1. **(C)** Representative flow cytometric analyses of fluorescence intensity and MUC1-C levels in HCECs transfected with the control vector (*left*) or the vector encoded with N-terminally truncated MUC1s (*right*) followed by fluorescein staining were shown in contour plots.

vectors, shMUC1-1 and shMUC1-2, both of which specifically targeted MUC1 but not MUC4 and MUC16 (Fig. 4A). Using the same approach as described above, such knocked-down HCECs were stained with fluorescein, incubated with an anti-MUC1 antibody, and then fixed for a flow cytometry analysis. The results showed that decreased expression of surface MUC1 was accompanied by a shift of cells toward high fluorescence, particularly for cells that had been transduced with shMUC1-2 (Fig. 4B).

Fluorescein Ingress is Mediated by Extracellular Domain of MUC1

Even though the diquafosol treatment or cell culture with MPM promotes MUC1 expression and decreases fluorescein ingress, this could possibly be a consequence of the pheno-typic transition caused by an increase in the cytoplasmic domain of MUC1 (MUC1-C) that involves multiple signaling cascades. Therefore, the idea that the extracellular domain of MUC1 physically blocking fluorescein ingress requires additional validation. To this end, the differentiation of HCECs grown in MPM were examined. We found that the expression of the Keratin 3 and Keratin 12 (the corneal epithelial markers) and Vimentin (the mesenchymal differentiation marker) were essentially unchanged (Fig. 5A), suggesting that the cultured cells maintained their corneal differentiation.[25] To confirm that the aforementioned intracellular fluorescein ingress was primarily influenced by the extracellular domain of MUC1, we overexpressed the N-terminally truncated MUC1, which lacked the extracellular domain (Fig. 5B), in HCECs and examined the fluorescence after fluorescein staining. We found that, although the amount of MUC1-C was significantly increased in transfected cells, the fluo-
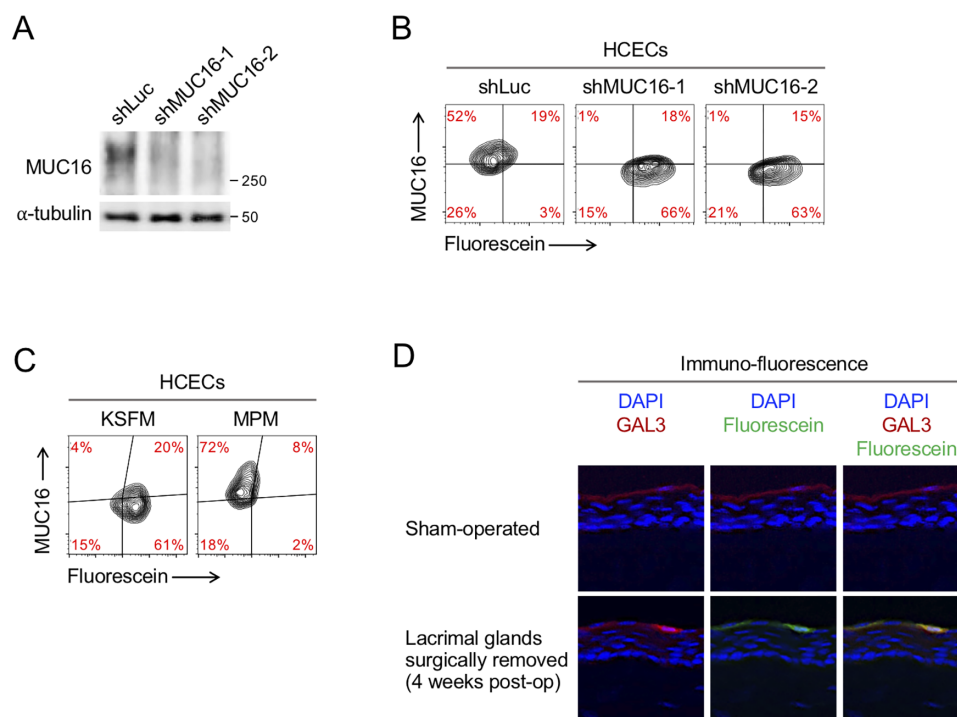
FIGURE 6. **(A)** The expression of MUC16 protein in HCECs separately transduced with two lentiviral-based shRNA vectors (shMUC16-1 or shMUC16-2) were examined through a Western blot analysis. The a-tubulin was used as a loading control. **(B)** Representative flow cytometric analyses of fluorescence intensity and MUC16 expressions in transduced HCECs followed by fluorescein staining were shown in contour plots. **(C)** Representative flow cytometric analyses of fluorescence intensity and MUC16 expression in HCECs cultured in KSFM (*left*) or MPM (*right*) followed by fluorescein staining were presented in contour plots. **(D)** The frozen tissue sections prepared from fluorescein-stained corneal epithelia were stained with GAL3 (*red*, immunofluorescence), and the cell nuclei were counterstained with DAPI (*blue*, immunofluorescence).

rescein ingress as evidenced by fluorescence intensity was not affected (Fig. 5C). Therefore MUC1 extracellular domain was involved in fluorescein ingress into HCECs.

MUC16 May Also Contribute to Blocking Fluorescein Ingress Into Corneal Epithelial Cells

To determine whether MUC16 also blocks fluorescein ingress into corneal epithelial cells, we performed knock-down with the same lentiviral-based shRNA vector targeting MUC16 in HCECs. As shown in Figure 6A, the expression of MUC16 was sufficiently downregulated by using two different MUC16 shRNA sequences. Consequently, we found that the decreased expression of surface MUC16 was accompanied by a shift of cells toward high fluorescence, thus suggesting an inhibitive role of MUC16 in fluorescein ingress (Fig. 6B). Using the MPM culture media to enhance mucin expression as described previously, we showed that the HCECs expressing high membrane-associated MUC16 exhibited reduced fluorescence (Fig. 6C). Ideally, these in vitro findings are validated in vivo as well with a dry eye animal model. However, because MUC16 is minimally expressed in the corneal epithelium of rabbits, we sought to examine the association of galectin-3 (GAL3), a MUC1 and MUC16 binding protein, with fluorescein ingress. Interestingly, the immunofluorescence revealed an increase in GAL3 expression in a number of the superficial corneal epithelial cells of the experimental dry eyes (Fig. 6D). Notably, the cells with high GAL3 expression were coincidently fluorescein stained (Fig. 6D, middle and right, immunofluorescence). This finding was also observed in other experimental replicates (Supplementary Fig. S2). Taken together, our findings support that the surface mucins and their associated proteins play a role in fluorescein ingress in dry eye disease.

DISCUSSION

Fluorescein staining has been proven very useful in the assessment of ocular surface diseases; however, the nature of fluorescein punctate staining on corneal surface remains disputed. Corneal fluorescein staining is traditionally thought to ensue when the tight junction of superficial cells is disrupted, resulting in fluorescein trapped in the paracellular/intercellular space.[15,16] Recent studies favor the idea that fluorescein stains individual cells, particularly those with altered physiological conditions.[14,17,26] Adhering to this idea, our study found that fluorescein ingress was reduced in HCECs that had been promoted to express MUC1, whereas a knockdown of MUC1 in HCECs enhanced fluorescein ingress. This effect was likely attributed to the MUC1 extracellular domain because overexpressing MUC1-C (the MUC1 cytoplasmic domain) failed to decrease fluorescein ingress in HCECs. Moving beyond the cell cultures, we used a dry eye animal model to show that the punctate spots on dry eyes after fluorescein staining consist of fluorescein-incorporated corneal epithelial cells. As such, the clinical presentation of fluorescein punctate staining may signify a loss of mucins on the ocular surface.

Although our results characterize MUC1 as a corneal epithelial barrier to fluorescein permeation, this notion demands a critical examination. Supporting evidence includes the *in vitro* studies showing that fluorescein-stained corneal epithelial cells became hyperfluorescent when exposed to culture media with higher osmolarity, which reportedly has a repressive effect on mucin expression on the ocular surface.[26–28] Likewise, the number of hyperfluorescent cells was significantly increased after exposure to multipurpose solution that has been known to decrease the expression of membrane-associated mucins of corneal epithelial cells.[29–31] Meanwhile, from a clinical perspective, fluorescein punctate staining is observed among patients with certain ocular surface diseases, including dry eye and superior limbic keratoconjunctivitis that are often presented with ocular mucin deficiency.[32–35] Although some dry eye patients do not present with fluorescein punctate staining, it is possible that those patients are most likely to have "mild" dry eye or lipid-deficiency "evaporative" dry eye, and their mucin expression remains sufficient to prevent fluorescein ingress. Herein, our study provides a molecular basis for these correlational studies, thus supporting that a mucin deficiency may lead to the loss of epithelial barrier to fluorescein permeation.

Concerning the biophysical properties of mucins, their density on the cell surface is a major factor that determines the penetration rate of fluorescein. In principle, the extracellular domains of mucins are assembled into mucus meshes, with a thickness of up to 800 μm and pore sizes around 100 to 200 nm, depending on the organ systems, pH values, and mucin volume. Importantly, as reported in the studies on cervical or gastric mucus, a 10- to 100-fold decrease in mucin content was expected to lead to pores that are substantially larger, potentially micrometer-sized.[36,37] Because fluorescein sodium was estimated to be approximately 2000 nm in diameter,[38] it is plausible that ocular mucins normally can limit fluorescein penetration, unless the concentration of mucins decrease to the levels that open up the microstructure of the tear film. Intriguingly, a previous study reported that precoated rabbit corneas with 1% porcine stomach mucins blocked the staining of rose bengal but not fluorescein.[16] Possibly, this discrepancy was attributed to the compositions of mucins, for example, soluble versus membrane-bound mucins that were used between studies.

Another issue of concern relates to the pattern of fluorescein staining that often appears as punctate fluorescent spots on the cornea, which is puzzling given that the epithelial cells throughout the ocular surface of a diseased eye are similarly affected, presumably resulting in a generalized loss of mucins presented as diffuse fluorescence. In fact, even in the interpalpebral area where the ocular surface is more exposed to air and shearing of eyelids, homogenous and diffuse fluorescence is not observed during the fluorescein staining of cornea.[14–16] Notably, our findings may provide a clue for this observation: Epithelial cells are staggered in overlapping layers of cornea, and, in some areas, the epithelial cells have not yet fully surfaced to confront desiccating stress. As we showed that fluorescein sodium only stains the most superficial epithelial cells, bridging several neighboring epithelial cells in the uppermost layer of dry eyes may appear as fluorescent spots, whereas the areas that are not covered by the epithelial cells of this layer may remain unstained, thus resulting in punctate appearance.

In addition to the decrease in MUC1, robust expression of GAL3 was also noted in several superficial cells in the dry eye model. This finding is consistent with a previous study that showed higher expression of GAL3 in the tears and superficial conjunctival epithelium of dry eye patients compared to healthy individuals.[39] Notably, we observed that the cells with high GAL3 expression were mostly fluorescein stained, which is intriguingly because reduced cell surface GAL3 has been linked to the increased corneal permeability to rose Bengal.[40] Nonetheless, these findings are not conflicting. Indeed, since GAL3 has been recognized as a danger-associated molecular pattern and pattern-recognition receptor, an increase in GAL3 is suggestive of a corneal injury, accompanied by a decrease in MUC1 expression, in dry eye.[41] Accordingly, fluorescein ingress occurs in corneal epithelial cells without sufficient surface mucins, even though the expression of GAL3 is increased.

Although our findings demonstrate a role of MUC1 in fluorescein ingress, the definitive investigation of the roles of other transmembrane mucins, including MUC4 and MUC16, was limited by the dry eye model of rabbit. Meanwhile, the MUC4 and MUC16 are very large glycoproteins, and therefore, manipulating their expression levels is technically challenging. However, as shown in another study that MUC16 functions as a barrier to bacterial invasion and rose bengal dye penetration,[42] it is plausible that the MUC16 or MUC4 also involves fluorescein ingress.

In summary, our study provides evidence that intact transmembrane MUC1 on ocular surface blocks fluorescein ingress into corneal epithelial cells. This finding helps clinical interpretations of fluorescein punctate staining and the underlying condition of corneal epithelium.

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