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A predominant form of C-terminally end-cleaved AQP0 functions as an open water channel and an adhesion protein in AQP0$^{\Delta C/\Delta C}$ mouse lens

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Abstract

The purpose of this investigation was to find out whether C-terminally end-cleaved aquaporin 0 **(AQP0)**, that is present predominantly in the lens mature fiber cells of the WT, functions as a water channel and a cell-to-cell adhesion **(CTCA)** protein in a knockin **(KI)** mouse model (AQP0$^{\Delta C/\Delta C}$) that does not express intact AQP0. A genetically engineered KI mouse model, AQP0$^{\Delta C/\Delta C}$, expressing only end-cleaved AQP0 was developed. This model expresses 1–246 amino acids of AQP0, instead of the full length 1–263 amino acids. Lens transparency of postnatal day 10 (P10) was analyzed qualitatively by dark field imaging. WT, AQP0$^{+/-}$ and AQP0$^{+/\Delta C}$ lenses were transparent; AQP0$^{-/-}$ and AQP0$^{\Delta C/\Delta C}$ mouse lenses displayed loss of transparency. Lens fiber cell membrane vesicles **(FCMVs)** were prepared from wild type (WT), AQP0 heterozygous (AQP0$^{+/-}$), AQP0 knockout (AQP0$^{-/-}$), AQP0$^{+/\Delta C}$ and AQP0$^{\Delta C/\Delta C}$; water permeability **(P_f)** was measured using the osmotic shrinking method. CTCA assay was performed using adhesion-deficient L-cells and FCMVs prepared from the abovementioned genotypes. FCMVs of AQP0$^{+/-}$ and AQP0$^{-/-}$ showed a statistically significant reduction ($P<0.001$) in P_f and CTCA compared to those of WT. AQP0$^{+/\Delta C}$ and AQP0$^{\Delta C/\Delta C}$ FCMVs exhibited no statistically significant alteration ($P>0.05$) in P_f compared to those of WT. However, CTCA of AQP0$^{+/\Delta C}$ AQP0$^{\Delta C/\Delta C}$ FCMVs was significantly higher ($P<0.001$) than that of WT FCMVs. Our experiments clearly show that C-terminally end-cleaved AQP0 can function both as a water channel and a CTCA molecule in the lens fiber cell membranes. Also, end-truncation plays an important role in increasing the CTCA between fiber cells.

Keywords

AQP0; C-terminally end-cleaved AQP0; water permeability; cell-to-cell adhesion

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1. Introduction

The crystalline lens is a unique tissue, which is meticulously designed to be transparent for focusing light precisely on the sensory retina. It has a monolayer of epithelial cells at the anterior surface and multiple layers of fiber cells that extend from the anterior to the posterior pole. Fiber cells are derived from the epithelial cells at the germinative zone. Of the two major regions in the lens, the cortex contains elongating and maturing secondary fiber cells whereas the nuclear region has the mature and the oldest fibers. The fiber cells of a lifetime are present in the lens, which lacks vasculature as an adaptation to avoid light scattering. A microcirculation comprising AQP water channels, solute transporters, co-transporters etc. nourish the lens and remove metabolic wastes [1–6] to uphold transparency and homeostasis.

The structure of the lens is critical for its function and a salient feature is the presence of all the fiber cells produced in a lifetime. The cellular proteins present undergo post-translational modifications including end-cleavage. AQP0, the most abundantly expressed membrane protein in the lens, is synthesized as a 28 kDa protein. When the fiber cells mature, the amino- (N) and/or carboxyl- (C) terminal ends cleave off to result in AQP0 smaller than its original molecular size. Water permeability [3] and cell-to-cell adhesion (CTCA) are the two important roles of AQP0 [6–10]. Does the end-cleaved AQP0 perform both functions? The effect of certain N- and C-terminal end truncations of AQP0 on water channel function and CTCA had been investigated using *in vitro/ex vivo* models [8,10–12] which showed the capability of cleaved forms to perform both functions. However, protein structural analyses proposed distinct roles for the intact and cleaved forms of AQP0; it has been postulated that posttranslational N- and/or C-terminal end cleavages result in the closure of water pore [13]. There are no *in vivo* investigations on the effect of end-cleavage of AQP0 on water channel and CTCA functions. To fill the gap, we developed a knockin mouse model ($AQP0^{\Delta C/\Delta C}$) expressing only end-cleaved AQP0 and investigated the water channel and CTCA functions.

2. Materials and methods

2.1. Animals

WT, $AQP0^{-/+}$ and $AQP0^{-/-}$ [14,15], and $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mouse models [16] used in this study are in C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) inbred strain background. These mice had been screened to verify the expression of the beaded filament protein CP49 without the natural mutation [15,16], following the methods described by Alizadeh et al. [17] and Simirskii et al.[18]. $AQP0^{\Delta C/\Delta C}$ mouse model was developed through inGenious Targeting Laboratory, Inc. (Ronkonkoma, NY). This mouse model expresses a major form of C-terminally end-cleaved AQP0 (that lacks 17 amino acids, 247–263) in the fiber cells. This form of AQP0 is observed in the nuclear region of human, bovine and mouse [6,19–22]. $AQP0^{\Delta C/\Delta C}$ mice do not express the intact AQP0 which has 1–263 amino acids in the WT. The creation of this mouse model and characterization as well as the lens optical quality of the lens had been described in a recent paper by Varadaraj and Kumari [16]. All procedures were performed following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health's (NIH;

Bethesda, MD, USA) “Guide for the Care and Use of Laboratory Animals” and protocols approved by Stony Brook University Animal Care and Use Committee.

2.2. Assessment of lens transparency

Lens transparency was studied using the method as described [15,16]. In brief, lenses of pups at postnatal day 10 (P10) from WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mice were dissected out into mammalian physiological saline that was prewarmed to 37°C. To qualitatively assess transparency, lenses were imaged under dark field using the same lighting and imaging conditions using a binocular microscope connected to a digital camera.

2.3. Mouse lens fiber cell membrane water permeability assay

Membrane water permeability (P_f) measurement was carried out using the rate of shrinking of lens fiber cell membrane vesicles (FCMVs), as done previously [3,23]. In brief, lens capsules of WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mice pups at P10 were gently removed and fiber cell bundles from the outer cortex were dissected out. FCMVs were prepared and incubated in hypertonic saline (450 mOsm) and the rate of shrinkage was imaged using digital video microscopy; P_f was calculated as described by Varadaraj et al. [3].

2.4. Mouse lens FCMV Adhesion Assay

Adhesion-deficient L-cells were cultured as described [8,12]. In brief, mouse fibroblast L-cells (ATCC, Manassas, VA) were cultured at 37°C in an incubator set with 5% CO_2. Lens FCMVs were prepared from WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mice pups at P10 as described in section 2.3 above. Adhesion assay was done as described previously [10]. The FCMVs were loaded with CellTracker Green and plated over a monolayer of parental L-cells, which lack endogenous adhesion protein and incubated for 1hr at 37 °C. The culture dishes were gently washed three times for 5 min each. Cells were imaged under a Zeiss epifluorescent microscope (excitation band filter of 465–495 nm; emission band filter of 515–555 nm). The number of fluorescent FCMVs attached to the lawn of cells was counted. Mean and standard deviations were calculated from the values of six repetitions and represented as a bar graph.

2.5. Statistical Analysis

SigmaPlot 10 software was used for Student’s t-tests. Statistical significance was calculated using a paired *t*-test. $P < 0.05$ was considered significant.

3. Results and Discussion

We tested the functional status of end-cleaved AQP0 in the KI mouse model that was genetically modified to lose 17 amino acids at the C-terminal end to result in a shortened AQP0 with 1–246 amino acids instead of the 1–263 amino acids (intact AQP0) present in the WT cortex. Our experiments corroborate that the end-cleaved AQP0 functions as an open water channel and a CTCA protein.

Knockout and mutations of AQP0 cause cataract due to loss of function or gain of function [14,24–28]. To find out the effects of the expressing only end-cleaved AQP0 in the lens on transparency, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ lenses were observed at a very early stage in development and compared with age-matched WT and $AQP0^{+/-}$ and $AQP0^{-/-}$ lenses. At postnatal day 10 (P10) wild type (WT), $AQP0^{+/-}$ and $AQP0^{+/\Delta C}$ mouse lenses remained transparent whereas $AQP0^{-/-}$ and $AQP0^{\Delta C/\Delta C}$ mouse lenses showed loss of transparency (Fig. 1).

Our previous *in vitro* studies have shown that C-terminally end-truncated AQP0 mutant (AQP0–1-246), which was chosen to develop the current knockin model, trafficked to the plasma membrane and showed both P_f and CTCA functions [12]. To test whether this is true in an *in vivo* model, we prepared lens outer cortical FCMVs of WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mice and carried out osmotic P_f measurements after subjecting them to a hypertonic medium. Membrane P_f was calculated using the rate of shrinking of each vesicle. FCMVs of WT which expresses naturally both intact AQP0 and cleaved AQP0 had an average P_f of 38.90 ± 8.91 μm/s (Table 1; Fig. 2). The P_f of FCMVs of $AQP0^{+/-}$ and $AQP0^{-/-}$ decreased 2 and 4-fold, respectively, compared with that of WT (Table 1; Fig. 2). The reductions were statistically significant ($P< 0.001$) and denoted with a star. However, loss of 17 amino acids at the C-terminal end of AQP0 in $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mice lenses did not significantly ($P>0.05$) alter the fiber cell P_f, when compared with WT lenses (Table 1 and Fig. 2). Using electron crystallographic analyses, it had been suggested that intact AQP0 functions as an open water channel; end-cleaved AQP0 is closed as a water pore but functions as a CTCA protein [13]. However, the present investigation and previous studies [11,12] show that both intact and cleaved forms of AQP0 can function as open water channels.

Next, we tested the CTCA capability of the different genotypes. FCMV adhesion assay was performed *ex vivo* to test and verify the adhesive property between lens fiber cell membranes. CellTracker Green-loaded WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ or $AQP0^{\Delta C/\Delta C}$ mice lens FCMVs were plated over a monolayer of parental L-cells and subjected to adhesion assay. FCMVs of $AQP0^{+/-}$ and $AQP0^{-/-}$ exhibited significantly less adhesion ($P< 0.001$) to parental L- cells compared with those of WT lens (Fig. 3). These data suggest that as the level of AQP0 decreases there is a proportional decrease in CTCA. AQP0 present in the WT is responsible for the increased level of vesicle adhesion to the parental L-cells. FCMVs from $AQP0^{+/-}$ showed comparatively less adhesion than those of WT, and significantly more than those of $AQP0^{-/-}$ indicating that AQP0-induced adhesion is due to the direct interaction of AQP0 with the plasma membrane of the L-cells. When we examined $AQP0^{+/\Delta C}$ or $AQP0^{\Delta C/\Delta C}$ FCMVs there was a significant increase ($P<0.05$) in adhesion compared to WT FCMVs. These data clearly demonstrate that the end-cleaved AQP0 is capable of CTCA. Further, the results show that the C-terminal end-cleavage of AQP0 during lens development helps to increase the adhesion between fiber cells, corroborating the previous *in vitro* [8,12] and *ex vivo* [10] studies.

In the WT lens, the outer cortex has the highest concentration of intact AQP0; inner cortical and outer nuclear regions contain a mixture of intact and end-cleaved AQP0 proteins [21,29,30] with a gradient reduction in intact AQP0; the concentration of cleaved AQP0

proportionally increases from the inner cortex towards the nuclear region. In the AQP0$^{\Delta C/\Delta C}$ mouse model the lenses, express only cleaved AQP0. The created C-terminally end-truncated AQP0 (AQP0–1-246) mimics the most prevalent natural cleavage of AQP0 [21,29] in the human lens. Several proteins in the lens such as AQP0 [20,29,30], connexins (Cx46 and 50; [31,32]), crystallins [33] and beaded filament proteins (filensin and CP49; [34,35]) undergo end-cleavage. Under normal conditions, this process does not affect lens transparency. The significance of the end-cleavage, the functions of the intact and cleaved forms and the necessity for the presence of intact and the end cleaved forms in the lens throughout the lifespan have kindled the curiosity of scientists. The functional status of intact and cleaved AQP0 i.e., whether both types can perform both functions or each type does a specific function, currently exists as a scientific debate. Electron diffraction structural studies indicate that C-terminal end-cleaved AQP0 functions as an adhesion protein but is closed as a water pore [13]. Another conclusion arrived by X-ray crystallographic analyses [36] is that intact AQP0 besides being an open water channel may also participate in CTCA. Michea et al. [37,38] conducted *in vitro* experiments using liposomes; AQP0 adhered to negatively charged liposomes demonstrating that intact AQP0 may participate in CTCA via electrostatic interaction. It has been suggested that the extracellular loops of AQP0 interact with the lipids of the opposing plasma membrane [36–40]. Our previous *in vitro* studies showed that intact AQP0 is capable of both water permeability and CTCA functions [8,16]. Recently, we found that FCMVs prepared from the WT attached to adhesion-deficient L-cells [10] corroborating the *in vitro* studies [10,12] and verifying the electrostatic mechanism of CTCA between the positively charged residues in the extracellular loops of AQP0 and the negatively charged lipids in the adjacent plasma membrane. The current results stretch the field even further; FCMVs with end-cleaved AQP0 showed water permeability comparable to that of the WT and significantly higher CTCA than the FCMVs of WT. These results demonstrate that intact and cleaved forms of AQP0 are capable of both functions and end-cleavage could be a necessary step for enhanced CTCA at the lens interior.

Correlating the current data to what happens at the inner cortical and nuclear regions of the lens, where the concentrations of the cleaved-AQP0 are more, our results reflect the physiological phenomenon of increased CTCA (Fig. 3) in these regions. Lens ultrastructural studies revealed that AQP0 can form thin junctions between fiber cell membranes. In the WT, while there are small areas of thin junctions in the outer cortex (11–13 nm; [39–42], large patches of square array thin junctions are formed at the inner cortical and nuclear regions which have mature fiber cells with cleaved AQP0 [39,40, 43,44]. Formation of thin junctions could impact compaction of the fiber cells and refractive index gradient (RING) adjustment of the continuously spherically growing lens to avoid spherical aberration [12]. We imaged and analyzed the lenses of the AQP0$^{+/\Delta C}$ and AQP0$^{\Delta C/\Delta C}$ mice and found that lenses of the latter had developed abnormal distortion aberration starting at the lens nuclear region by P5, and by P20 developed cataract which was more severe than in AQP0$^{-/-}$ [16]. The appearance of distortion aberrations while focusing is a consequence of the loss of RING. It is paradoxical that it is the cortical region of AQP0$^{\Delta C/\Delta C}$ lens which does not possess its normal share of intact (full length) AQP0; nonetheless, the distortion aberration occurs first at the nuclear region (Fig.1). This observation highlights the necessity for the

spatial distribution of intact and end-cleaved AQP0 from the outer cortex to the inner nucleus in the appropriate ratio for lenticular homeostasis. Presence of cleaved AQP0 at the cortex, which normally contains intact AQP0 and a few small areas of thin junctions, might be now allowing the formation of large areas of square array thin junctions with enhanced adhesion thereby tipping the balance for the conducive adhesion from the cortex to the nucleus and jeopardizing the development of a proper RING. In the $AQP0^{+/\Delta C}$ lens, there is no cataract at P10 (Fig. 1) indicating that presence of intact AQP0 must have allowed the creation of a favorable ratio of intact to cleaved AQP0 from the outer cortex to the nucleus at that early age. From our experimental data, it is rational to infer that truncations of N- and C-terminal ends of AQP0 are natural events in the fiber cell maturation process to adjust lens RING [12,30] and biomechanics [45] for precise focusing. C-terminal post-translational cleavages were present in the human lens as early as 2 years of age [29] and in mouse at P10 [16] suggesting that AQP0 end-truncation is a normal fiber cell-maturation-related event. The end-cleavage could be a mechanism to compact the old fiber cells because new fiber cells are being added throughout the lifespan. Since end cleavages occur after trafficking of AQP0 to the membrane in the WT, the ends could be important for trafficking and spacial distribution; as seen from the experiments end-cleavage does not affect the P_f and CTCA functions.

In conclusion, data collected using the $AQP0^{\Delta C/\Delta C}$ mouse lens FCMVs on Pf and adhesion functions show that post-translationally end-cleaved AQP0 can function as a water channel and a CTCA molecule. End-cleavages help to modulate the CTCA function of AQP0 to establish appropriate RING in the lens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AQP0	aquaporin 0
CTCA	cell-to-cell adhesion
P10	postnatal day 10
AQP0–1-246	C-terminally end-cleaved-AQP0
WT	wild type
$AQP0^{+/-}$	AQP0 heterozygous
AQP0-KO	AQP0 knockout
KI	knockin

AQP0$^{\Delta C/\Delta C}$	C-terminally end-cleaved-AQP0 KI homozygous mouse model
AQP0$^{+/\Delta C}$	C-terminally end-cleaved-AQP0 KI heterozygous mouse model
P_f	water permeability
FCMV	fiber cell membrane vesicle
Cx	connexin
CP49	phakinin
RING	refractive index gradient

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Highlights

- Lenses expressing only C-terminally end-cleaved AQP0 show loss of transparency
- C-terminally end-cleaved AQP0 does not affect membrane water permeability
- C-terminally end-cleaved AQP0 significantly increased fiber cell-to-cell adhesion

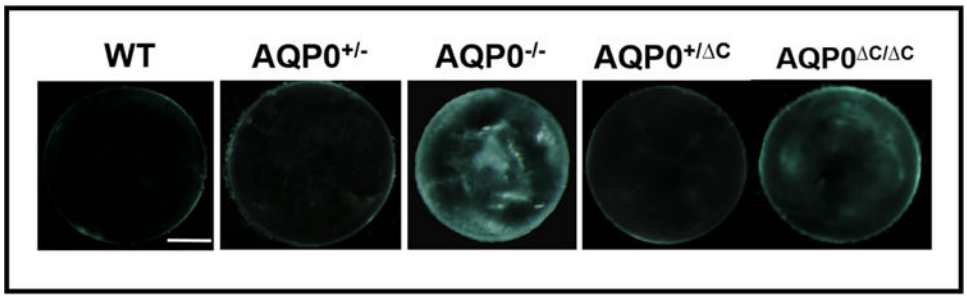

Fig. 1.

Lens transparency. Lenses from WT, AQP0$^{+/-}$, AQP0$^{-/-}$, AQP0$^{+/\Delta C}$ and AQP0$^{\Delta C/\Delta C}$ mice pups at P10 were photographed using darkfield illumination. Bar, 425 μm.

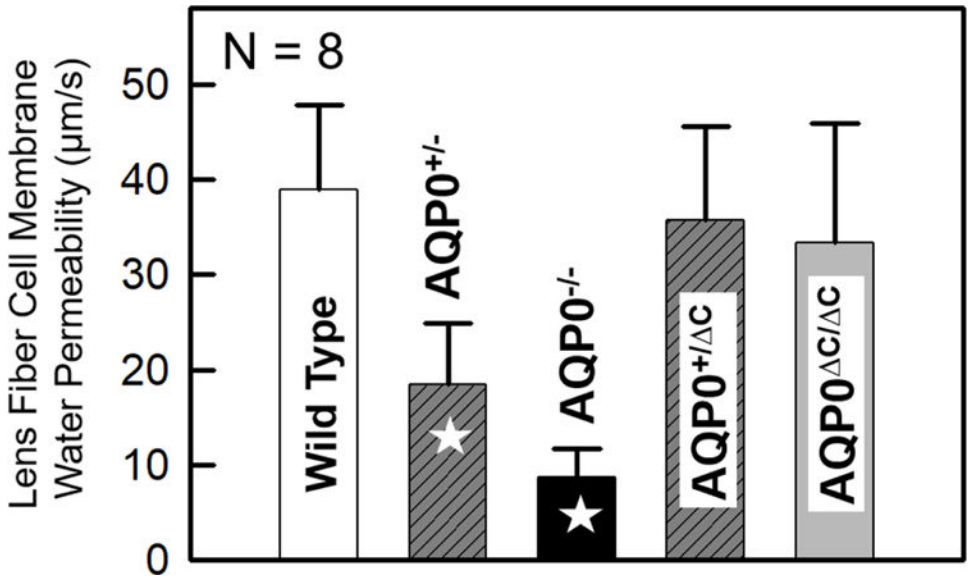

Fig. 2.

Lens fiber cell membrane water permeability. Lens fiber cell membrane vesicles from WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ pups at P10 were tested. Each bar represents mean ± SD. Star represents the degree of significance in comparison, $P < 0.001$.

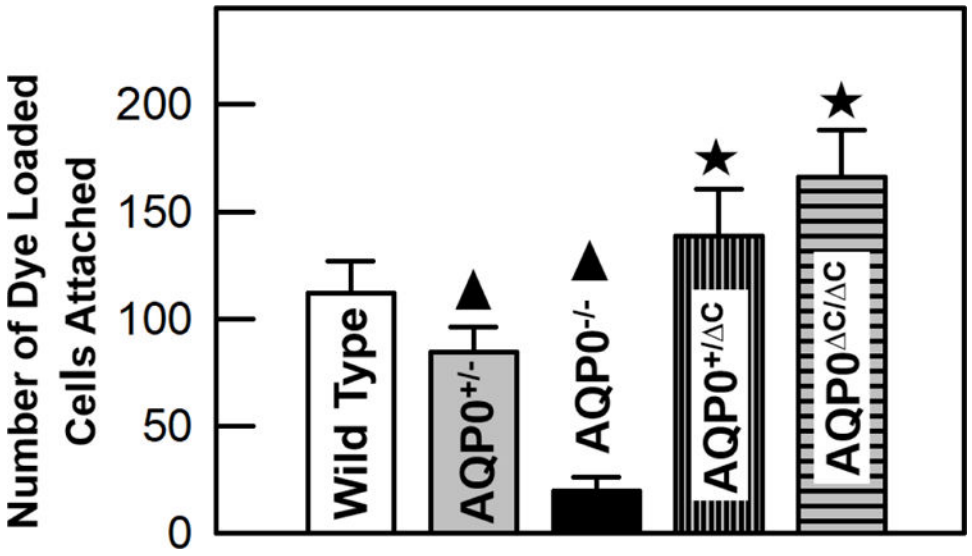

Fig. 3.

Lens fiber cell membrane vesicle (FCMV) adhesion assay. Adhesion of WT, $AQP0^{+/-}$, $AQP0^{-/-}$, $AQP0^{+/\Delta C}$ and $AQP0^{\Delta C/\Delta C}$ mouse lens FCMVs to a monolayer of parental L-cells. Each bar represents mean ± SD. Star represents the degree of significance in comparison, $P < 0.001$.

Table 1.

Lens fiber cell membrane water permeability (P_f) of WT, AQP0$^{+/-}$, AQP0$^{-/-}$, AQP0$^{+/\Delta C}$ and AQP0$^{\Delta C/\Delta C}$ mice (C57 strain).

Genotype	Fiber Cell Membrane Water Permeability (μm/s)
Wild Type	39.90 ± 8.91
AQP0$^{+/-}$	19.20 ± 6.13 *
AQP0$^{-/-}$	8.70 ± 3.0 *
AQP0$^{+/\Delta C}$	35.70 ± 9.89
AQP0$^{\Delta C/\Delta C}$	34.40 ± 12.49

* P< 0.001