• Basic Research •

Knockout of *TMEM206* in mice associated with a loss of corneal transparency

Zi-Jian Yang[1], *Shou-Yue Huang*[1], *Yu-Feng Zhou*[1], *Shun-Chang Sun*[2]

[1]Department of Ophthalmology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201801, China
[2]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201801, China

Correspondence to: Shun-Chang Sun. Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 201801, China. shunchangsun@aliyun.com

Received: 2024-02-07 Accepted: 2024-07-04

Abstract

● **AIM:** To investigate the role of transmembrane protein 206 (TMEM206) in corneal edema in mice by knockout the *TMEM206* gene using CRISPR/Cas9 editing technology.

● **METHODS:** *TMEM206*-knockout mice were generated using the CRISPR-Cas9 system. Variations in ophthalmic pathology were observed using slit lamp microscope and optical coherence tomography (OCT), intraocular pressure (IOP) was measured using a TonoLab Rebound Tonometer, and the ultrastructure of the corneal was observed using a transmission electron microscope.

● **RESULTS:** Corneal opacity was observed in 4/18 homozygous *TMEM206*$^{-/-}$ mice whereas a similar change was not observed in heterozygous *TMEM206*$^{+/-}$ mice and wild-type littermates. OCT examination showed that the mean central cornea thickness was 125±5.4 μm in 4 homozygous *TMEM206*$^{-/-}$ mice developed corneal edema and 115±1.2 μm in wild-type mice (t=3.468, P<0.05) at 43wk. The mean IOP was 12.08±0.07 mm Hg in four right eyes with corneal edema and 12.03±0.03 mm Hg in three normal left eyes (P>0.05). Transmission electron microscopy revealed a disruption in the organization of the collagen fibrils in the central part of the cornea in homozygous *TMEM206*$^{-/-}$ mice.

● **CONCLUSION:** TMEM206 is associated with corneal edema which caused organizational disruption of collagen fibrils in corneas of mice.

● **KEYWORDS:** transmembrane protein 206; knockout; cornea; edema; mouse

DOI:10.18240/ijo.2024.11.01

Citation: Yang ZJ, Huang SY, Zhou YF, Sun SC. Knockout of *TMEM206* in mice associated with a loss of corneal transparency. *Int J Ophthalmol* 2024;17(11):1967-1972

INTRODUCTION

Proteins of the transmembrane protein family are present in various cell membranes, such as those of the endoplasmic reticulum, mitochondria, lysosomes, and Golgi, and are involved in important physiological processes[1]. A number of transmembrane proteins act as channels that allow the transport of specific substances across biological membranes[2]. Transmembrane protein 206 (TMEM206) was previously confirmed by whole-cell patch-clamp recording to be a proton-activated Cl^- (PAC) channel activated by a highly acidic extracellular pH (~5.5 to 6.0). This channel shows a strong outwardly rectifying current-voltage relationship that is also referred to as a proton-activated outwardly rectifying anion channel or acid-sensitive outwardly rectifying anion channel[3]. The PAC channel is activated at a highly acidic extracellular pH that may be limited to pathological conditions (for example, in cancer and ischemic stroke) in which the PAC currents may contribute to cell death induced by high levels of tissue acidosis. In addition to its pathological role in acidosis, the broad tissue expression profile of TMEM206 suggests that it may also play an important functional role[4].

Normal vision relies on the maintenance of corneal transparency, which can be struck a balance if the inner and outer restricting endothelial and epithelial layers cannot compensate the tendency of the stromal layer to imbibe water and swell[5]. The corneal endothelium and epithelium transfer ions and water between the stroma, front aqueous chamber and tear fluid, respectively[6]. The corneal epithelium transfers chloride from the basal part to the tear part, and sodium from the tear to the basal part[7]. The primary role of the corneal endothelium is to keep corneal transparency *via* the active transfer of ions and water[8]. Several types of chloride transporting molecules have been identified in corneal epithelial cells[9]. However, little is known about TMEM206 in the cornea. Corneal edema is common in corneal endothelial dysfunction and is caused by surgery, trauma and inflammation. The cornea transparency is dependent

on ion transport and endothelial cell number in the corneal endothelium; furthermore, chloride channels are implicated in corneal edema. TMEM206 is a novel chloride channel. In this study, we focus on the impact of the TMEM206 channel on corneal edema. We discovered that the loss of corneal transparency was detected in *TMEM206* knockout mice. Our study provides insight into the mechanisms underlying corneal edema and identifies a potential target for the therapeutic intervention for corneal edema.

MATERIALS AND METHODS

Ethical Approval All experiments involving the care and use of animals were carried out in accordance with the U.S. National Institutes of Health guidelines (NIH, revised 1996) and the Animal Research: Reporting *In Vivo* Experiments guidelines.

Generation of *TMEM206* Knockout Mice C57BL/6 mice were purchased from Gempharmatech Co., Ltd. (Nanjing, China) and housed under standard conditions with a 12h light/dark cycle on a daily. Next, we generated a *TMEM206* knockout mouse model using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated nuclease 9 (Cas9) technology[10]. The co-injection of single guide RNAs targeting exon 2 and exon 5, and Cas9 protein, was performed in 1-cell-stage C57BL/6J zygotes. The single guide RNAs sequences were as follows: gRNA1: 5'-ctagcttctgctgattaccc-3'; gRNA2: 5'-ccttaaagatgtaccccaag-3'; gRNA3: 5'-caagaaggctcacggaccac-3' and gRNA4: 5'-agtgaagcctgaagctcggc-3'. These experiments successfully generated mice carrying mosaic heterozygous indel modifications between exon 2 and exon 5.

The *TMEM206* knockout mice were created by Gempharmatech Co., Ltd. (Nanjing, China). Founders were backcrossed to generate homozygous *TMEM206* knockout offspring. *TMEM206* knockout mutation was confirmed by polymerase chain reaction (PCR) screening and Sanger sequencing using genomic DNA extracted from tail biopsies.

Evaluation of Corneal Opacity Corneal opacity and the integrity of the corneal epithelium were evaluated individually using a slit lamp microscope (Topcon, Japan) in 30-week-old mice without anesthesia. The degree of cornea opacity for each eye was scored on a numerical scale of 0-4, as follows: 0, clear cornea; 1, mild stromal opacity; 2, moderate stromal opacity; 3, severe corneal opacity; and 4, opaque cornea. Digital images of each cornea were acquired by slit lamp microscopy. Central corneal thickness was measured by optical coherence tomography (OCT, Zeiss, Germany) in 43-week-old mice under intraperitoneal anesthesia. Intraocular pressure (IOP) was measured using a TonoLab Rebound Tonometer (Icare, Finland) under intraperitoneal anesthesia.

Electron Microscopy Features of Mice Corneas Mice were anaesthetized systemically by an intraperitoneal injection with 40 μL of ketamine and 10 μL chlorpromazine hydrochloride in 0.5 mL of 0.9% sodium chloride solution. The cornea was divided into blocks and fixed in 2.5% glutaraldehyde and osmium tetroxide to observe the corneal ultrastructure by transmission electron microscopy (Hitachi H9500, Japan). Images of corneal ultrastructure were obtained at magnifications of ×13 500 to ×50 000. In brief, the vacuum around the sample was approximately 10 Pa. Backscattered electrons originating from the specimen's surface were detected by a detector at an accelerating voltage of 80 kV in accordance with the manufacturer's instructions.

Statistical Analysis The data were expressed as means±standard deviation (SD), and statistical analysis was performed using GraphPad Prism V7 software (GraphPad, California, USA). One-way analysis of variance was performed for analysis of normal distribution data in different groups, and Kruskal-Wallis test was employed for data analysis that did not show normal distribution. Student's *t*-test was used to compare difference between two groups. A value of $P<0.05$ was considered as a significant difference.

RESULTS

Generation of *TMEM206* Knockout Mice Constitutive knockout of the *TMEM206* allele was obtained by inserting a stop codon in exon 2 of the gene by CRISPR-Cas9-mediated gene editing (Figure 1A). Mutated F0 founders were identified by genotyping and sequencing. Tail tissues were genotyped by conventional PCR with two specific primers. The primer sequences used for conventional PCR were as follows: KO-F: 5'-ctgagtggactctaagccacatttcc-3'; KO-R: 5'-gtgcattatacactggt gaaccagttg-3'; WT-F: 5'-gagaccaagatggtggagcaaa-3' and WT-R: 5'-tgtcactcagggtcaaagtggg-3'. The six F0 founders were genotyped by PCR followed by Sanger sequencing analysis (Figure 1B-1E). One of the F0 founders was selected for expansion into heterozygote and homozygote breeding pairs. Heterozygous breeding pairs were then selected to produce *TMEM206*$^{-/-}$ mice and wild-type littermates.

***TMEM206*$^{-/-}$ Mice Developed Corneal Opacity** Sixty-nine F1 generation mice were obtained using founders that were backcrossed from *TMEM206*$^{+/-}$ mice. Sixty-nine mice from the F1 generation featured 18 *TMEM206*$^{-/-}$ mice, 36 *TMEM206*$^{+/-}$ mice and 15 wild-type C57BL/6 mice. In week 30 postburn, the corneal opacity score was 4 in three homozygous *TMEM206*$^{-/-}$ mice (Figure 2A, 2C, and 2D) and 3 in one homozygous *TMEM206*$^{-/-}$ mouse (Figure 2B). The corneal opacity score was 0 in heterozygous *TMEM206*$^{+/-}$ mice (Figure 2E) and wild-type littermates (Figure 2F). Variable ophthalmic pathology was observed in four out of the 18 homozygous *TMEM206*$^{-/-}$ mice; these changes were not

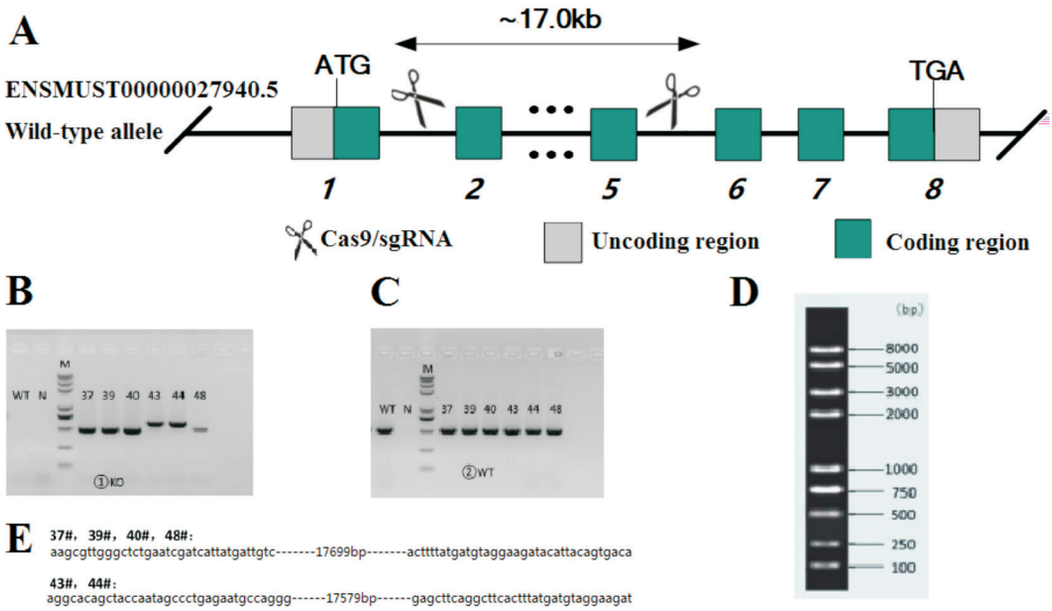

Figure 1 Genotypical analysis of *TMEM206* knockout F0 founder mice A: Schematic diagram of *TMEM206* knockout *via* CRISPR-Cas9-mediated gene editing; B: PCR products amplified from the targeted region of genomic DNA revealed *TMEM206* knockout genotypes (KO); C: PCR products amplified from the targeted region of genomic DNA revealed wild-type *TMEM206* genotypes (WT); D: The precise length of DNA ladders; E: Two different types of knockout segments in the *TMEM206* gene from the 6 F0 founders. PCR: Polymerase chain reaction.

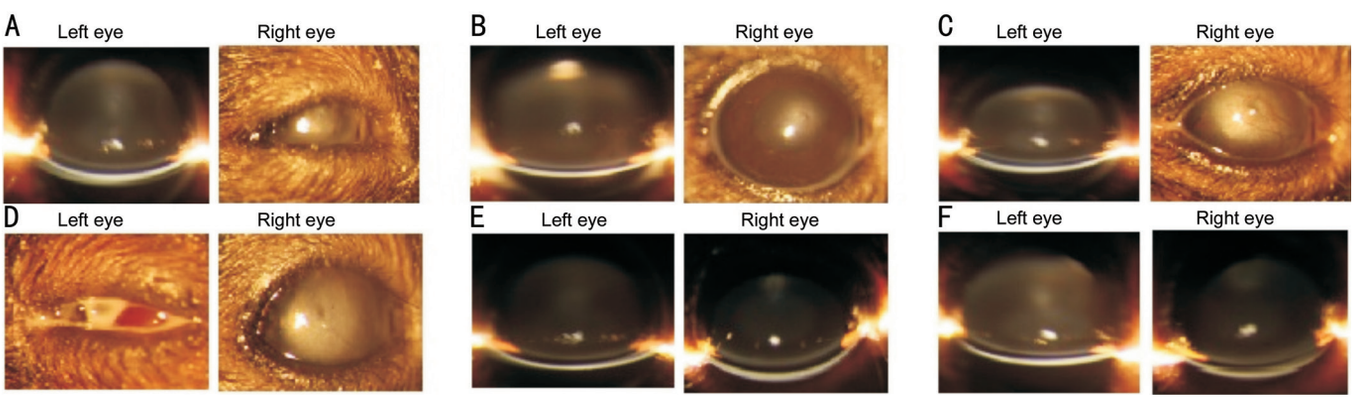

Figure 2 Anterior imaging by slit lamp microscopy in 30-week mice A-D: Four homozygous *TMEM206*$^{-/-}$ mice; E: Heterozygous *TMEM206*$^{+/-}$ mice; F: Wild-type C57BL/6 mice.

observed in heterozygous *TMEM206*$^{+/-}$ mice and their wild-type littermates. Corneal edema was observed in the right eye in four *TMEM206*$^{-/-}$ mice and the lens capsule was collapsed in the left eye of one *TMEM206*$^{-/-}$ mouse. Corneal opacity scores did not differ significantly when compared between heterozygous *TMEM206*$^{+/-}$ mice and wild-type mice. OCT examination showed that the mean central cornea thickness was 125±5.4 μm for the edematous cornea in 4 out of the 18 homozygous *TMEM206*$^{-/-}$ mice developed corneal edema; this compared to 115±1.2 μm in wild-type mice. The central cornea of *TMEM206*$^{-/-}$ mice was significantly thicker than that in wild-type C57BL/6 mice (t=3.468, P<0.05) according to the measurement of 4/18 homozygous *TMEM206*$^{-/-}$ mice with corneal edema at 43wk (Figure 3). All samples of corneal epithelium were smooth and intact except for in one damaged eye. The mean IOP was 12.08±0.07 mm Hg in four right eyes with corneal edema and 12.03±0.03 mm Hg in three normal left eyes. There was no obvious difference between the IOP of eyes with corneal edema when compared with the IOP of normal eyes. Collectively, our findings showed that *TMEM206* knockout is associated with corneal edema in mice.

Electron Microscopy Features of Cornea Corneal transparency is dependent upon the arrangement of collagen fibrils. Transmission electron microscopy of the corneas from experimental mice revealed disruption in the organization of the collagen fibrils in the central part of the cornea in homozygous *TMEM206*$^{-/-}$ mice (Figure 4A) when compared to the collagen fibrils of wild-type mice with normal corneas (Figure 4B). These results demonstrated that disruption in the organization of collagen fibrils in the cornea was associated with the corneal edema caused by *TMEM206*-knockout in mice.

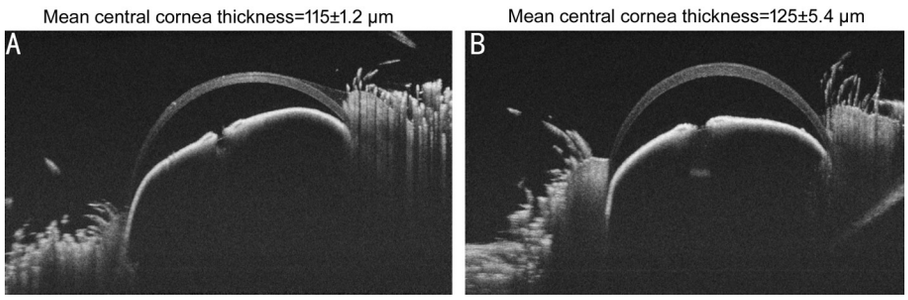

Figure 3 Corneal thickness, as determined by optical coherence tomography in 43-week mice A: The corneal thickness of wild-type C57BL/6 mice; B: The corneal thickness of homozygous *TMEM206*$^{-/-}$ mice with corneal edema.

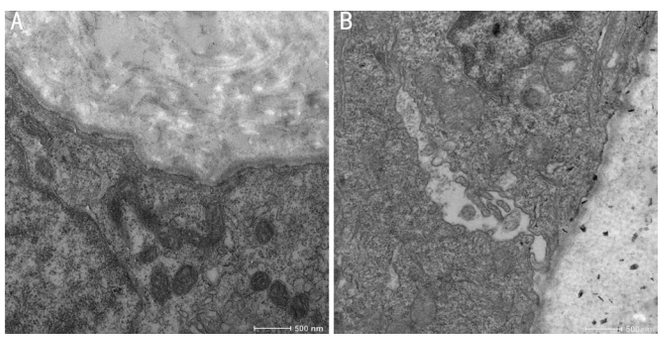

Figure 4 Electron micrographs of the corneas in 43-week mice A: Collagen fibrils in the corneas of *TMEM206*$^{-/-}$ mice; B: Collagen fibrils in the corneas of wild-type C57BL/6 mice.

DISCUSSION

The corneal stroma extracellular matrix consists mainly of collagen with minor amounts of proteoglycans. The deficient synthesis of proteoglycan can produce blindness by disrupting the arrangement of the collagen fibrils in human[11]. Hydration of the corneal stroma is kept predominantly by a balance between its trend to imbibe water and swell and an active process of ion transport throughout the corneal endothelium[12]. The tendency to absorb water and swell is owing to the presence of negatively charged macromolecules such as glycosaminoglycans in the stromal collagenous matrix. Ion transport throughout the corneal endothelium is considered to involve the active transfer of anions from the stroma to the aqueous humor, followed by the passive dispersion of cations[13]. The cornea loses its clarity at hydration levels out of the normal range. Thus, faulty endothelial ion transport will contribute to stromal swelling, corneal edema and damaged vision[14]. Chloride ions are the most abundant anions; the movement of chloride ions across cell membranes, mediated by chloride ions channels, is central to certain cellular functions, such as the regulation of cell volume and the acidification of intracellular vesicles in cells. Several types of chloride ion transporting channels have been detected in corneal epithelial cells[15]. The TMEM206 protein channel has been characterized as an acid-sensitive ion channel in the plasma membrane[16]. However, little is known about TMEM206 in corneal endothelial and epithelial cells.

The *TMEM206* gene encodes a new type of chloride channel activated by protons[3]. TMEM206 is a transmembrane protein that is widely expressed in mammalian cells[3]. The wide expression profile of TMEM206 suggests a broad role for this chloride channel in physiological and pathological processes[17]. In the present study, the results of *TMEM206*-knockout in mice demonstrated ophthalmic pathology in 4/18 homozygous *TMEM206*$^{-/-}$ mice; this pathology was not observed in heterozygous *TMEM206*$^{+/-}$ mice. Corneal edema and collapse of the lens structure were the key aspects of ophthalmic pathology in *TMEM206*$^{-/-}$ mice. OCT examination confirmed that the common ophthalmic pathology was corneal edema. Corneal edema was detected in four homozygous mice while the collapse of the lens structure was only in one homozygous mouse out of 18 homozygous *TMEM206*$^{-/-}$ mice. Eye infection can cause the collapse of the lens structure. In the present study, one homozygous *TMEM206*$^{-/-}$ mouse showed ophthalmic pathology in its left eye with the collapse of the lens structure and corneal edema in the right eye; there was no evidence of eye infection in its littermates. This study suggested that lens structure collapse might be caused by *TMEM206* knockout. This study suggests that corneal edema is associated with the knockout of *TMEM206* in mice. Based on the results of this study, it may be speculated that TMEM206 also serves a fundamental role in the corneal endothelium and the transport of water by the epithelium. The regular arrangement of collagen fibrils is necessary for corneal transparency[11]. Corneal transparency depends on the orderly packing of small size collagen fibrils. The distinctive arrangement of the collagen fibrils is a chief determinant of their function[13]. Other extracellular matrix molecules such as hyaluronan are also related to the reduction of light scattering[13]. A previous study concerning developing embryos suggested that there was a relation between glycosaminoglycans in the stromal collagenous matrix and the appearance of an exactly organized collagen lattice during the achievement of corneal transparency[18].

To improve our understanding of the role of TMEM206 in the cornea, we aimed to investigate the arrangement of collagen

fibrils in the corneal stromal layer by transmission electron microscopy. Parallel bundles of collagen fibers are known to be tightly packed in lamellae lying parallel to the surface of the corneal stroma[19]. The precise temporal and spatial regulation of the extracellular matrix assembly is dependent on matrix-matrix and matrix-cell interactions during corneal development. Sulfate groups on the proteoglycans bind water and act as a reserve for hydration[20]. In the present study, organizational disruption of collagen fibrils occurred in the central part of the cornea in homozygous *TMEM206*$^{-/-}$ mice. *TMEM206* knockout resulted in the transport of chloride ions and water into the extracellular matrix, thus affecting stromal hydration and the structural organization of the collagen fibrils. Therefore, the disruption of collagen fibrils may be caused by *TMEM206* knockout subsequently affecting chloride transport. Further studies are now necessary to fully elucidate the role of TMEM206 in the structure and function of the cornea. There are several types of chloride ion channels in corneal epithelial cells; other chloride channels could compensate for abnormalities in the function of PAC channels. Therefore, the knockout of *TMEM206* could not have been responsible for corneal edema in all mice.

Normal vision requires transparency of the cornea. However, the cornea transparency is in part dependent on the capability of the corneal epithelial layer to induce fluid flow from the stromal side into the tear film side; however, these flows are not as important as those mediated by the inside endothelial layer[21]. The corneal epithelial layer is the front layer while the corneal endothelium is the rear layer; the corneal stroma locates between these two layers. Damage to the corneal endothelium or corneal epithelium can result in stromal edema. TMEM206 was previously confirmed to be a PAC channel activated by a highly acidic extracellular pH in a range of cells. Tissue acidosis is a wide-spread pathological feature associated with many diseases, including ischemia, cancer, infection and inflammation[22]. The threshold for PAC channel activation is too acidic to be found under physiological conditions except for endocytic pathways and secretory vesicles, which are known to have an acidic extracellular pH[23]. The presence of corneal edema in some of the *TMEM206* knockout mice in the present study suggested that TMEM206 may be expressed in corneal endothelium and epithelium and play an essential role in chloride ion transport across the corneal endothelium and epithelium. Therefore, the results of the present study suggested that TMEM206 may also play an important function under some physiological conditions with a pH between 7.2 and 7.5.

In summary, *TMEM206* knockout in mice disrupts organization of the collagen lattice and results in corneal edema. However, there are several types of chloride channels in the corneal epithelia and endothelium. The maintenance of corneal transparency *via* the transport of ions and fluid can be compensated for through other chloride channels. Therefore, the loss of corneal transparency was only observed in four of the 18 *TMEM206* knockout mice.

ACKNOWLEDGEMENTS

Foundation: Supported by National Natural Science Foundation of China (No.31571294).

Conflicts of Interest: Yang ZJ, None; **Huang SY,** None; **Zhou YF,** None; **Sun SC,** None.

REFERENCES

1 Zhang Z, Luo S, Barbosa GO, Bai M, Kornberg TB, Ma DK. The conserved transmembrane protein TMEM-39 coordinates with COPII to promote collagen secretion and regulate ER stress response. *PLoS Genet* 2021;17(2):e1009317.

2 Xu J, Su Z, Ding Q, Shen L, Nie X, Pan X, Yan A, Yan R, Zhou Y, Li L, Lu B. Inhibition of proliferation by knockdown of transmembrane (TMEM) 168 in glioblastoma cells *via* suppression of Wnt/β-catenin pathway. *Oncol Res* 2019;27(7):819-826.

3 Mihaljević L, Ruan Z, Osei-Owusu J, Lü W, Qiu Z. Inhibition of the proton-activated chloride channel PAC by PIP_2. *eLife* 2023;12:e83935.

4 Zhang L, Liu SY, Yang X, Wang YQ, Cheng YX. TMEM206 is a potential prognostic marker of hepatocellular carcinoma. *Oncol Lett* 2020;20(5):174.

5 Liu X, Li H, Qu S, Yu Q, Lin H, Bi YL. Different compression sutures combined with intracameral air injection for acute corneal hydrops. *Int J Ophthalmol* 2022;15(9):1538-1543.

6 Sella R, Zangwill LM, Weinreb RN, Afshari NA. Repeatability and reproducibility of corneal epithelial thickness mapping with spectral-domain optical coherence tomography in normal and diseased cornea eyes. *Am J Ophthalmol* 2019;197:88-97.

7 Olivieri M, Cristaldi M, Pezzino S, Rusciano D, Tomasello B, Anfuso CD, Lupo G. Phenotypic characterization of the SIRC (Statens Seruminstitut Rabbit Cornea) cell line reveals a mixed epithelial and fibroblastic nature. *Exp Eye Res* 2018;172:123-127.

8 Che DY, Chan ZL, Zhou JB, Zhu DQ. Silicone oil as a corneal lubricant to reduce corneal edema and improve visualization during vitrectomy. *Int J Ophthalmol* 2024;17(1):92-96.

9 Foster JW, Jones RR, Bippes CA, Gouveia RM, Connon CJ. Differential nuclear expression of Yap in basal epithelial cells across the cornea and substrates of differing stiffness. *Exp Eye Res* 2014;127:37-41.

10 Petazzi P, Menéndez P, Sevilla A. CRISPR/Cas9-Mediated Gene Knockout and Knockin Human iPSCs. *Methods Mol Biol* 2022;2454:559-574.

11 Espana EM, Birk DE. Composition, structure and function of the corneal stroma. *Exp Eye Res* 2020;198:108137.

12 Bu J, Yu J, Wu Y, Cai X, Li K, Tang L, Jiang N, Jeyalatha MV, Zhang M, Sun H, He H, Quantock AJ, Chen YX, Liu ZG, Li W. Hyperlipidemia affects tight junctions and pump function in the corneal endothelium. *Am J Pathol* 2020;190(3):563-576.

13 Petrela RB, Patel SP. The soil and the seed: the relationship between Descemet's membrane and the corneal endothelium. *Exp Eye Res* 2023;227:109376.

14 Numa K, Imai K, Ueno M, Kitazawa K, Tanaka H, Bush JD, Teramukai S, Okumura N, Koizumi N, Hamuro J, Sotozono C, Kinoshita S. Five-year follow-up of first 11 patients undergoing injection of cultured corneal endothelial cells for corneal endothelial failure. *Ophthalmology* 2021;128(4):504-514.

15 Mellmer MA, Sanpitakseree C, Demir B, Ma K, Elliott WA, Bai P, Johnson RL, Walker TW, Shanks BH, Rioux RM, Neurock M, Dumesic JA. Effects of chloride ions in acid-catalyzed biomass dehydration reactions in polar aprotic solvents. *Nat Commun* 2019;10(1):1132.

16 Deng Z, Zhao Y, Feng J, Zhang J, Zhao H, Rau MJ, Fitzpatrick JAJ, Hu H, Yuan P. Cryo-EM structure of a proton-activated chloride channel TMEM206. *Sci Adv* 2021;7(9):eabe5983.

17 Xu XY, Zhang FF, Gan J, Zhang MY, Shen ZS, Guo Q, Teng Y, Ji JW, Cao JL, Tang QY, Zhang Z. Identification of the acid-sensitive site critical for chloral hydrate (CH) activation of the proton-activated chloride channel. *J Neurosci* 2023;43(4):526-539.

18 Chen S, Mienaltowski MJ, Birk DE. Regulation of corneal stroma extracellular matrix assembly. *Exp Eye Res* 2015;133:69-80.

19 Wang C, Polovitskaya MM, Delgado BD, Jentsch TJ, Long SB. Gating choreography and mechanism of the human proton-activated chloride channel ASOR. *Sci Adv* 2022;8(5):eabm3942.

20 Li L, Kang Q, Wang S, Zheng X. Effects of androgen on ultrastructure of corneal epithelium and function of the tear film in BALB/c mice. *Cornea* 2015;34(3):334-341.

21 Massoudi D, Malecaze F, Galiacy SD. Collagens and proteoglycans of the cornea: importance in transparency and visual disorders. *Cell Tissue Res* 2016;363(2):337-349.

22 Osei-Owusu J, Yang J, Leung KH, Ruan Z, Lü W, Krishnan Y, Qiu Z. Proton-activated chloride channel PAC regulates endosomal acidification and transferrin receptor-mediated endocytosis. *Cell Rep* 2021;34(4):108683.

23 Osei-Owusu J, Yang J, Del Carmen Vitery M, Tian M, Qiu Z. PAC proton-activated chloride channel contributes to acid-induced cell death in primary rat cortical neurons. *Channels(Austin)* 2020;14(1):53-58.