CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Aquaporin 5 Facilitates Corneal Epithelial Wound Healing and Nerve Regeneration by Reactivating Akt Signaling Pathway

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Aquaporins (AQPs) are a highly conserved group of membrane proteins that play critical roles in water and small solute transport across epithelial and endothelial barriers. The aim of this study was to determine whether AQP5, a well-known water channel protein, also plays a role in corneal epithelial wound healing. AQP5 knockout ($AQP5^{-/-}$) mice were generated using CRISPR/Cas9. A corneal wound healing model was established using epithelial debridement of corneas. The time to corneal epithelial and nerve regeneration was significantly delayed in the $AQP5^{-/-}$ mice. Reduction in Ki-67–positive cells and nerve growth factor (NGF) expression was confirmed in the $AQP5^{-/-}$ mice during healing. Epithelial and nerve regeneration rates were significantly increased in the $AQP5^{-/-}$ mice after treatment with NGF, which was accompanied by recovered levels of phosphorylated Akt. NGF treatment also improved the recovery of corneal nerve fiber density and sensitivity in the $AQP5^{-/-}$ mice. While the promotion of NGF induced corneal epithelial and nerve regeneration, Akt inhibitor reversed Akt reactivation. A significant impairment of corneal wound healing in the $AQP5^{-/-}$ mice resulted from distinct defects in corneal epithelial cell proliferation and nerve regeneration. These results provide evidence for the involvement of aquaporin in cell proliferation and suggest that AQP5 induction could be a potential therapy for accelerating the resurfacing of corneal defects. (*Am J Pathol* 2021, 191: 1974–1985; <https://doi.org/10.1016/j.ajpath.2021.07.010>)

The cornea acts as a barrier against external stimuli. Failure to heal a corneal wound in time may result in pathogen invasion, causing corneal inflammation, turbidity, ulcer, or even blindness.¹ Cell proliferation and migration are essential in corneal epithelial wound repair.^{2–4} Stem cells around the cornea repair corneal epithelial defects by providing progenitor cells to the corneal epithelium.^{5,6} The cornea is also one of the most densely distributed tissues, and is rich in nerve fibers from the trigeminal ganglion.⁷ Normal and appropriate corneal nerves are responsible for maintaining corneal sensation, blink reflex, and ocular surface homeostasis, and they participate in the process of corneal wound healing.⁷

The transmembrane proteins aquaporins (AQPs) play a significant role in maintaining cell water homeostasis.⁸ In mammals, 13 kinds of aquaporins (AQP0 to AQP12) have

been detected. The AQP superfamily is composed of channels that allow water molecules or water and some solutes (eg, glycerol and urea) to diffuse through the cell membrane.⁹ Dysfunctional AQP is related to many eye diseases, such as keratoconus, bullous keratopathy, Fuchs dystrophy, cataract, and glaucoma.^{10–13} AQP1, AQP3, and AQP5 are expressed in the cornea, and are related to many eye diseases. In the cornea, AQP1 plays an important role in cell migration, whereas AQP3 promotes cell migration

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and proliferation during wound healing.^{14,15} Previous studies have shown that AQP5 plays an important role in maintaining corneal homeostasis. Shankardas et al¹⁶ found that down-regulation of AQP5 resulted in the increased proliferation and migration of human corneal epithelial cell lines (CEPI17). However, previous studies have also shown that AQP5 plays an important role in promoting cell migration and ion proliferation, thereby accelerating the process of corneal reepithelialization.¹⁷ In addition, after blast exposure, the expression of AQP5 in the cornea is up-regulated, and its localization in corneal epithelial cells is changed.¹⁸ Drugs targeting AQP5 up-regulation may be a possible therapy for dry eye.¹⁹ However, the specific mechanism of AQP5 in promoting the corneal wound healing in mice is still unclear.

The nerve growth factor (NGF), which is the most important class in the nerve growth factor family, exists in various tissues and organs of the body. Increasing evidence shows that NGF plays a key role in corneal wound healing. Previous studies have confirmed that exogenous supplementation of NGF can promote corneal nerve regeneration and corneal wound healing in diabetic mice.²⁰ In addition, mesencephalic astrocyte-derived neurotrophic factor and ciliary neurotrophic factor have also been reported to play an important role in promoting corneal nerve regeneration and corneal wound healing.^{21,22} Various growth factors have also been considered to coordinate the function of epithelial cells in the process of tissue repair. Among them, NGF and its receptors, p75 and tropomyosin receptor kinase A, participate in the maintenance of stem cells with the high proliferation potential of corneal limbal basal cells.^{23–26} In mouse retina, NGF promotes Müller cell proliferation by activating the phosphatidylinositol 3-kinase/Akt signal.²⁷

Several signaling pathways participate in the process of corneal epithelial wound healing. Diquafosol and chitosan promote corneal epithelial wound healing by activating the extracellular signal-regulated kinase pathway.²⁸ The Shp2-mediated mitogen-activated protein kinase pathway promotes corneal epithelial wound healing by regulating ΔNp63.²⁹ IL-1β or tumor necrosis factor-α promotes corneal epithelial wound healing by inhibiting the activity of STAT3 through p16^{Ink4a}.³⁰ In addition, recent studies have shown that the activation of the Akt signaling pathway by inhibiting phosphatase and tensin homolog promotes corneal epithelial wound healing in diabetic mice.³¹

Previous findings showed that AQP5 deficiency causes corneal epithelial punctate defects.³² Therefore, the present hypothesis is that AQP5 plays an important role in one or more stages of corneal epithelial regeneration. The possibility that AQP5 plays a role in corneal epithelial regeneration was explored in *AQP5*^{−/−} mice. The findings confirmed that AQP5 accelerates the process of corneal epithelial regeneration and promotes the regeneration of corneal epithelial nerve fibers.

Materials and Methods

Animals

Using CRISPR/Cas9 technology, AQP5 knockout (*AQP5*^{−/−}) mice were generated by the high-flux electric transfer of fertilized eggs obtained from Cyagen Biosciences Inc. (Guangzhou, China). *AQP5*^{−/−} mouse model is shown in [Supplemental Figure S1](#). The genotypes of the experimental mice were identified according to previous description.³³ The mice used in the present study were obtained from C57BL/6N strain. A total of 75 male *AQP5*^{+/+} mice and 189 male *AQP5*^{−/−} mice (aged 10 to 12 weeks) were used. All experiments and animal care procedures were conducted under the guidance of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Care and Use Committee of Qingdao University (Qingdao, China).

Corneal Epithelial Debridement Wounds

The mice were anesthetized using 5% chloral hydrate. Under a stereomicroscope, a 2-mm-diameter injury was made in the corneal epithelium using an Algerbrush II corneal rust ring remover (Alger Co, Lago Vista, TX). As per previous description, the corneal epithelial wound healing process was evaluated by sodium fluorescein staining under a slit lamp (66 Vision Tech, Suzhou, China).³⁴

To explore the role of NGF in corneal wound healing in the *AQP5*^{−/−} mice, 50 ng NGF (R&D Systems, San Diego, CA) was administered to *AQP5*^{−/−} mice in the NGF group by subconjunctival injection at 0 hours after scraping the corneal epithelium. To explore the role of phosphorylated Akt (p-Akt) in corneal wound healing in mice, 640 ng Akt inhibitor (Akti; Merck Millipore, Darmstadt, Germany) was administered to *AQP5*^{−/−} mice by subconjunctival injection at 24 hours before corneal epithelial scraping and 0 and 24 hours after corneal scraping, and 50 ng NGF was added at 0 hours after corneal scraping. These mice formed the NGF + Akti group. The method used to generate the model is shown in [Supplemental Figure S2](#).

Corneas were fixed in optimal cutting temperature compound for immunofluorescence staining, and corneal epithelial cells were collected for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot analysis.

Corneal Whole Mount Staining for Nerve Fibers

Mouse eyes were fixed in a Zamboni fixative solution for 2 hours on ice, and the corneas were dissected around the scleral-limbal region. The corneas were blocked in phosphate-buffered saline containing 0.1% Triton X-100, 2% goat serum, and 2% bovine serum albumin for 2 hours at room temperature. They were then incubated overnight in

Alexa Fluor 488—conjugated neuronal class III β -tubulin antibody (Merck Millipore) in tris-buffered saline containing 0.1% Triton X-100, 2% goat serum, and 2% bovine serum albumin. After cleaning the cornea six times, the cornea was cut into six petals and observed under a fluorescence microscope. Image J version 1.44p software (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>, last accessed July 3, 2021) was used to calculate the coverage rate of the corneal nerve in the unit area of the corneal epithelium.

Corneal Sensitivity Measurement

Corneal sensitivity was measured by a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) in mice not under anesthesia. The test was started at the maximum length of nylon filament (6 cm) and then decreased by 0.5 cm until the corneal contact threshold was reached. The longest length of nylon filament that caused a positive reaction was considered the threshold, and each result was verified three times.

Immunofluorescence Staining

After the mice were sacrificed by cervical dislocation, their eyes were fixed in OCT, cut into 7- μ m slices, and mounted on poly-L-lysine—coated glass slides. The corneas were fixed with 4% paraformaldehyde for 15 minutes and blocked with 5% bovine serum albumin for 1 hour. The corneas were stained with AQP5 (1:200; Abcam, Cambridge, MA), Ki-67 (1:200; Abcam), NGF (1:100; ABclonal, Wuhan, China), and p-Akt (1:400; Cell Signaling Technology, Danvers, MA), followed by fluorescein-conjugated secondary antibodies (1:200; Life Technologies, Grand Island, NY). The staining was observed under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Western Blot Analysis

After the mice were sacrificed by cervical dislocation, corneal epithelial cells were collected, and two corneal epithelia were put together as one sample. The corneal epithelium was lysed in radioimmunoprecipitation assay buffer, and total protein was extracted. The sample was run in 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Merck Millipore), which were probed with antibodies against AQP5 (1:5000; Abcam), NGF (1:300; Affinity Biosciences, Zhenjiang, China), p-Akt (1:2000; Cell Signaling Technology), and Akt (1:1000; Cell Signaling Technology), followed by fluorescein-conjugated secondary antibodies (1:4000; Zhongshan Jinqiao Biotech, Beijing, China). The images were collected using an automatic chemiluminescence image analysis system (Tanon, Shanghai, China). ImageJ software was used to calculate the expression level of the target protein.

RNA Extraction and qRT-PCR Analysis

After the mice were sacrificed by cervical dislocation, corneal epithelial cells were collected, and two corneal epithelia were put together as one sample. The total RNA was extracted using a GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA) as per the instruction manual. cDNA was synthesized using the PrimeScript RT Reagent Kit (Takara, Dalian, China). ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) and Bio-Rad CFX96 Real-Time Systems (Bio-Rad, Hercules, CA) were used for qRT-PCR. The expression was determined using a C_T , and the relative expression level was calculated using the $2^{-\Delta\Delta C_T}$ method. Data were collected from three independent experiments. The primers used in qRT-PCR are specified in Table 1.

Data Analysis

The results are expressed as means $\pm$ SD. Statistical analysis was performed using a one-way analysis of variance, and differences were considered significant at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. All experimental data were analyzed using GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA). The data are representative of at least three experiments.

Results

Expression of AQP5 in Corneal Epithelium

To verify the localization and expression of AQP5 in mouse cornea, corneal sections of $AQP5^{+/+}$ and $AQP5^{-/-}$ mice were stained with anti-AQP5 antibody. AQP5 was located on the membrane in the central and marginal parts of the corneal epithelium, and the expression of AQP5 was abundant

Table 1 Primers Used in quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Gene	Primer type	Primer sequence
NGF	Forward	5'-GCCAAGGACGCAGCTTTCTA-3'
	Reverse	5'-TTCAGGGACAGAGTCTCCTTCTG-3'
PEDF	Forward	5'-TGCCCCCTCTGGAGAAGTCCTA-3'
	Reverse	5'-CCCACTGCCCCCTTGAAGTAA-3'
GDNF	Forward	5'-CCGGTAAGAGGCTTCTCGAA-3'
	Reverse	5'-TCAGTCTTTTAATGGTGGCTTGAA-3'
CNTF	Forward	5'-GCCGCTCTATCTGGCTAGCA-3'
	Reverse	5'-TCATCTCACTCCAGCGATCAGT-3'
BDNF	Forward	5'-GGGTACACAGCGGCAGATAAA-3'
	Reverse	5'-TGCAGCCTTCCTTGGTGTAAC-3'
TAC-1	Forward	5'-GACCCAAGCCTCAGCAGTTC-3'
	Reverse	5'-CCATTAGTCCAACAAAGGAATCTGT-3'
GAPDH	Forward	5'-AAGGTCATCCCAGAGCTGAA-3'
	Reverse	5'-CTGCTTCACCACCTTCTTGA-3'

Figure 1 Loss of AQP5 inhibits reepithelialization of debrided mouse cornea. **A:** Immunofluorescence staining indicating AQP5 expression in the cornea of *AQP5*^{+/+} mice. **B:** Immunofluorescence staining indicating AQP5 expression in the cornea of *AQP5*^{-/-} mice. **C:** Frozen sections were immunostained with rabbit IgG antibody as a negative control group. **D:** Western blot analysis was used to confirm the level of AQP5 protein in the corneal epithelium of *AQP5*^{+/+} and *AQP5*^{-/-} mice. The three lanes represent one sample each, with two corneal epithelia pooled to form one sample. **E:** Fluorescein sodium staining was used to observe the corneal epithelial defects in *AQP5*^{+/+} and *AQP5*^{-/-} mice at 0, 12, 24, and 36 hours after corneal epithelial scraping. **F:** The histogram represents the ratio of the residual defect area/the original defect area. $n = 6$ (**F**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 150 μm (**A–C**). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(green) (Figure 1A). However, AQP5 showed almost no fluorescence staining in the *AQP5*^{-/-} mice and the negative control group (Figure 1, B and C). The expression of AQP5 in the corneal epithelium of the *AQP5*^{-/-} mice was significantly lower than that in the *AQP5*^{+/+} mice (Figure 1D).

Loss of AQP5 Inhibits Reepithelialization of Debrided Mouse Cornea

To investigate the role of AQP5 in the corneal epithelial wound healing, the healing of central corneal epithelial wound

in the *AQP5*^{-/-} mice was evaluated. The central cornea of the *AQP5*^{+/+} mice and the *AQP5*^{-/-} mice was scraped to cause a 2-mm diameter injury, the repair of which was recorded at 0, 12, 24, and 36 hours. There was a significant difference between the *AQP5*^{+/+} mice and the *AQP5*^{-/-} mice in the rate of corneal epithelial healing from 24 to 36 hours after corneal epithelial scraping (Figure 1E). The corneal epithelial defect area in the *AQP5*^{-/-} mice (24 hours: 21.357% $\pm$ 4.512%; 36 hours: 8.098% $\pm$ 3.623%) was more significantly decreased than in the *AQP5*^{+/+} mice (24 hours: 13.524% $\pm$ 5.8%; 36 hours: 0.546% $\pm$ 0.948%) (Figure 1F).

AQP5 Deficiency Inhibits Corneal Epithelial Cell Proliferation and Corneal Nerve Growth

Proliferation of corneal epithelial cells was examined to explore the effects of AQP5 deficiency on inhibiting corneal wound healing in mice. Immunofluorescence staining showed that the number of Ki-67 positive-stained cells in the corneal epithelium in the *AQP5*^{-/-} mice (unwounded: 2.667 ± 0.516 ; wounded: 7.25 ± 1.768) was significantly lower than that in the *AQP5*^{+/+} mice (unwounded: 5 ± 1.095 ; wounded: 14.167 ± 1.472), regardless of whether the corneal epithelium was scraped or not (Figure 2, A and B).

Next, the effects of AQP5 deficiency on corneal nerve regeneration were evaluated. Corneas of the *AQP5*^{+/+} and *AQP5*^{-/-} mice and those of the *AQP5*^{+/+} and *AQP5*^{-/-} mice 48 hours after scraping were stained with neuronal class

III β -tubulin antibody (Figure 2, C and D). The results showed that the density of corneal nerves in *AQP5*^{-/-} mice (unwounded: $9.0\% \pm 4.5\%$; wounded: $4.414\% \pm 1.907\%$) was sparser than in *AQP5*^{+/+} mice (unwounded: $18.56\% \pm 4.534\%$; wounded: $8.66\% \pm 3.383\%$).

Corneal sensitivity in the *AQP5*^{-/-} mice (unwounded: 4.25 ± 0.316 ; wounded: 2.083 ± 0.97) was lesser than that in the *AQP5*^{+/+} mice (unwounded: 5.917 ± 0.204 ; wounded: 4.292 ± 0.246) (Figure 2E).

AQP5 Affects Corneal Nerve Regeneration by Affecting NGF

To explore the effects of AQP5 on corneal nerve regeneration, qRT-PCR was performed on NGF and several other nerve growth factors, including pigment epithelium-derived factor

Figure 2 AQP5 deficiency inhibits corneal epithelial cell proliferation and corneal nerve growth. **A:** Immunofluorescence staining with anti-Ki-67 antibody in *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 24 hours after central corneal scraping. **B:** Immunofluorescence staining with anti-Ki-67 antibody per unit area. **C:** Immunofluorescence staining for β -tubulin III in *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 48 hours after central corneal scraping. Images of the entire cornea are shown in the **top panels**, and central cornea images are shown in the **bottom panels**. **D:** Nerve densities of the entire cornea (**top panels** in **C**) were calculated on the basis of areas stained positive for β -tubulin III using ImageJ software (version 1.44p). **E:** Histogram of corneal sensitivity in *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 48 hours after central corneal scraping. $n = 6$ (**B**, **D**, and **E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 50 μ m (**A**); 400 μ m (**C**, **bottom panels**). UW, unwounded; W, wounded.

(PEDF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and tachykinin 1 (TAC-1). The expression of NGF in the corneal epithelium in the *AQP5*^{-/-} mice (unwounded: 0.523 ± 0.08 ; wounded: 0.896 ± 0.07) was significantly lower than that in the *AQP5*^{+/+} mice

(unwounded: 1.003 ± 0.1 ; wounded: 2.334 ± 0.313) (Figure 3A).

The expression of NGF in the *AQP5*^{-/-} mice was less than that in the *AQP5*^{+/+} mice, regardless of whether the corneal epithelium was scraped or not (Figure 3, B and C). Immunofluorescence staining showed that NGF was widely

Figure 3 AQP5 affects corneal nerve regeneration by affecting nerve growth factor (NGF). **A:** The expression levels of NGF, pigment epithelium-derived factor (PEDF), glial cell-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), and tachykinin 1 (TAC-1) in *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 24 hours after central corneal scraping were evaluated by real-time fluorescence quantitative PCR. **B:** Western blot analysis was used to confirm the level of NGF protein in the corneal epithelium of *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 24 hours after central corneal scraping. Two corneal epithelia were pooled into one sample. **C:** Quantification of the intensities of Western blot analysis bands of NGF compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **D:** Immunofluorescence staining with anti-NGF antibody in *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 24 hours after central corneal scraping. $n = 3$ (C). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bar = 50 μ m (D). UW, unwounded; W, wounded.

localized in the corneal epithelium of the *AQP5*^{+/+} mice but less localized in the *AQP5*^{-/-} mice. However, corneal epithelial injury (24 hours after wounding) could have led to an increase in NGF expression (Figure 3D).

AQP5 Affects Akt Activation

To elucidate the effects of AQP5 deficiency on inhibiting corneal epithelial repair and nerve regeneration, the phosphorylation levels of Akt were first analyzed by Western blotting. Compared with those in *AQP5*^{+/+} mice, the levels of p-Akt in the *AQP5*^{-/-} mice before and after corneal scraping were reduced by 40% and 33%, respectively (Figure 4, A and B). Immunofluorescence staining showed that p-Akt was widely localized in the corneal epithelium of the *AQP5*^{+/+} mice but less localized in the *AQP5*^{-/-} mice. However, corneal epithelial injury (24 hours after wounding) may have led to an increase in p-Akt expression (Figure 4C).

NGF Promotes Corneal Epithelial Wound Healing and Nerve Regeneration in *AQP5*^{-/-} Mice

To determine the role of NGF in the corneal epithelial wound healing, NGF was injected into the subconjunctival space after the corneal epithelium was scraped off in the *AQP5*^{-/-} mice. As shown in Figure 5A, the application of NGF accelerated the rate of the corneal epithelial wound healing. The percentage of corneal epithelial defects decreased from 24.465% ± 4.341% (24 hours) and 7.867% ± 4.072% (36 hours) in the negative control group to 9.725% ± 2.308% (24 hours) and 0.076% ± 0.121% (36 hours) in the NGF group (Figure 5B). Immunofluorescence staining showed that the number of Ki-67 positive-stained cells in the corneal epithelium (24 hours after scrape) in the NGF group was significantly increased compared with the negative control group (Figure 5, C and D). Corneal whole mount staining showed that corneal nerve density was

Figure 4 AQP5 affects Akt activation. **A:** Western blot analysis was used to confirm the level of phosphorylated Akt (p-Akt) protein in the corneal epithelium of *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 24 hours after central corneal scraping. Two corneal epithelia were pooled into one sample. **B:** Quantification of the intensities of Western blot analysis bands of p-Akt compared with the total amount of Akt. **C:** Immunofluorescence staining with anti-p-Akt antibody in *AQP5*^{+/+} and *AQP5*^{-/-} mice before and 24 hours after central corneal scraping. *n* = 3 (**B**). **P* < 0.05, ***P* < 0.01. Scale bar = 50 μm (**C**). UW, unwounded; W, wounded.

Figure 5 Nerve growth factor (NGF) promotes corneal epithelial wound healing and nerve regeneration in *AQP5*^{-/-} mice. *AQP5*^{-/-} mice were wounded by epithelium debridement (2-mm diameter). At 0 hours after corneal epithelial scraping, phosphate-buffered saline [negative control group (NC)] or NGF (NGF group) was injected into the subconjunctival area of *AQP5*^{-/-} mice. **A:** Corneal epithelial defect areas in NC and NGF groups were observed by fluorescein sodium staining at 0, 12, 24, and 36 hours after corneal epithelial scraping. **B:** The histogram represents the ratio of the residual defect area/the original defect area. **C:** Immunofluorescence staining with anti-Ki-67 antibody in NC and NGF groups at 24 hours after central corneal scraping. **D:** Immunofluorescence staining with anti-Ki-67 antibody per unit area. **E:** Immunofluorescence staining for β -tubulin III in NC and NGF groups at 72 hours after central corneal scraping. Images of the entire cornea are shown in the **top panels**, and central cornea images are shown in the **bottom panels**. **F:** Nerve densities of the entire cornea (**top panels** in **E**) were calculated on the basis of areas staining positive for β -tubulin III using ImageJ software (version 1.44p). **G:** Histogram of corneal sensitivity in NC and NGF groups at 48 hours after central corneal scraping. $n = 6$ (**B**, **D**, **F**, and **G**). * $P < 0.05$, *** $P < 0.001$. Scale bars: 50 μ m (**C**); 400 μ m (**E**, **bottom panels**).

significantly increased after the subconjunctival injection of NGF (Figure 5E). The density of the corneal nerve increased from $4.414\% \pm 1.854\%$ in the negative control group to $15.08\% \pm 3.857\%$ in the NGF group (72 hours

after scrape) (Figure 5F). The results of the corneal sensitivity measurement showed that NGF promoted the recovery of corneal sensitivity (72 hours after scrape) (Figure 5G).

NGF Promotes Corneal Epithelial Wound Healing and Nerve Regeneration by Affecting Akt Activation

To determine the mechanism of NGF in regulating the repair rate of corneal epithelial injury, in addition to NGF, an Akt inhibitor was used to block the activation of Akt phosphorylation in the *AQP5*^{-/-} mice. The results of the Western blot analysis showed that the level of p-Akt in the corneal epithelium in the NGF group was increased 24 hours after corneal scraping. However, the level of p-Akt in the corneal epithelium in the NGF + Akti group did not change significantly (Figure 6, A and B). As shown in Figure 6C, subconjunctival injection of Akt inhibitor significantly reduced the promotion effect of NGF on corneal epithelial wound healing. The percentages of corneal epithelial defects were $9.83\% \pm 2.522\%$ (24 hours) and $0.045\% \pm 0.11\%$ (36 hours) in the NGF group and $22.577\% \pm 7.717\%$ (24 hours) and $9.594\% \pm 9.291\%$ (36 hours) in the NGF + Akti group (Figure 6D). The results of immunofluorescence staining showed that the number of Ki-67 positive-stained cells in the corneal epithelium (24 hours after scrape) in the NGF + Akti group was significantly decreased compared with that in the NGF group (Figure 6, E and F). Corneal whole mount staining showed that corneal nerve density was significantly decreased after the subconjunctival injection of the Akt inhibitor (Figure 6G). The density of the corneal nerve decreased from $15.43\% \pm 3.745\%$ in the NGF group to $6.467\% \pm 1.61\%$ in the NGF + Akti group (72 hours after scrape) (Figure 6H). The results of the corneal sensitivity measurement showed that the use of the Akt inhibitor blocked the effects of NGF on the recovery of corneal sensitivity (72 hours after scrape) (Figure 6I).

Discussion

AQP5 is an effective water channel expressed in many cells.³⁵ The water permeability of secretory cells is generally higher than that of nonsecretory cells. Previous studies have shown that AQP5 promotes the process of corneal wound healing, but its specific mechanism remains to be studied.¹⁷ AQP5 deficiency reduces the water permeability across the cornea in mice.³⁶ In addition to being an effective water transporter, AQP5 promotes cell migration and proliferation in corneal epithelial wound healing.³⁵ AQP5 mediated the effects on cell-cell adhesion through interactions with junctional protein, independently of AQP5-mediated water transport.³⁷ Previous studies have provided evidence that AQP5 is associated with innervation. After parasympathetic denervation, the expression of AQP5 in rat submandibular gland decreased.³⁸ However, further potential role(s) of AQP5 in the cornea remain to be identified.

The mouse corneal epithelium consists of five to six layers of cells.^{39–41} A single layer of mitotic columnar basal cells is located on the basement membrane. The basal cells are covered by one to three layers of pterygoid cells,

followed by two to three layers of flat squamous cells. Maintenance of the epithelial layer and its continuous regeneration are essential in the microbial barrier function, the replacement of damaged corneal surfaces, and corneal transparency.¹⁷ Corneal nerve fibers play an important role in the nutrition of the corneal epithelium and contribute to the maintenance of a healthy ocular surface. Several neurotrophic factors, such as NGF, glial cell-derived neurotrophic factor, and ciliary neurotrophic factor, can stimulate the proliferation, migration, and immune protection of corneal epithelial cells through their receptors in the corneal epithelium.^{20,22} The findings of the present study show a significant impairment in corneal wound healing in the *AQP5*^{-/-} mice due to distinct defects in corneal epithelial cell proliferation and nerve regeneration.

Some members of the aquaporin superfamily are involved in damage repair in different tissues. AQP1, AQP3, and AQP5 play an important role in maintaining corneal transparency and internal environment stability.⁴² AQP1 is also involved in the migration of different cell types, such as kidney cells, gastric epithelial cells, endothelial cells, melanoma cells, glioma cells, corneal cells, and corneal endothelial cells.^{14,16,43–46} After an optic nerve crush, the inhibition of AQP4 can increase the damage to ganglion cells.⁴⁷ AQP5 is involved in promoting cell migration of lung adenocarcinoma cells and non-small-cell lung cancer cells, as well as the proliferation of colon, lung, and ovarian cancer cells.^{48–52} In addition, down-regulation of AQP5 may inhibit the migration and proliferation of ectopic endometrial epithelial cells through the Akt pathway.⁵³

In the present study, the expression of AQP5 in the cornea of the *AQP5*^{-/-} mice was verified. The results of immunofluorescence staining and the Western blot analysis showed that AQP5 was abundant in the corneal epithelium of the *AQP5*^{+/+} mice but not in the corneal epithelium of the *AQP5*^{-/-} mice. Compared with the *AQP5*^{+/+} mice, the *AQP5*^{-/-} mice showed slower corneal wound healing and nerve regeneration. Number of Ki-67-positive cells and nerve density in the corneas of the *AQP5*^{-/-} mice were significantly lower than that in the *AQP5*^{+/+} mice, regardless of whether the corneal epithelium was scraped or not. These results suggest that AQP5 deficiency inhibits the proliferation of corneal epithelial cells and nerve fibers in the cornea. The expression of several nerve growth-related factors in the corneal epithelium of the mice was detected by qRT-PCR. These nerve growth-related factors were differentially expressed in the corneal epithelium of the mice and had different expression patterns. Only the expression pattern of NGF was closely related to AQP5 deletion and corneal epithelial wound healing. Therefore, NGF was selected for analysis in subsequent experiments. The expression of NGF and p-Akt in the corneal epithelium decreased after AQP5 knockout, regardless of whether the corneal epithelium was scraped or not. Interestingly, these results were consistent with the previous results of Ki-67 and corneal whole mount staining of nerve fibers. These

Figure 6 Nerve growth factor (NGF) promotes *AQP5*^{-/-} corneal epithelial wound healing and nerve regeneration by affecting Akt activation in mice. *AQP5*^{-/-} mice were wounded by epithelium debridement (2-mm diameter). At 0 hours after corneal epithelial scraping, phosphate-buffered saline [negative control group (NC)] or NGF (NGF group) was injected into the subconjunctival area of *AQP5*^{-/-} mice. For Akt inhibition [Akti; NGF + Akti group (NGF + Akti)], subconjunctival injection of Akti was administered to *AQP5*^{-/-} mice 24 hours before corneal epithelial scraping and 0 and 24 hours after corneal epithelial scraping, and NGF was supplemented at 0 hours after corneal epithelial scraping. **A**: Western blot analysis was used to confirm the level of phosphorylated Akt (p-Akt) protein in the corneal epithelium at 24 hours after central corneal scraping. Two corneal epithelia were pooled into one sample. **B**: Quantification of the intensities of Western blot analysis bands for p-Akt compared with the total amount of Akt. **C**: The corneal epithelial defect areas in the NGF and NGF + Akti groups were observed by fluorescein sodium staining at 0, 12, 24, and 36 hours after corneal epithelial scraping. **D**: Histogram representing the ratio of the residual defect area/the original defect area. **E**: Immunofluorescence staining with anti-Ki-67 antibody at 24 hours after central epithelial scraping. **F**: Immunofluorescence staining with anti-Ki-67 antibody per unit area. **G**: Immunofluorescence staining for β -tubulin III at 72 hours after central corneal scraping. Images of the entire cornea are shown in the **top panels**, and central cornea images are shown in the **bottom panels**. **H**: Nerve densities of the entire cornea (**top panels** in **G**) were calculated on the basis of the areas staining positive for β -tubulin III using ImageJ software (version 1.44p). **I**: Histogram of corneal sensitivity in NGF and NGF + Akti groups at 48 hours after central corneal scraping. $n = 3$ (**B**); $n = 6$ (**D**, **F**, **H**, and **I**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars: 50 μ m (**E**); 400 μ m (**G**, **bottom panels**).

results indicate that AQP5 deficiency may reduce the expression of NGF in the corneal epithelium and inhibit the activation of Akt, which affects the corneal epithelial wound healing.

To verify the hypothesis, activation of NGF and Akt was studied in the *AQP5*^{-/-} mice to observe corneal wound healing in this group. After exogenous supplementation of NGF, the speed of corneal wound healing accelerated, and the expression of Ki-67 and nerve fibers in the corneal epithelium increased. However, the inhibition of Akt activation in *AQP5*^{-/-} mice supplemented with NGF, inhibited the effects of NGF on the speed of wound healing, the expression of Ki-67, and the nerve fibers in the corneal epithelium.

This study shows, for the first time, that AQP5 deficiency can affect the nerve regeneration of mice by affecting NGF and the activation of Akt signaling pathways. However, AQP5 and NGF may not be regulated by direct interaction. Therefore, a future study will aim to clarify the specific mechanism between AQP5 and NGF.

In the present study, the significant damage to corneal wound healing in the *AQP5*^{-/-} mice occurred due to the obvious defects in corneal epithelial cell proliferation and nerve regeneration, which may have played a role in the activation of Akt. These results provide evidence for the involvement of aquaporins in cell proliferation and nerve regeneration, and suggest that AQP5 induction is a potential therapeutic method for accelerating the repair of corneal defects.

Author Contributions

P.C. and G.D. designed the experiments; Y.L., Y.W., D.C., X.C., and G.D. performed the experiments; Y.L., Y.W., D.C., X.C., and P.C. analyzed the data; Y.L., P.C., and G.D. wrote the manuscript; all authors reviewed the manuscript and gave their approval of the final version.

Supplemental Data

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