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Aquaporin 0 plays a pivotal role in refractive index gradient development in mammalian eye lens to prevent spherical aberration

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ABSTRACT

Aquaporin 0 (AQP0) is a transmembrane channel that constitutes ~45% of the total membrane protein of the fiber cells in mammalian lens. It is critical for lens transparency and homeostasis as mutations and knockout cause autosomal dominant lens cataract. AQP0 functions as a water channel and as a cell-to-cell adhesion (CTCA) molecule in the lens. Our recent *in vitro* studies showed that the CTCA function of AQP0 could be crucial to establish lens refractive index gradient (RING). However, there is a lack of *in vivo* data to corroborate the role of AQP0 as a fiber CTCA molecule which is critical for creating lens RING. The present investigation is undertaken to gather *in vivo* evidence for the involvement of AQP0 in developing lens RING. Lenses of wild type (WT) mouse, AQP0 knockout (heterozygous, $AQP0^{+/-}$) and AQP0 knockout lens transgenically expressing AQP1 (heterozygous $AQP0^{+/-}/AQP1^{+/-}$) mouse models were used for the study. Data on AQP0 protein profile of intact and N- and/or C-terminal cleaved AQP0 in the lens by MALDI-TOF mass spectrometry and SDS–PAGE revealed that outer cortex fiber cells have only intact AQP0 of ~28 kDa, inner cortical and outer nuclear fiber cells have both intact and cleaved forms, and inner nuclear fiber cells have only cleaved forms (~26–24 kDa). Knocking out of 50% of AQP0 protein caused light scattering, spherical aberration (SA) and cataract. Restoring the lost fiber cell membrane water permeability (P_f) by transgene AQP1 did not reinstate complete lens transparency and the mouse lenses showed light scattering and SA. Transmission and scanning electron micrographs of lenses of both mouse models showed increased extracellular space between fiber cells. Water content determination study showed increase in water in the lenses of these mouse models. In summary, lens transparency, CTCA and compact packing of fiber cells were affected due to the loss of 50% AQP0 leading to larger extracellular space, more water content and SA, possibly due to alteration in RING. To our knowledge, this is the first report identifying the role of AQP0 in RING development to ward off lens SA during focusing.

1. Introduction

Eye lens is a relatively transparent living tissue that focuses images on the retina. It has a unique property of continuously adjusting the refractive index of this constantly growing tissue to assure accurate focusing. Lens consists of ~66% water and ~33% proteins. The cytosolic protein concentration and free water content in the fiber cells are maintained in a reciprocal gradient to adjust refractive index. Studies have shown that lens refractive index does not change abruptly but increases gradually from the periphery to the center [1]. Human lens has a Refractive Index Gradient (RING) which is 1.386–1.406 from the lens cortex to the nucleus [2,3] and in mouse it is 1.35–1.55 [4].

To get a clear image of an object, all light rays need to be focused at the same point on the retina. Spherical aberration (SA) is a phenomenon in which lens fails to image central and peripheral light rays at the same focal point resulting in multiple focal points and generating a fuzzy image. It is caused by a change in lens curvature or a change from a normal refractive index gradient (RING) to a homogeneous or irregular RING [5]. In the eye lens with a gradient refractive index, SAs are minimized as the incident light ray continues within the lens fiber cells through extracellular space and cytoplasm. It has been suggested that lens RING is dependent on the major fiber cell cytoplasmic protein, crystallin, and water [6,7]. SA of human eye and corrective measures are receiving significant attention [8,9]. Understanding of the proteins responsible for aging-related SA in humans which is a malady caused by alterations in lens RING would help to find strategies to treat it successfully. Aquaporin 0 (AQP0) expressed in lens fiber cells could be a potential target.

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AQP0 is abundantly expressed in the lens fiber cells. Low levels of AQP0 mRNA and protein have been identified in retina [10], liver [11] and sertoli cells of testis [12]. AQP0 contributes ~45% of total membrane protein in the lens. Mutation or deletion of a single or both copies of AQP0 resulted in autosomal dominant bilateral lens cataract in mouse highlighting the significance of this protein for maintaining lens transparency and homeostasis. Functional studies showed that AQP0 plays a significant role in plasma membrane water permeability (P_f) and fiber cell to fiber cell adhesion (CTCA) [13–17].

Three AQPs are present in mammalian lens. AQP1 and AQP5 are identified in the epithelial cells, and AQP0 and AQP5 in the fiber cells. AQP0 is the least efficient water channel. AQP1 expressed in anterior and equatorial epithelial cells is 40 times more efficient than AQP0 [18]. The purpose of the prolific expression and the low P_f of AQP0 has puzzled scientists for a long time. Besides, the N- and/or C-terminal ends of AQP0 and several other lens proteins are cleaved as the fiber cells mature but the shortened forms remain in the lens throughout life. The reason behind these events also remained enigmatic. Based on our previous experiments and available literature, we hypothesized that AQP0's low P_f, capability for CTCA, interaction with other proteins, progressive cleavage of N- and C-terminal ends in the mature fibers of the inner cortical and nuclear regions and expression of the more efficient AQP5 in the fiber cells [19–21], all of these could be a prelude for lens establishing RING to prevent SA during focusing [17].

In the current study, we investigated *in vivo*, the possible unique role of AQP0 in developing RING, using lenses of WT, AQP0 knockout and AQP0 knockout mouse expressing AQP1 transgene in the fiber cells. Our data strongly suggest that intact AQP0 promotes CTCA *in vivo* and is involved in preventing SA by participating in lens RING development.

2. Materials and methods

2.1. Animals

Wild type C57BL/6J (WT), AQP0 knockout (from Dr. Shiels, Washington University, MO) heterozygous ($AQP0^{+/-}$) and $TgAQP1^{+/-}/AQP0^{+/-}$ [14,16] heterozygous were used for the study. Homozygous AQP0 knockout lenses have much severe cataract and were excluded from the study.

2.2. MALDI-TOF MS analysis of mouse lens membrane protein

Lenses were dissected out of capsule and carefully partitioned as outer cortex (OC), inner cortex (IC), outer nucleus (ON) and inner nucleus (IN). The tissues were homogenized in 4 mM Tris–HCl, 5 mM DTT, 5 mM EDTA, pH 8.0 with protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 110,000*g* for 30 min at 4 °C. Pellets were extracted with 7 M urea in homogenizing buffer and spun down as above. Membrane pellets were washed with dH_2O and dissolved in a mixture of formic acid and isopropanol (7:3), then a solution of 50% *acetonitrile*/0.1%TFA containing sinapinic acid (20 mg/ml) was added. The mixture of each sample (0.5 μl) was dried on sample target for analysis and run on a Voyager-DE STR (Applied Biosystems) MALDI-TOF mass spectrometer system operated in linear mode. The mass scale (*m/z* 5000–35,000) was calibrated with a mixture of myoglobin and carbonic anhydrase. Approximately 150 laser shots were used to produce each spectrum.

2.3. Analysis of fiber cell AQP0

Lens membranes pellets prepared as for MALDI-TOF analysis were extracted in 1% SDS, mixed with Bio-Rad Laemmli sample buffer and fractionated (15 μg) using Bio-Rad 10% precast gel. Coomassie blue stained gel was imaged using ProteinSimple (Santa Clara, CA) and bands were quantified using AlphaView software.

2.4. Quantification of lens transparency

Images of lenses were captured under the same lighting and imaging conditions, converted to gray scale (Adobe Photoshop 9) and processed using ImageJ software (NIH). A single horizontal line of pixels was selected through the center of the lens and plot profile function was selected to create values of pixel brightness intensity along the selected horizontal line. Sigma Plot 10 software was used to plot a graph of the pixel brightness intensity data.

2.5. Spherical aberration studies

To evaluate the role played by AQP0 in correcting longitudinal SA, a qualitative assessment of lens SA was done using optical grid focusing. Photographs of whole lenses placed on electron microscope specimen grid background were used to gain a qualitative estimate of light scatter and defocusing effects of RING alteration and SA.

2.6. Lens water content determination

Wet weight of 2.5-month-old mice lenses was measured using an analytical microbalance. Lenses were dried at 90 °C for 24 h and dry weights were measured. Percentage of lens water content was calculated thus: Lens water content $(\%) = \frac{\text{wet weight(mg)} - \text{dry weight(mg)}}{\text{wet weight(mg)}} \times 100$. Data were analyzed for statistical significance using Student's *t*-test.

2.7. Electron microscopy of mouse lenses

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) of WT, $AQP0^{+/-}$ and $AQP1^{+/-}/AQP0^{+/-}$ mouse lenses were carried out as described previously [16]. Digital images were acquired with an AMT XR-60 CCD Camera system and compiled using Adobe Photoshop (version 9).

3. Results and discussion

In vitro investigations on intact [14,16,18] and N- or C-terminal cleaved AQP0 [17] showed both forms capable of functioning as water channels. *In vivo* studies using mouse lens corroborated the function of AQP0 as a water channel [13–16] that facilitates water movement across plasma membranes in response to osmotic gradients. It exists as intact form in the lens outer cortex (Fig. 1A and B). However, as the fiber cells mature, N- and/or C-terminal end-cleaved AQP0 begin to appear at the inner cortex. In several mammalian lenses, the major cleaved form measures ~26 kDa [22–24]. Human fetal lenses showed no detectable level of truncated AQP0. However, cleaved forms of AQP0 were observed at the age of 2 [23]; inner nuclear region of 5 year-old lenses showed large quantities of cleaved AQP0 [24]. These observations suggest that cleavage occurs very early during development and growth of the lens. Similar changes occur in several other lens proteins also. However, the significance of protein truncation has remained a mystery, other than widely recognized as a lens fiber cell maturation event.

In order to understand the significance of the presence of intact and cleaved forms of AQP0 in the lens and the possible roles of these forms in CTCA and RING we performed *in vivo* studies. Adult WT mouse lenses were dissected into four regions namely, outer cortex (OC), inner cortex (IC), outer nucleus (ON) and inner nucleus

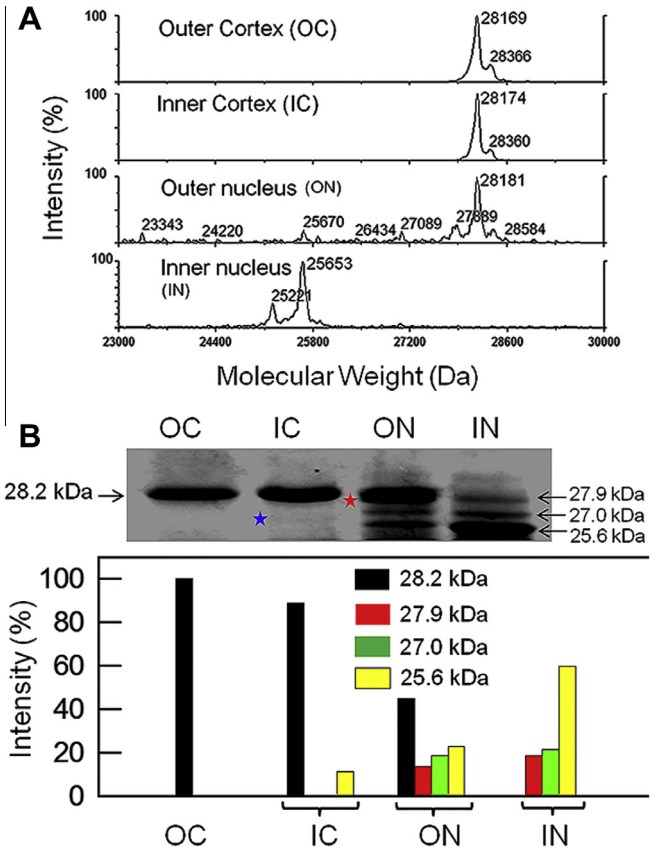

Fig. 1. Analysis of mouse lens fiber cell membrane proteins. (A) Representative MALDI-TOF spectra and (B) SDS–PAGE of fiber cell membrane proteins from the outer cortex, inner cortex, outer nucleus and inner nucleus. Red star indicates the 27.9 kDa band merged with the 28.2 kDa band; Blue star indicates 25.6 kDa band. Protein band intensity was quantified and represented below the gel image as a bar graph. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(IN) as described for human lens studies [24]. Fiber cell membrane fractions were prepared and subjected to MALDI-TOF mass spectrometry and SDS–PAGE (Fig. 1A and B). Spectra of OC and IC showed one major ion peak of ~28 kDa corresponding to intact AQP0. A major peak of ~28 kDa and several minor peaks of cleaved AQP0 were observed in ON spectrum. Interestingly, IN spectrum lacked the prominent 28 kDa peak and showed one major peak of ~26 kDa and several minor peaks. Corresponding to MS data (Fig. 1A), four major bands of ~28 (intact), 27.9, 27 and 25.6 kDa were resolved by SDS–PAGE (Fig. 1B); Western blotting showed immunoreactivity of these bands to extracellular loop-specific anti-AQP0 antibody (data not shown). The ratio of intact to truncated AQP0 was determined using densitometry scanning of the SDS–PAGE bands and values were plotted using SigmaPlot 10 (Fig. 1B). WT mouse lens intact and cleaved AQP0 showed similar pattern of spatial distribution as observed in human lenses [22,24]. Due to the abundance of intact AQP0 which provided the major peak, the less abundant cleaved forms were not detected by mass spectrometry of IC and ON samples (Fig. 1A).

Previously, we have demonstrated *in vitro* that intact AQP0 promotes CTCA [25]. Using WT, AQP0 heterozygous knockout ($AQP0^{+/-}$) and AQP0 heterozygous knockout mouse lens transgenically expressing adhesion-deficient AQP1 ($TgAQP1^{+/-}/AQP0^{+/-}$) in the fiber cells we demonstrate that AQP0 is a fiber CTCA promoting protein *in vivo*, as observed *in vitro* studies [17,25,26]. Fig. 2A shows lenses of $AQP0^{+/-}$ and $TgAQP1^{+/-}/AQP0^{+/-}$ mouse models having severe light scattering compared to WT lens. $TgAQP1^{+/-}/AQP0^{+/-}$ lenses expressing AQP1 still showed reduced light scattering even after plasma membrane P_f lost by the knockout of AQP0 was compensated [14,16]. $AQP0^{+/-}$ and $TgAQP1^{+/-}/AQP0^{+/-}$ lenses showed intense light scattering and cataract in the suture area (Fig. 2A) of the outermost fiber cells which have only intact AQP0 (OC, Fig. 1). In mammalian lenses, fiber cell sutures are organized along the optical axis and greatly influence the optical quality [27]. Knockout of AQP0 greatly altered suture formation [28,29]. Fig. 2B shows light scattering in the lenses imaged under dark field based on the pixel brightness intensity along a horizontal line selected through the center of each lens. The higher the pixel intensity, the greater the light scattering. Compared to the WT, there is significant increase in light scattering in $AQP0^{+/-}$ and $TgAQP1^{+/-}/AQP0^{+/-}$ lenses starting from the outer cortex and throughout the lens. The lenses of the mouse models have 50% less AQP0 compared to the WT. Loss of 50% AQP0 could be causing RING alteration from the outer cortex where only intact AQP0 is expressed (Fig. 1A and B) to the lens nucleus where cleaved forms are present suggesting the quantity of intact and cleaved AQP0 as

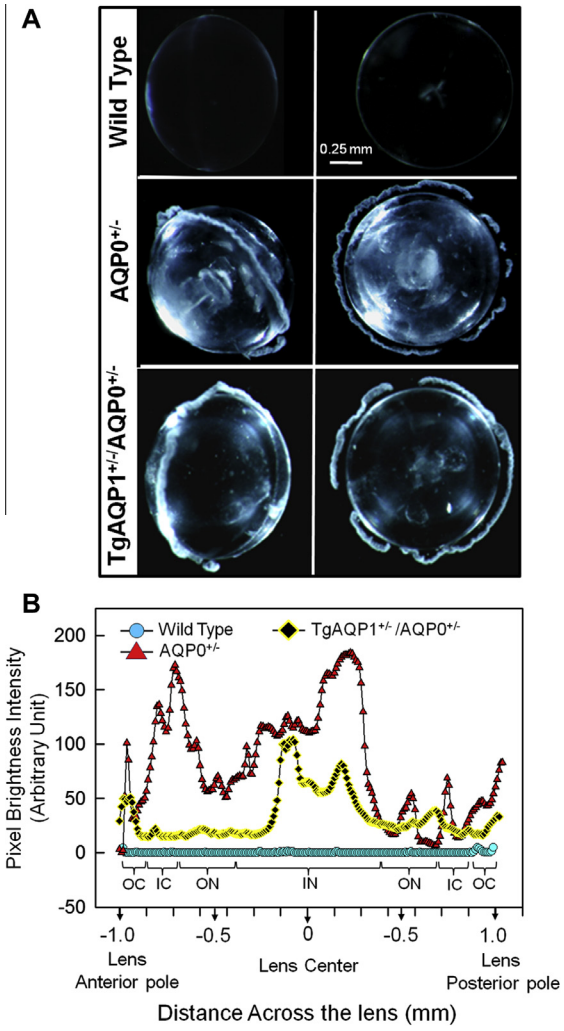

Fig. 2. Mouse lens transparency. (A). In all lenses, a thin layer of light scattering was observed from the capsule and the anterior epithelial cells. Except WT lens, both $AQP0^{+/-}$ and $TgAQP1^{+/-}/AQP0^{+/-}$ lenses showed higher levels of light scattering that was seen throughout the lens. Column 1: Lenses were imaged equatorial side facing up. Column 2: Lenses were imaged anterior pole facing up to show 'Y' suture area which is visible in the WT and not visible in the other lenses due to reduction fiber cell CTCA causing suture abnormalities and cataract. (B). Quantification of lens transparency of WT, $AQP0^{+/-}$ and $TgAQP1^{+/-}/AQP0^{+/-}$ mice. OC – outer cortex; IC – inner cortex; ON – outer nucleus; IN – inner nucleus.

expressed in the WT is necessary for strong CTCA and proper RING development to avoid light scattering.

SA occurs when refractive index of the lens is not in perfect gradient [7,30]. WT, AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ lenses were imaged under dark field with or without electron microscope specimen grid. WT lens is relatively transparent and able to focus the grid sharply (Fig. 3A). AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ mouse lenses were less transparent and unable to focus the grid sharply. The fuzzy images (Fig. 3A) could be due to SA caused by alteration in the RING. These lenses failed to focus light rays at the same focal point due to alterations in the angle of refraction caused by changes in RING, possibly a consequence of the presence of only 50% of AQP0 in the heterozygous condition compared to WT lens. This phenomenon of SA is schematically represented as Fig. 3B. Lens SA is caused by alteration in RING [31]. Lens RING is determined by lens proteins and water content [32]. AQP0 in mammals has established a reciprocal gradient distribution of intact and N- and/or C-terminal end cleaved AQP0 protein in the lens from the outer cortex to the nucleus. The gradient of intact AQP0 correlates well with the water content gradient [33,34] and that of the cleaved forms correlates well with the RING in the lens. Outermost fiber cells have more water both in the cytoplasm and in the extracellular space in contrast to the central fibers. Refractive index is very low at the outer cortex, only slightly higher than water, and increases towards the center of the lens; water distribution determines the lens RING.

In order to investigate the cause for lens SA in the AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ mouse models, we quantified the free water content and extracellular space of the lenses and compared with those of WT lenses. Whole water content of the AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ mouse lenses was significantly higher than that of WT lenses (Fig. 3C). TEM and SEM images of WT lens sections exhibited tightly packed fiber cells with very narrow extracellular spaces (Fig. 4Aa and Ba,b) ranging between 0.5 and 0.7 nm. In AQP0$^{+/-}$ (Fig. 4Ab and Bc,d) and TgAQP1$^{+/-}$/AQP0$^{+/-}$ (Fig. 4Ac and Be,f) lenses, extracellular spaces were wider and ranged between 0.8 and 2.0 nm. The increase in extracellular space was observed starting from outer cortical fiber cells where AQP0 is expressed as intact protein suggesting intact AQP0 also facilitates CTCA. Extracellular space of TgAQP1$^{+/-}$/AQP0$^{+/-}$ is similar to that in AQP0$^{+/-}$, possibly a consequence of the lack of adhesion property of AQP1 transgene. In other words, fiber cell AQP1 transgene was able to replace the role/s of AQP0 only partially even after compensating the lens membrane P_f in the AQP0 knockout mouse.

Lens grows continuously peripherally which necessitates compact packing of the fiber cells and adjustment of RING, which should be low in the cortex and high in the nucleus to avoid SA. Distribution of AQP0 thin junctions between fiber cell membranes [35] could play a significant role in the compact packing of the fiber cells by providing strong CTCA and adjusting the water content within and between fiber cells. To our knowledge, this is the first investigation showing *in vivo* that AQP0 possibly plays a significant role in mammalian lens RING development to prevent SA.

In our recent publication [17], we hypothesized that intact and post-translationally N- and C-terminal cleaved AQP0 could

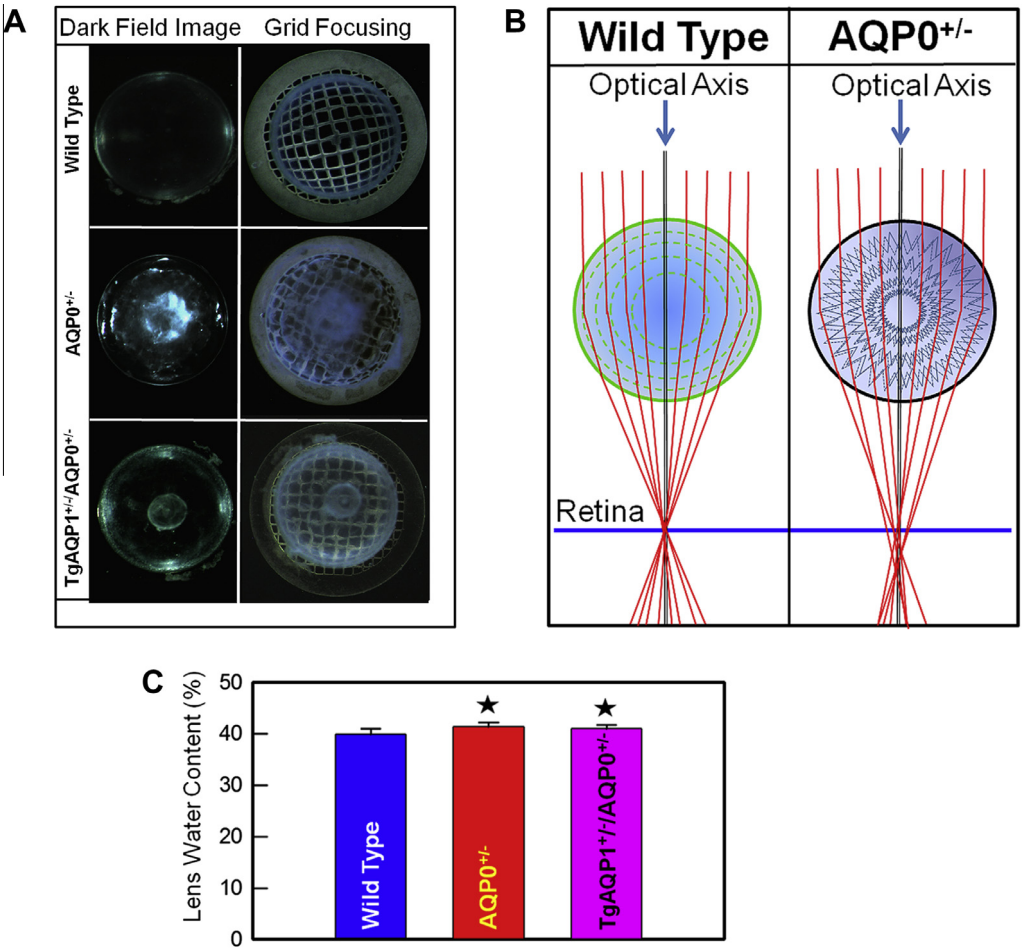

Fig. 3. (A) Lenses of WT, AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ mice imaged under dark field without or with an electron microscope specimen metal grid. In all lenses, a thin layer of light scattering was observed from the capsule and the anterior epithelial cells. Compared to WT, both AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ lenses showed higher levels of light scattering and SA. (B) Schematic representation of SA in WT and AQP0$^{+/-}$ observed in panel 3A. (C) Water content (%) in the lenses of WT, AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ mice.

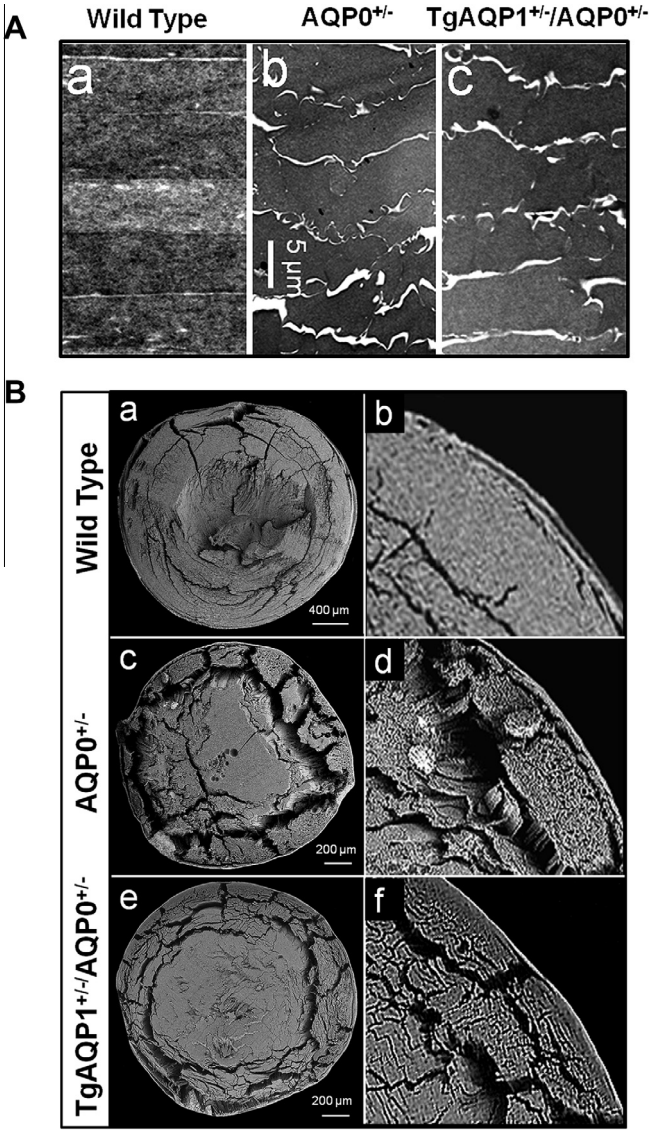

Fig. 4. Electron microscopic analyses of 2-month-old mouse lenses. (A) TEM analysis of WT (a), AQP0$^{+/-}$ (b) and TgAQP1$^{+/-}$/AQP0$^{+/-}$ (c) lenses sectioned along polar axis. (B) SEM of WT (a and b), AQP0$^{+/-}$ (c and d) and TgAQP1$^{+/-}$/AQP0$^{+/-}$ (e and f) lenses split along the polar axis. In A and B, lens outer cortical region of WT shows tightly packed fiber cells and that of AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ lenses is not tightly packed and shows more extracellular space between fiber cells.

participate in lens RING development by regulating the extracellular space between fiber cells, providing firm CTCA and carrying out fiber cell dehydration. Based on our current investigation, we propose that expression of the full amount of AQP0 in the fiber cells (as in the WT) is necessary to achieve perfect RING to focus images on the retina. Further, distribution of intact and N- and C-terminal ends cleaved AQP0 in a proper ratio from outer cortex to center of the lens in reciprocal gradients appears necessary to achieve ideal lens RING. AQP0$^{+/-}$ knockout mouse lenses expressing AQP1 in the fiber cells showed SA even after restoring lost membrane P_f, due to lack of 50% AQP0. This could be due to failure in sufficient AQP0 distribution and the consequent lack of AQP0 to form enough small patches of thin junctions (6.6 nm repeats) in the outer cortex and large patches of thin junctions in the inner cortex and nucleus to exert CTCA conducive for the development of suitable RING.

In vertebrate lenses, two functional types of AQP0 have been identified. AQP0 expressed in aquatic animals like fish has high P_f. AQP0 in killifish [36] and zebrafish [37] has P_f close to that of AQP1. Lenses of the aquatic animals provide more optical power than those in terrestrial animals because power of their corneas is significantly reduced in water. AQP0 expressed in terrestrial tetrapod vertebrates has low P_f. Review of literature shows no report of functional fiber cell AQPs other than AQP0 in fish. In mammalian lens, AQP5 which is 20 times more efficient than AQP0 is expressed both in epithelial and fiber cells. Zebrafish genome analysis showed that AQP5 exists as a pseudogene [38]. In terrestrial mammalian lens, AQP0 has possibly evolved with a low P_f to establish RING and AQP5 might have evolved with a high P_f to function as an osmoregulator [20]. The functional difference between AQP0 in aquatic animals like fish and terrestrial vertebrates like mouse and human could be due to the evolutionary adaptations undergone to thrive in their respective environments.

To summarize, our data suggest that the quantity of AQP0, ~45% of the total membrane protein consisting of intact and N and/or C-terminal ends cleaved AQP0, is required for RING development, adjustment and maintenance. The data presented also indicate that loss of 50% of intact and N and/or C-terminal ends cleaved AQP0 severely alter water content, RING and lens transparency leading to cataract. SA in the AQP0$^{+/-}$ and TgAQP1$^{+/-}$/AQP0$^{+/-}$ mouse lenses could be due to lack of proper CTCA and fiber cell compaction. These cause increased extracellular space and water content leading to altered RING in the constantly growing lens. In conclusion, the prolific expression AQP0 as in the WT lens and its existence as intact and cleaved forms could be prerequisites for eye lens to establish RING conducive to avoid SA for proper focusing and attaining visual acuity.

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