

1 **Dynamic functional contribution of the water channel AQP5 to the water permeability**
2 **of peripheral lens fiber cells**

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12 **Running title:** AQP5 insertion increases lens fiber cells water permeability

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25

1 ABSTRACT.

2 Although the functionality of the lens water channels, AQP1 (epithelium) and AQP0 (fiber
3 cells), are well established less is known about the role of AQP5 in the lens. Since in other
4 tissues AQP5 functions as a regulated water channel with a water permeability (P_{H_2O}) some
5 20x higher than AQP0, AQP5 could function to modulate P_{H_2O} in lens fiber cells. To test this
6 possibility a fluorescence dye dilution assay was used to calculate the relative P_{H_2O} of
7 epithelial cells and fiber membrane vesicles isolated from either the mouse or rat lens, in the
8 absence and presence of $HgCl_2$ - an inhibitor of AQP1 and AQP5. Immunolabeling of lens
9 sections and fiber membrane vesicles from mouse and rat lenses revealed differences in the
10 subcellular distributions of AQP5 in the outer cortex between species - with AQP5 being
11 predominantly membranous in the mouse, but predominantly cytoplasmic in the rat. In
12 contrast, AQP0 labelling was always membranous in both species. This species-specific
13 heterogeneity in AQP5 membrane localisation was mirrored in measurements of P_{H_2O} , with
14 only fiber membrane vesicles isolated from the mouse lens exhibiting a significant Hg^{2+} -
15 sensitive contribution to P_{H_2O} . When rat lenses were first organ cultured, immunolabeling
16 revealed an insertion of AQP5 into cortical fiber cells, and a significant increase in Hg^{2+} -
17 sensitive P_{H_2O} was detected in membrane vesicles. Our results show that AQP5 forms
18 functional water channels in the rodent lens, and suggests that dynamic membrane insertion
19 of AQP5 may regulate water fluxes in the lens by modulating P_{H_2O} in the outer cortex.

20

1 INTRODUCTION

2 The aquaporin (AQP) family of water channels mediate the passive diffusion of water across
3 cell membranes and, as such, play major roles in many physiological and pathological
4 processes (32). Thirteen AQP isoforms have been identified in humans, and ongoing research
5 has shown that differences in water permeability, transcriptional regulation, post-translational
6 modification, protein stability and polarized membrane distribution between the different
7 AQP isoforms accounts for the functional differences in water permeability observed at the
8 cellular level in different tissues (1, 9, 25). In the ocular lens, AQPs are considered essential
9 to the lens internal microcirculation system that generates circulating ionic and fluid fluxes
10 which, in the absence of a blood supply, deliver nutrients to and remove waste products from
11 the lens centre faster than would occur by passive diffusion alone (39, 40).

12 The microcirculation system and specialized architecture of the lens maintain its transparency
13 and homeostasis. The lens anterior surface is covered with a single layer of epithelial cells
14 while the bulk of the lens is formed of highly elongated fiber cells organized in concentric
15 growth rings (4). The lens continues to grow throughout life by the addition of new fiber cells
16 formed by the terminal differentiation of epithelial cells localized at the lens equator. In this
17 way, the older and organelle-free mature fiber cells are encapsulated into the deeper core of
18 the lens, whereas the youngest and metabolically-active differentiating fiber cells are found in
19 the peripheral outer cortex of the lens (38, 41). Superimposed upon this gradient of lens
20 development, differentiation and growth are spatial differences in the expression and post-
21 translational modification of at least three AQP isoforms. AQP0 (19), AQP1 (49) and most
22 recently AQP5 expression in the lens has been confirmed in multiple species (20, 34), and
23 changes in the expression patterns during mouse lens development and growth have been
24 mapped (44, 49).

1 AQP1 is abundantly expressed in a variety of tissues (2), and in the eye is found in the
2 corneal endothelium, amacrine and glial cells of the retina, and in the lens epithelium (8, 24,
3 31). During development, AQP1 is firstly expressed in lens epithelial cells at embryonic day
4 17 in the mouse lens. AQP1 expression progressively increases during postnatal
5 development, which coincides with an increase in the size of the lens (50). This increased
6 expression of AQP1 translates into an increase in the mercury-sensitive water permeability of
7 isolated lens epithelial cells (10, 50). Deletion of AQP1 in the lens epithelium resulted in an
8 approximately three-fold reduction of the water permeability of AQP1 knock-out mouse
9 lenses (46). Although lack of AQP1 expression did not affect lens morphology and
10 transparency, it was found that incubation of lenses from AQP1 knockout mice in high
11 glucose solutions resulted in accelerated loss of lens transparency relative to that seen in wild
12 type lenses. Therefore, AQP1 is required to maintain the transparency of the lens, especially
13 following exposure to stress conditions such as hyperglycemia and osmotic imbalance.

14

15 AQP0, previously known as major intrinsic protein 26 (MIP26), is a highly abundant protein
16 which constitutes over 60% of the total membrane protein content of the lens (7). AQP0 was
17 thought to be exclusively expressed in in lens fiber cells, although recent reports show weak
18 immunoreactivity in bipolar, amacrine and ganglion cells of the retina (11, 23). AQP0 is a
19 low-conductance water channel, with water permeability some 30- and 20-fold lower than
20 AQP1 and AQP5 respectively (10, 42, 57). AQP0 can double its permeability in mildly acidic
21 environments (48). Unlike AQP1 and AQP5, AQP0 is not sensitive to inhibition by mercury
22 (42), due to the absence of a cysteine residue contained within the water pore which, in other
23 AQP isoforms, renders the water channel sensitive to mercurial inhibition (33, 47). As the
24 lens grows and ages, AQP0 undergoes differentiation-dependent post-translational
25 modifications to its C-terminal domain, including phosphorylation, truncation, glycation and

1 deamidation (3). While the effect of glycation and deamidation on AQP0 water permeability
2 is unclear, the most abundant modifications, phosphorylation and truncation, are thought to
3 alter the regulation of its water permeability. Several sites of phosphorylation have been
4 identified (45), while extensive C-terminal truncation has been well characterized in the core
5 of the lens (19, 54). Phosphorylation of AQP0 at Serine 235, the most abundant
6 phosphorylation site, occurs via PKA and is mediated by AKAP2 (16). The phosphorylation
7 of the C-terminal tail has been shown to disrupt calmodulin binding (45), and therefore
8 changes the regulation of AQP0 activity in the presence of elevated Ca^{2+} . Structurally, AQP0
9 have been shown to exist in two conformations suggesting a change of AQP0 function from
10 a water pore in the periphery to an adhesion protein in the core of the lens through its
11 identification within 11-13 nm “thin lens junctions” (17, 18, 21).

12

13 AQP5 is a water channel recently identified in epithelial and fiber cells of the ocular lens at
14 the gene (43, 55) and protein (5, 54) levels. Most recently, its expression was verified not
15 only in our laboratory (20) but also by other researchers (34). Interestingly,
16 immunohistochemical mapping of AQP5 distribution across the lens radius (20), revealed a
17 differentiation-dependent change in the sub-cellular localization of AQP5 from a cytoplasmic
18 to membranous labeling pattern with fiber cell differentiation, and therefore radial position
19 within the lens. Furthermore, the labeling pattern observed for AQP5 in the outer cortex of
20 the lens was very different to that observed for AQP0, which is always membranous in the
21 young, differentiating fiber cells present in this region (19). These differences in membrane
22 localization of AQP5 relative to AQP0 raises questions about the relative functional roles of
23 the two AQPs in the different regions of the lens, especially since AQP5 has a ~20 fold
24 higher water permeability than AQP0 (57), although AQP0 is 100 times more abundant than
25 AQP5 (5).

1 To determine whether AQP5 contributes to the water permeability of peripheral lens fiber
2 cells, we have in this study employed a fluorescence dye-dilution assay that utilizes fiber cell
3 membrane vesicles isolated from the outer cortex of either the mouse or rat lens. In parallel
4 we show species-specific differences in AQP5 membrane labeling levels, and hence
5 differences in the potential contribution of AQP5 to overall water permeability. To
6 distinguish the relative contributions of AQP5 and AQP0 to overall water permeability,
7 vesicles from both species were incubated in the absence or presence of mercury (HgCl_2),
8 which blocks AQP5, but not AQP0 water channels. Our results suggest not only that AQP5
9 contributes to the water permeability of cortical fiber cells, but also that the water
10 permeability in this region can be dynamically upregulated by the insertion of AQP5 from a
11 cytoplasmic pool into the membranes of differentiating fiber cells.

1 **MATERIALS AND METHODS**

2 **Reagents**

3 An affinity-purified anti-AQP5 antibody directed against the final 17 amino acids (rat
4 sequence) of the C-terminus of the protein, was obtained from Millipore (Billerica, MA,
5 Cat#: AB15858). Affinity-purified anti-AQP0, directed against the final 17 amino acids
6 (human sequence) of the C-terminus of the protein, was obtained from Alpha Diagnostic
7 International, Inc. (San Antonio, TX, Cat#: AQP01-A). Secondary antibodies (goat anti-
8 rabbit-Alexa 488), fluorophore-conjugated wheat germ agglutinin (WGA-Alexa Fluor 594)
9 for labelling of cell membranes, and calcein acetoxymethyl ester (calcein-AM) used for
10 fluorescent loading of epithelial cells and fiber cell membrane vesicles were each obtained
11 from Thermofisher Scientific (Waltham, MA, USA). DAPI, obtained from Sigma Aldrich (St
12 Louis, MO), was used for fluorescent labeling of cell nuclei. Phosphate buffered saline (PBS,
13 pH 7.4) was prepared fresh from PBS tablets. Unless otherwise stated all other chemicals
14 were from Sigma Aldrich.

15 **Collection and preparation of lenses**

16 Adult mouse and rat lenses were removed from C57BL/6 mice or Wistar rats immediately
17 following death, using procedures in accordance with the ARVO Statement for the Use of
18 Animals in Ophthalmic and Vision Research, which were approved by the University of
19 Auckland Animal Ethics Committee (#001303). Following extraction, lenses were either
20 immediately placed in fixative and subsequently processed for immunohistochemistry, placed
21 in warm PBS prior to preparation of isolated epithelial cells or fiber cell vesicles, or prepared
22 for organ culturing from 2 to 17 hours in Artificial Aqueous Humour (AAH: containing 125
23 mM NaCl, 0.5 mM MgCl₂, 4.5 mM KCl, 10 mM NaHCO₃, 2 mM CaCl₂, 5 mM glucose, 10
24 mM sucrose, 10 mM HEPES, pH 7.4, 300 mOsmL⁻¹). At the end of the incubation period,

1 organ cultured lenses were either immediately fixed in 0.75% paraformaldehyde and
2 processed for immunohistochemistry, or used to isolate fiber cell vesicles.

3 **Isolation of lens epithelial cells and fiber cell membrane vesicles**

4 Immediately following extraction from the eye, or after organ culture in AAH, a pair of
5 sharpened forceps was used to separate and remove the lens capsule and adherent epithelial
6 cells from the underlying fiber cell mass. Forceps were then used to peel off small clumps of
7 differentiating fiber cells from the outer cortical fiber cell mass. The lens capsule was
8 incubated in collagenase dissociation buffer (150 mM Na-gluconate, 4.7 mM KCl, 5 mM
9 glucose, 5 mM HEPES, 0.125% Type IV collagenase, pH 7.4) for 30 minutes at 37 °C to
10 isolate lens epithelial cells. After the first 15 minutes of incubation, 4 μM calcein-AM was
11 added to the dissociation buffer to label dissociated epithelial cells with fluorescent calcein
12 during the remaining 15 minutes of the dissociation incubation period. Epithelial cells were
13 pelleted at 150 g for 3-5 minutes using a bench-top microcentrifuge and washed twice with
14 300 mOsmL⁻¹ isotonic saline (139 mM NaCl, 4.7 mM KCl, 1 mM MgCl₂, 5 mM glucose, 5
15 mM HEPES, pH 7.4, 300 mOsmL⁻¹). To form fiber cell membrane vesicles, clumps of
16 differentiating fiber cells were isolated from the outer cortex of the lens with forceps and
17 transferred to isotonic saline which contained 5 mM CaCl₂. Clumps of fiber cells were
18 incubated for 30 minutes at room temperature (20 °C), which induced spontaneous formation
19 of vesicles (51). To load these fiber cell-derived membrane vesicles with fluorophore, 6 μM
20 calcein-AM was added to the Ca²⁺-containing isotonic saline for the duration of the 30
21 minutes incubation time. Membrane vesicles were then pelleted at 150 g for 3-5 minutes and
22 washed twice in Ca²⁺-free isotonic saline for immediate use in the fluorescence dye-dilution
23 assay.

1 **Immunolabeling of lens sections and fiber cell membrane vesicles**

2 Lenses, either immediately post-extraction from the eye, or after organ culture, were fixed in
3 0.75% paraformaldehyde at room temperature for 24 hours, and prepared for cryosectioning
4 using previously-published protocols (28). For sectioning, whole lenses were mounted
5 encased in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek,
6 Zoeterwoude, The Netherlands), and snap frozen in liquid nitrogen for 15-20 seconds. Lenses
7 were cryo-sectioned at -19°C using a cryostat (CM3050, Leica Microsystems, Germany),
8 and 14 μm -thick equatorial cryosections were transferred onto plain microscope slides.
9 Isolated fiber membrane vesicles were plated for immunolabeling experiments on plain
10 microscope glass slides, before being fixed for 30 minutes in 2% paraformaldehyde. Both
11 lens sections and fiber-derived membrane vesicles were subjected to the same established
12 immunohistochemistry protocols (19). Briefly, fixed lens sections or fiber membrane vesicles
13 were first incubated in blocking solution (3% bovine serum albumin, 3% normal goat serum
14 in PBS pH 7.4) for 1 hour at room temperature. After washing in PBS pH 7.4, samples were
15 incubated in primary antibody in blocking solution (1:100) overnight at 4°C . Samples were
16 washed in PBS pH 7.4 and incubated for 1.5 hours, at room temperature in the dark with
17 fluorescent secondary antibodies in blocking solution (1:200), or contained $0.125\mu\text{g/ml}$ DAPI
18 to stain cell nuclei of lens sections. Where necessary, sections were then incubated with
19 WGA-Alexa Fluor 594 in PBS pH 7.4 (1:100) for 1 hour (room temperature) to label cell
20 membranes. Lens sections and membrane vesicles were cover-slipped using VectaShield
21 HardSetTM anti-fade mounting medium (Vector Laboratories, Burlingame, CA).
22 Immunofluorescent images were acquired using a laser scanning confocal microscope
23 (Olympus FV1000, Tokyo, Japan) equipped with FluoView 2.0b software. For presentation,
24 labeling patterns were pseudo-colored and combined using Adobe Photoshop CS6 (Adobe
25 Systems Inc., San Jose, CA).

1 **Fluorescence dye-dilution assay for water permeability**

2 Calcein-AM-loaded epithelial cells or fiber cell membrane vesicles were attached to the
3 bottom of a custom-made recording chamber using Cell-Tak™ adhesive agent (Corning, Two
4 Oak, Bedford, USA) for 30 minutes at room temperature. To remove loosely-attached cells or
5 vesicles, 300 mOsmL⁻¹ isotonic saline was washed through the recording chamber for 5
6 minutes using a gravity-fed perfusion system which allowed the composition of the bath to be
7 completely changed with a time constant of 2 seconds. The chamber was mounted on a Nikon
8 Eclipse TE 300 inverted epifluorescence microscope, equipped with a 40x CFI Plan Fluor
9 NA 0.75 objective and a filter set (Nikon, B-2A) that matched the excitation and emission
10 spectra of calcein-AM. Differential Interference Contrast (DIC) and epifluorescence images
11 were collected using an electron-multiplying CCD camera (Cascade II 512B emCCD,
12 Photometrics, Tucson, USA), suitable for imaging in low-light applications with rapid frame
13 rates. Imaging protocols consisted of collecting an initial DIC image of epithelial cells or
14 fiber cell membrane vesicles to allow accurate measurement of their initial cross sectional
15 area in isotonic saline. Subsequently, a series of fluorescence images were collected at 2Hz to
16 record the time-course of change in fluorescence intensity in response to a 390 mOsmL⁻¹
17 hypertonic challenge, induced by switching incoming bathing solution from isotonic to
18 hypertonic saline, which contained 185 mM NaCl (Figure 1A). Images were acquired using
19 Imaging Workbench 5.2 (Indec BioSystems, Los Altos, USA). Regions of interest were
20 created within the boundaries of individual epithelial cells or fiber-derived vesicle, thereby
21 enabling changes in fluorescence intensity in response to alterations in the volume of an
22 epithelial cell or vesicle to be captured in real time (Figure 1B). Hypertonic challenge was
23 performed either in the absence or presence of 300 μM HgCl₂, an irreversible inhibitor of
24 AQP5 and AQP1 channels (33, 47), but not AQP0 water channels (42). The effect of mercury
25 was examined by a 5 minute pre-incubation of lens epithelial cells or fiber cell membrane

1 vesicles in isotonic saline containing 300 μM HgCl_2 , before applying identical hypertonic
 2 challenge in the presence of 300 μM HgCl_2

3 **Calculation of water permeability.**

4 The water permeability of epithelial cells and fiber cell-derived membrane vesicles have been
 5 determined by modifying the equation adopted from a paper published by Varadaraj et al. in
 6 1999 (51)

$$7 \quad P_{H_2O} = \frac{1}{S_m} \frac{C_{H_2O}}{C_i(0) - C_0} \frac{dV_i(0)}{dt} \quad (1)$$

8 Equation 1 is based on the assumption that water flows across a cell membrane in response to
 9 the gradient of osmolarity, $\frac{C_{H_2O}}{C_i(0) - C_0}$ established across the membrane, at a rate determined by
 10 the surface area, S_m , and rate of change of volume, $\frac{dV_i(0)}{dt}$. Since in our method we used a
 11 fluorescent tracer to follow the change in volume, instead of monitoring the change in cross
 12 sectional area, we modified the Equation 1 to fit our assay system which measures the time
 13 course of the rate in change of fluorescence intensity in response to hypertonic challenge.
 14 This response is given by Equation 2

$$15 \quad P_{H_2O} = \frac{1}{S_m} \frac{C_{H_2O}}{C_i(0) - C_0} \frac{dF_i(0)}{dt} \quad (2)$$

16
 17 To calculate the water permeability the membrane surface area (S_m) was measured using DIC
 18 images that captured the initial morphology of the epithelial cell or fiber cell membrane
 19 vesicles showing near perfect spherical shape for these objects (Figure 1A), and then
 20 calculated using Equation 3.

$$21 \quad S_m = 4\pi r^2 \quad (3)$$

1 where the radius (r , μm) was obtained using the cross sectional area (A) calculated by tracing
 2 the pixels within the defined region of interest using Image J software, (Equation 4).

$$3 \quad r = \sqrt{\frac{A}{\pi}} \quad (4)$$

4
 5 Imaging Workbench 5.2 software was used off-line to quantify the change in fluorescence
 6 intensity in response to hypertonic challenge within individual calcein-AM loaded epithelial
 7 cells or fiber membrane vesicles. Correction for photo-bleaching was applied by normalizing
 8 data against a monoexponential function fitted to the initial, pre-challenge, region of the
 9 timecourse using Clampfit 9.2 (Molecular Devices, Sunnyville, CA) to produce a bleach-
 10 corrected timecourse showing the change in fluorescence intensity caused in response to
 11 hyperosmotic challenge (Figure 1B). A monoexponential of the form $y = Ae^{-\tau t} + C$ was
 12 fitted to the fluorescence response to challenge (Figure 1C), and the time constant (τ)
 13 extracted to quantify the rate of change of fluorescence using the best fit values for the initial
 14 and final fluorescence F1 and F2 (Equation 5):

$$15 \quad \frac{dF_i(0)}{dt} = \frac{F2-F1}{\tau} \quad (5)$$

17 The rate of change in fluorescence intensity (Equation 5) along with the external osmotic
 18 gradient and initial resting area were fitted into Equation 2 to calculate the relative water
 19 permeability of epithelial cells and fiber cell membrane vesicles expressed in arbitrary units
 20 (AU), where $C_{\text{H}_2\text{O}} \approx 55\text{M}$ is the concentration of water and $C_{i(0)} = 300 \text{ mOsmL}^{-1}$ is the initial
 21 bathing solution, assumed to be equal to the initial intracellular osmolarity, and $C_0 = 390$
 22 mOsmL^{-1} . We applied this approach for calculation of the water permeability of epithelial
 23 cells and membrane vesicles since it has been shown that using mono-exponential fits of

1 normalized fluorescence data is more accurate and mathematically stable, for calculation of a
2 decay rate (30, 56), whereas usage of multi-exponential and parameterised interpretation of
3 fluorescent decays is inaccurate (35, 36, 56). Following this rationale we have chosen to
4 normalize our dataset of change of fluorescence time course and use arbitrary units (AU) for
5 our rate calculations.

6

7 **Statistical analysis**

8 To evaluate the water permeability of lens epithelial cells or fiber cell membrane vesicles,
9 data were obtained from at least 3 animals performed through at least 3 separate experiments.
10 Mean data of experiments are given \pm standard error of the mean (SEM). Statistical
11 significance was tested with the Mann-Whitney U test, using GraphPad Prism (La Jolla,
12 California, USA). * indicates statistically significant differences at the $\alpha=0.05$ level..

13

14

1 **RESULTS**

2 **Species differences in the membrane localization of AQP5 in rodent lenses and fiber-** 3 **cell-derived membrane vesicles**

4 In previous studies, we have used immunohistochemistry to map the distribution of AQP0 in
5 the rat lens (19), and AQP0 and AQP5 in the mouse lens (20, 44). In these previous studies
6 we, noted subtle species differences in the distribution of AQP5 in the outer cortex. In the
7 present study, we applied our immunohistochemistry mapping approach to axial sections to
8 examine more closely how these differences in distribution of AQP5 in the adult mouse and
9 rat lens manifest as fiber cells proceed through the different stages of differentiation and
10 elongation to reach the anterior and posterior poles of the lens (Figure 2). We confirmed our
11 previous observation, that in differentiating fiber cells AQP5 is predominately cytoplasmic in
12 peripheral fiber cells, but undergoes a distinctive translocation to the membranes of
13 elongating fiber cells at distinct stages of fiber cell differentiation. In the current study, we
14 also show that the radial location of this transition is species-dependent. In the mouse lens,
15 the pool of cytoplasmic AQP5 labelling was confined to a narrow, superficial zone located to
16 the lens modiolus at the equator (Figure 2, Region 1B). Either side of the modiolus region,
17 AQP5 is inserted into the membranes of elongating fiber cells (Figure 2A), with AQP5
18 labeling becoming strongly associated with the membranes of fiber cells that reach both the
19 anterior (Figure 2, Region 2B) and posterior poles of the lens (Figure 2, Region 3B). In
20 contrast, in the rat lens, AQP5 exhibited predominately cytoplasmic labeling throughout the
21 entire equatorial region (Figure 2, Region 1C), remaining cytoplasmic in differentiating fiber
22 cells as they elongated to reach both the anterior (Figure 2, Region 2C) and posterior (Figure
23 2, Region 3C) poles of the lens.

1 These observed differences of expression of AQP5 in the axial direction in rodent lenses
2 reinforced earlier descriptions along the continuum of differentiation in the equatorial
3 orientation (Figure 3), (20). Mouse AQP5 labeling was cytoplasmic in peripheral fiber cells
4 as they differentiated from epithelial into fiber cells at the equator of the lens (Figure 3C). At
5 deeper locations, associated with later stages of fiber cell differentiation, AQP5 labeling
6 abruptly became associated with the plasma membrane, with this membranous labeling then
7 being retained in the rest of the outer cortex of the mouse lens (Figure 3E). In contrast, in the
8 rat lens, AQP5 labeling remained predominately cytoplasmic in differentiating fiber cells
9 throughout the outer cortex (Figure 3J&I). AQP0, on the other hand, at these location, was
10 always membranous in both the mouse (Figure 3B&D) and rat lenses (Figure 3F&H). Based
11 on these observed regional differences in the membrane distribution of AQP5 and AQP0 in
12 the mouse and rat lenses we might expect that the relative contributes of the two water
13 channels to the water permeability of peripheral fiber cells of the outer cortex would be
14 different between the two species.

15 To test this hypothesis, the water permeability of mouse and rat fiber cells needed to be
16 directly compared. However, because of the terminally-differentiated state of lens fiber cells
17 and their thin and extremely elongated morphology measuring water permeability in fiber
18 cells is difficult. Previous studies (6) (51, 52) have presented measurements of the water
19 permeability of lens fiber cells using fiber-cell-derived membrane vesicles, which are
20 spontaneously formed when fiber cells are isolated from the lens in presence of Ca^{2+} ions. To
21 illustrate this process, a series of DIC images is presented from an isolated cluster of rat lens
22 fiber cells that undergoes cell swelling and subsequent formation of individual vesicles after
23 30 minutes of incubation in Ca^{2+} -containing isotonic saline (Figure 4A). To test whether the
24 distribution of AQP5 and AQP0 observed in fixed lens sections remains preserved in fiber-
25 cell-derived vesicles, mouse and rat fiber vesicles were labeled with specific C-terminal anti-

1 AQP5 and -AQP0 antibodies. Quantification of labeling distribution between cytoplasmic
2 and membrane-bound AQP0/5 was compromised by collapse of the vesicles following
3 paraformaldehyde fixation, however the labeling distribution qualitatively observed in fiber
4 cell membrane vesicles corresponded with results obtained from rat and mouse lens
5 cryosections. Mouse vesicles showed a predominant membrane localization of AQP5 (Figure
6 4Bi) although occasionally vesicles with cytoplasmic localization were also identified (Figure
7 4Bii), while AQP0 labeling was always associated with the membrane (Figure 4Biii&iv). In
8 contrast, the majority of membrane vesicles derived from the rat lens displayed predominant
9 cytoplasmic labeling (Figure 4Ci), with a small number of vesicles displaying some
10 membrane labeling (Figure 4Cii). However, like the mouse AQP0 labeling in fiber cell
11 membrane vesicles derived from the rat lens was found associated with the membrane
12 (Figure 4Ciii&iv). Based on observed differences in the relative abundances, subcellular
13 distributions and relative permeability's of AQP0 and AQP5 water channels, we would
14 predict functional heterogeneity in water permeability in fiber-derived membrane vesicles,
15 and furthermore that species differences may be apparent in the relative contribution of
16 AQP5 conductance to overall water permeability.

17 **Measuring the water permeability of mouse and rat fiber cell membrane vesicles.**

18 Two approaches were used to distinguish between the relative contribution of AQP5 and
19 AQP0 to water permeability of lens differentiating fiber cells. Firstly, we utilized the
20 differential sensitivity of AQP5 and AQP0 to Hg^{2+} (33) as a pharmacological tool to delineate
21 the relative contributes of the two water channels to overall water permeability. Secondly, we
22 exploited the observed species-dependent distribution of AQP5 in the outer cortex of the
23 mouse and rat lenses to make measurements where the Hg^{2+} -sensitive AQP5 contribution
24 would be expected to be lower (cytoplasmic AQP5) or higher (membranous AQP5) for fiber
25 vesicles derived from the rat or mouse, respectively.

1 To validate the use of mercury as a pharmacological blocker of AQP5, it was first applied to
2 mouse lens epithelial cells, which express the mercury-sensitive aquaporins, AQP1 and
3 AQP5. The average P_{H_2O} of mouse epithelial cells ($11.45 \pm 0.6 \times 10^{-3}$ AU, $n = 23$) was halved
4 ($5.4 \pm 0.3 \times 10^{-3}$ AU, $n = 9$) after inhibition of AQP1/5 with $300 \mu\text{M}$ HgCl_2 (Figure 5).
5 Relative to epithelial cells, fiber cell membrane vesicles from the mouse had an average P_{H_2O}
6 ($7.5 \pm 0.8 \times 10^{-3}$ AU, $n = 38$), some 32% less than the P_{H_2O} of epithelial cells. It was found
7 that P_{H_2O} of mouse fiber cell vesicles displayed significant variability. While one third of
8 mouse fibre-derived membrane vesicles gave P_{H_2O} values that were similar in magnitude to
9 those measured in mouse lens epithelia, a second population showed a lower water
10 permeability that was some 73% lower than the P_{H_2O} of epithelial cells, a result that was
11 similar to what had previously been reported in the literature (50, 51). Pre-incubated mouse
12 fiber-derived membrane vesicles exposed to Hg^{2+} significantly reduced the observed average
13 P_{H_2O} ($4.6 \pm 0.6 \times 10^{-3}$ AU, $n = 19$, $P < 0.02$) and eliminated the sub-group of vesicles with
14 higher P_{H_2O} values seen in the absence of mercury (Figure 5).

15 The same functional dissection of mercury-sensitive water permeability was performed on rat
16 lenses (Figure 6). As seen in the mouse, rat epithelial cells had a high P_{H_2O} ($14.4 \pm 0.9 \times 10^{-3}$
17 AU, $n = 30$) that was inhibited ($6.8 \pm 0.9 \times 10^{-3}$ AU, $n = 19$) in presence of mercury (Figure
18 6). The average P_{H_2O} of rat fiber-derived membrane vesicles ($4.8 \pm 0.6 \times 10^{-3}$ AU, $n = 30$),
19 was ~3-fold lower than the water permeability measured in rat lens epithelial cells. However,
20 unlike the mouse, rat fiber-derived membrane vesicles consisted of one large group of
21 vesicles exhibiting this low P_{H_2O} , with only 5 vesicles out of 30 that exhibited higher P_{H_2O}
22 values that mirrored those seen in rat epithelial cells. Incubation with Hg^{2+} of rat fiber-derived
23 membrane vesicles showed a small, but not significant, reduction in P_{H_2O} ($3.9 \pm 0.4 \times 10^{-3}$ AU,
24 $n = 15$) and the absence of the sub-group of vesicles which exhibited higher P_{H_2O} values. In
25 summary, the comparative mercury sensitivities of mouse and rat fiber-derived membrane

1 vesicles seem to be consistent with their immunohistochemical localizations within both
2 fixed lens sections (Figure 2&3) and membrane vesicles (Figure 4). Taken together, it
3 appears that AQP5 is present in the membrane and contributes, along with AQP0, to the
4 overall water permeability of fiber cells in the outer cortex of the mouse lens. In the rat lens,
5 where AQP5 exists predominately as a pool of cytoplasmic water channels, AQP5 does not
6 appear to make a significant contribution to overall water permeability of cortical fiber cells,
7 as evidenced by the Hg²⁺-insensitivity of the P_{H₂O} measured in rat fiber cell membrane
8 vesicles. This suggests that in the outer cortex of the rat lens that AQP0 mediates water
9 permeability.

10 **Membrane insertion of AQP5 increases the mercury-sensitive water permeability of rat** 11 **fiber-derived membrane vesicles**

12 In other tissues, AQP5 has been shown to be a regulated water channel which, in response to
13 osmotic challenge, increases water permeability through translocation to the plasma
14 membrane (26, 27, 58). We therefore investigated whether the process of organ culturing
15 lenses under conditions of osmotic challenge could also induce an insertion of AQP5 into the
16 membranes of fiber cells in the outer cortex of the lens (Figure 7), and whether such an
17 insertion would subsequently increase the mercury-sensitive water permeability of fiber cell
18 membrane vesicles isolated from organ cultured lenses (Figure 8). Interestingly, we found
19 that simply organ-culturing lenses in isotonic AAH was sufficient to induce an increase in the
20 membrane localization of AQP5 in both the mouse (Figure 7B-E) and rat (Figure 7F-I)
21 lenses, relative to what was observed when lenses were fixed immediately following removal
22 from the eye. Incubating mouse lenses for two hours in isotonic AAH induced translocation
23 of AQP5 to the membranes of superficial fiber cells in the localized bow region of the outer
24 cortex (Figure 7C) in comparison to immediately fixed lenses where AQP5 was only
25 cytoplasmic (Figure 7B). There was no additional change in the localization of AQP5 in the

1 deeper regions of the outer cortex where AQP5 remained membranous in lenses that were
2 fixed immediately upon removal from the eye (Figure 7D) and organ cultured mouse lenses
3 (Figure 7E). As previously described, the subcellular distribution of AQP5 labeling in
4 immediately-fixed rat lenses was predominantly cytoplasmic throughout the outer cortex
5 (Figure 7F&H). However, an overnight incubation of rat lenses in isotonic AAH resulted in a
6 switch in AQP5 localization into the membrane in peripheral differentiating fiber cells
7 (Figure 7G), with this membrane localization becoming even more prominent in fiber cells
8 located in the deeper regions of the outer cortical region (Figure 7I). While we do not yet
9 understand the signaling mechanisms responsible for this apparent insertion of AQP5 from a
10 cytoplasmic pool into the membrane, we can use the observed phenomenon to investigate
11 whether this increased membrane insertion of AQP5 alters the mercury-sensitive fraction of
12 the water permeability measured in fiber-derived membrane vesicles. Unfortunately, it is
13 technically challenging to derive fiber cell vesicles from the small (~1-5% of total outer
14 cortex area) superficial outer cortical region of the mouse lens that exhibits membrane
15 insertion of AQP5 in response to organ culture. Hence, measurements of water permeability
16 post-organ culture were only performed on rat fiber-derived vesicles where the biggest
17 increase in Hg^{2+} -sensitive $P_{\text{H}_2\text{O}}$ would be expected to be observed following organ culture.

18

19 Fiber-derived membrane vesicles were therefore prepared from the outer cortex of organ-
20 cultured rat lenses, and their water permeability tested using the fluorescence-based
21 functional assay for water permeability (Figure 8). We found that in comparison to native rat
22 vesicles ($P_{\text{H}_2\text{O}} = 4.8 \pm 0.6 \times 10^{-3}$ AU, $n = 30$), vesicles derived from organ-cultured rat lenses
23 had a significantly higher average $P_{\text{H}_2\text{O}}$ ($7.2 \pm 0.6 \times 10^{-3}$ AU, $n=31$, $P < 0.01$). The observed
24 increase in average $P_{\text{H}_2\text{O}}$ appeared to be due to AQP5 insertion, since the addition of Hg^{2+} to
25 vesicles prepared from organ cultured lenses produced a significant reduction in $P_{\text{H}_2\text{O}}$ ($4.2 \pm$

1 0.6 x 10⁻³ AU, n = 18, P < 0.015) to values that were found to be not significantly different to
2 the average P_{H2O} measured in acutely-isolated lenses, where AQP5 was predominantly
3 located in the cytoplasm of peripheral fiber cells. These results suggest that in the rat lens
4 AQP5 can be dynamically recruited to the membranes of differentiating fiber cells to increase
5 water permeability in the outer cortex of the lens.

6

7 **DISCUSSION**

8 In this study we have extended our previous observations (20) of species differences in the
9 subcellular location of AQP5 in mouse and rat lenses, and in addition have shown that AQP5
10 contributes to the water permeability of fiber cell membranes. Furthermore, we have shown
11 that this contribution of AQP5 to overall water permeability can be increased by the
12 trafficking of AQP5 from a cytoplasmic pool to the fiber cell membrane. In both rat and
13 mouse lenses, AQP5 could be found in distinct cytoplasmic or membranous pools in the outer
14 cortex, but the extent of cytoplasmic AQP5 labeling was more extensive in the rat than the
15 mouse lens (Figures 2 &3). In contrast, AQP0, was always found to be associated with the
16 membranes of cortical fiber cells in both species (Figure 3&4). These species-specific
17 differences in the subcellular distribution of AQP5 appeared to be maintained in the fiber cell
18 membrane vesicles isolated from the outer cortex of the mouse and rat lens (Figure 4). From
19 this observation, we expected that the functional measurements of P_{H2O} in the vesicles
20 isolated from the mouse and rat lens would exhibit heterogeneity in P_{H2O} values that reflect
21 the differences in the subcellular location of AQP5, the higher protein abundance of AQP0
22 relative to AQP5, and the higher P_{H2O} of water channels formed from AQP5 relative to
23 AQP0.

1 Consistent with previous reports (50), measured P_{H_2O} in epithelial cells isolated from mouse
2 (Figure 5) and rat (Figure 6) lenses were both significantly inhibited by Hg^{2+} , and were
3 higher than the measured P_{H_2O} of fiber-derived membrane vesicles. However, the current
4 study revealed significant Hg^{2+} -sensitive water permeability in fiber-derived membrane
5 vesicles isolated from the mouse lens (Figure 5). The observed range of P_{H_2O} values recorded
6 in this study tends to mirror the heterogeneity in membrane vs cytoplasmic labeling of AQP5
7 and membrane labelling of AQP0 observed immunohistochemically in both lens sections, and
8 in vesicles isolated from cortical fiber cells of the mouse lens. Since a subset of mouse fiber
9 cell membrane vesicles displayed P_{H_2O} values equivalent to those observed in the
10 morphologically distinct epithelial cells (Figure 1A) and this subset of high vesicles with high
11 P_{H_2O} was eliminated in the presence of Hg^{2+} , we can conclude that these vesicles contain in
12 addition to AQP0, the Hg^{2+} -sensitive water channels AQP1/5. Furthermore, since AQP1
13 expression has been shown to be restricted to the lens epithelium (46) we can assume that the
14 high P_{H_2O} permeability observed in this subset of mouse vesicles is mediated by presence of
15 AQP5 in the membranes of these vesicles. The remaining vesicles exhibited a lower, Hg^{2+} -
16 insensitive P_{H_2O} , which we attribute to the sole presence of AQP0 water channels in these
17 particular vesicles. Thus relative to an earlier study that analyzed P_{H_2O} in the mouse lens
18 using a small number of vesicles (51), the larger sample size of our current study provides a
19 more accurate representation of the heterogeneity in abundance and membrane distribution of
20 AQP0 and AQP5 now known to exist in the mouse lens. Our measurements appear to
21 confirm that, in the mouse lens, AQP5 forms a functional water channel which in
22 combination with AQP0, makes a significant contribution to the water permeability of fiber
23 cells in the outer cortex.

24 In the rat lens, immunolabeling in lens sections (Figure 2 and 3) and fiber cell membrane
25 vesicles (Figure 4) showed that AQP5 was predominantly localized in the cytoplasm.

1 Functional measurement of P_{H_2O} were consistent with this cytoplasmic location of AQP5,
2 since P_{H_2O} measurements in rat fiber cell membrane vesicles were dominated by a lower
3 Hg^{2+} -insensitive basal P_{H_2O} , which can be assigned to AQP0 (Figure 6). Interestingly, the
4 magnitude of Hg^{2+} -sensitive water permeability measured in rat fiber-derived membrane
5 vesicles was altered by organ culturing rat lenses in isotonic AAH overnight, and this could
6 be correlated with insertion of AQP5 into cortical fiber cell membranes (Figure 7). AQP5
7 insertion increased the mean P_{H_2O} values, which were subsequently shown to be sensitive to
8 Hg^{2+} (Figure 8). Taken together, our localization and functional studies in lenses of two
9 rodent species demonstrate that AQP5 located in the membrane contributes a Hg^{2+} -sensitive
10 component to the water permeability of cortical fiber cells, that is additional to the basal
11 contribution of AQP0 to P_{H_2O} . Furthermore, we show that AQP5 exists in the rat lens as a
12 cytoplasmic pool of water channels, which can be dynamically recruited to increase the P_{H_2O}
13 of fiber cell membranes in the outer cortex of the lens.

14 These conclusions raise two questions that deserve further discussion. Firstly, the observation
15 that organ culture of lenses can stimulate the dynamic insertion of AQP5 into the membranes
16 of differentiating fiber cells in both the mouse and rat lens (Figure 7), raises questions over
17 the pathways that regulate AQP5 membrane insertion in the lens. In other tissues, AQP5 has
18 been found to insert into the apical plasma following phosphorylation of the channel through
19 the cAMP-dependent PKA pathway (58). In addition, exposure to hypertonic challenge was
20 shown to upregulate AQP5 protein expression through an Extracellular signal-Regulated
21 Kinase (ERK) dependent pathway in mouse lung epithelial (MLE-15) cells (22). Together,
22 these studies suggest that phosphorylation of AQP5 might be an important molecular
23 mechanism regulating AQP5 membrane insertion. Although phosphorylated AQP5 has been
24 reported in the mouse lens (34), only one of the two predicted phosphorylation sites of AQP5
25 (Serine 156, Threonine 259) has been detected in lens tissue (53). Further investigation is

1 required to establish the phosphorylation status of cytoplasmic and membranous pools of
2 AQP5, in order to understand the mechanisms that control the dynamic insertion of AQP5 in
3 specific regions of the lens.

4 Secondly, our demonstration that membrane insertion of AQP5 into the differentiating fiber
5 cell membranes increases P_{H_2O} does not address the physiological requirement for the lens to
6 dynamically modulate P_{H_2O} in the outer cortex. It is interesting to speculate that modulation
7 of P_{H_2O} may be required to maintain the gradient in hydrostatic pressure that has been
8 measured in all lenses studied to date (13). This hydrostatic pressure gradient is generated by
9 the flow of water through gap junction channels and ranges from 0 mmHg in the periphery to
10 335 mmHg in the lens center (14). The pressure gradient is thought to drive intracellular flow
11 of fluid from the lens core to the periphery and is remarkably preserved in lenses from several
12 different species (13). This conservation of the pressure gradient amongst species has led to
13 the suggestion that the gradient is actively modulated to control the water content in the lens
14 core thereby setting the water/protein ratio which, in turn, determines the gradient of
15 refractive index that contributes to the optical properties of the lens (13). Recently, Gao *et al.*
16 demonstrated a link between activation of TRP channels, the alteration in Na/K ATPase
17 activity, and the modulation of hydrostatic pressure in the mouse lens (15). Intriguingly,
18 recent reports have revealed a synergistic association between the mechano-sensitive TRPV4
19 and aquaporin water channels to affect changes in fluid transportation to preserve cell volume
20 (37), (29),(12). As a working hypothesis it is intriguing to speculate that the dynamic
21 regulation of AQP5 membrane trafficking alters the water permeability of outer cortical fiber
22 cells to modulate the hydrostatic pressure gradient and thereby maintain the optical properties
23 of the lens. Testing this hypothesis will be the focus of future work.

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4

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1 **FIGURE LEGENDS**

2 **Figure 1: Fluorescent assay used to calculate the rate of change in cell/vesicle volume.**

3 (A) Representative images of a single rat epithelial cell (*top panel*) and fiber cell membrane
4 vesicle (*bottom panel*) loaded with calcein, before and after exposure to hypertonic (390
5 mOsmL⁻¹) saline. A single DIC image was taken prior to the exposure to hypertonic saline,
6 and subsequently used to calculate the initial area of the epithelial cell or membrane vesicle
7 and also to differentiate between epithelial cells and vesicles by the presence or absence of a
8 cell nucleus. Representative images from the initial exposure to isotonic (25 s), hypertonic
9 (75 s) and back to isotonic (145 s) saline are shown. (B) Representative bleach-corrected time
10 course, showing the normalized change in calcein fluorescence intensity in response to a
11 hypertonic challenge. Fluorescence intensity was collected from a central region of interest
12 within individual vesicles. (C) The initial change in fluorescence intensity of two vesicles in
13 response to hypertonic challenge, in either the absence and presence of mercury, fitted with
14 monoexponential equations to extract the rate of change in cell volume (τ), used to calculate
15 the water permeability (P_{H_2O} , Equation 2). Scale bar 5 μm .

16

17 **Figure 2: Comparison of the subcellular localization of AQP5 in axial sections taken**

18 **from mouse and rat lenses.** (A) A low-power overview of an axial mouse lens section,
19 labeled with the general membrane marker WGA (red) and the nuclear stain DAPI (blue) to
20 define three regions where the subcellular location of AQP5 (green) is subsequently
21 compared between mouse (B) and rat (C). (B) Higher magnification images from the mouse
22 lens reveal cytoplasmic labeling of AQP5 which is restricted to a narrow and superficial
23 region localized around the equator, designated by the dotted line (Region 1B). Beyond this
24 region AQP5 was found to be associated with the membrane at both the anterior (Region 2B)
25 and posterior (Region 3B) poles of the lens. (C) Higher magnification images from the rat

1 lens showed that AQP5 labeling remained cytoplasmic in the equator (Region 1C), in the
2 anterior (Region 2C) and posterior (Region 3C) poles of the lens. For clarity of display a
3 mirror image, in which WGA labeling is omitted, is presented on the right hand side next to
4 each double- or triple-labeled image.

5

6 **Figure 3: Comparison of the membrane localization of AQP0 and AQP5 in the outer**
7 **cortex of the mouse and rat lenses.** (A) An equatorial section of rat lens labeled with the
8 membrane marker WGA (red) and the nuclear stain DAPI (blue) showing the regions (white
9 boxes) from which high magnification images of the subcellular location of AQP0 and AQP5
10 were obtained from the mouse (B-E) and rat (F-I) lens. To account for the differences of the
11 sizes of the mouse and rat lenses, labeling for AQP0 and AQP5 was obtained from equivalent
12 regions represented with an r/a ratio - where at $r/a=1$ indicates a region adjacent to the
13 capsule and $r/a=0$ corresponds to the lens core. For both the mouse and rat lenses $r/a=0.98$ for
14 the region next to the capsule (B, C, F and G). In the deeper outer cortical region $r/a=0.90$ for
15 the mouse lens (D, E) and $r/a=0.77$ for the rat lens (H, I). In the mouse (B) and rat (F) lens,
16 AQP0 (green) is expressed around the entire membrane of differentiating fiber cells localized
17 adjacent to the capsule while AQP5 (green), in the same region, in both the mouse (C) and rat
18 (G) lenses, was localized only in the cytoplasm. In deeper regions of the outer cortex in both
19 the mouse (D) and rat (H) lenses AQP0 continued to be membranous. In the same region
20 changes in the expression of AQP5 were observed since in the mouse lens (E) AQP5 labeling
21 (green) was translocated exclusively to the membranes of differentiating fiber cells while in
22 the rat lenses (I) AQP5 labeling continued to be strongly cytoplasmic. n, nuclei; blue, DAPI.

23

24

1 **Figure 4: The subcellular distribution of AQP0 and AQP5 in membrane vesicles formed**
2 **from fiber cells isolated from the mouse or rat lens.** (A) A clump of fiber cells isolated
3 from the rat lens undergoes a process of vesiculation to spontaneously form a cluster of
4 membrane vesicles after 30 minutes of incubation in the presence of Ca^{2+} . (B) In membrane
5 vesicles isolated from the mouse lens AQP5 (green) was predominantly associated with the
6 membranes (Bi), but could occasionally be also found in the cytoplasm (Bii). In double
7 labeled vesicles, AQP0 (red) was always membranous and could be found co-expressed with
8 either higher (Biii) or lower levels of AQP5 labeling (Biv). (C) In the rat, AQP5 was
9 predominately cytoplasmic (Ci), although some vesicles exhibited membrane labeling for
10 AQP5 (Cii). In double labeled rat vesicles, AQP0 was always membranous while AQP5
11 labeling was usually cytoplasmic (Ciii), although vesicles that co-expressed both AQP0 and
12 AQP5 in the membrane were occasionally detected (Civ). Scale bar in A, B and C is $5\mu\text{m}$.

13

14 **Figure 5: Water permeability of epithelial cells and fiber cell membrane vesicles in the**
15 **mouse lens.** Scatter plots showing the water permeability ($P_{\text{H}_2\text{O}}$) of mouse epithelial cells and
16 fiber cell membrane vesicles in the presence and absence of $300\ \mu\text{M}$ HgCl_2 . The addition of
17 HgCl_2 significantly reduced the $P_{\text{H}_2\text{O}}$ of mouse epithelial cells and fiber cell membrane
18 vesicles. *** ($P < 0.0001$), *($P < 0.02$); (n) number of cells or membrane vesicles; Bars show
19 Mean \pm SEM.

20

21 **Figure 6: Water permeability of epithelial cells and fiber cell membrane vesicles in the**
22 **mouse lens.** Scatter plots showing the water permeability ($P_{\text{H}_2\text{O}}$) of rat epithelial cells and
23 fiber cell membrane vesicles in the presence and absence of $300\ \mu\text{M}$ HgCl_2 . The addition of
24 HgCl_2 significantly reduced the $P_{\text{H}_2\text{O}}$ of rat epithelial cells but not rat fiber-derived membrane

1 vesicles. *** ($P < 0.0001$); NS, not significantly different; (n) number of cells or membrane
2 vesicles; Bars show Mean \pm SEM

3 **Figure 7: Dynamic insertion of AQP5 in the cell membranes of organ-cultured rat and**
4 **mouse lenses incubated in isotonic AAH.** (A) Low power overview of an equatorial section
5 of a lens labeled with the membrane marker WGA (*red*) showing two regions (*white boxes*)
6 in the outer cortex where high magnification images were obtained from a mouse (B-E) or rat
7 (F-I) lens, fixed either immediately after removal from the eye (B, D, F, H) or after organ
8 culture in AAH (C, E, G, I). In immediately fixed mouse lenses AQP5 labeling was
9 predominately cytoplasmic in the superficial (B) region, but was membraneous in the deeper
10 regions (D) of the outer cortex. In sections taken from mouse lenses organ cultured for 2
11 hours, AQP5 labeling was found to colocalize with the membranes of differentiating fiber
12 cells in a discrete zone in the lens periphery (C, arrowhead), and retained its membraneous
13 location in deeper (E) regions of the outer cortex. In immediately fixed rat lenses, AQP5
14 labeling was predominately cytoplasmic in both the superficial (F) and deeper regions (H) of
15 the outer cortex. In contrast, in sections from rat lenses organ cultured for 17 hours, AQP5
16 labeling was found in discrete punctae, that colocalized with the membranes of differentiating
17 fiber cells in both peripheral (C) and deeper (E) regions of the outer cortex.

18

19 **Figure 8: Effect of organ culture on the Hg^{2+} -sensitive water permeability of rat fiber**
20 **cell membrane vesicles.** Scatter plots comparing the water permeability (P_{H_2O}) of fiber cell
21 membrane vesicles prepared from lenses fixed either immediately after removal from the eye,
22 or after organ culture in AAH for 17 hours. The addition of 300 μM $HgCl_2$ significantly
23 reduced the P_{H_2O} of membrane vesicles prepared from organ cultured rat lenses. **($P < 0.01$);
24 *($P < 0.015$); (n) number of cells or membrane vesicles; Bars show Mean \pm SEM.