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The Water Permeability of Lens Fibre Cells: The Relative Role of AQP0 and AQP5

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Abstract

Transportation of water is essential for the maintenance of lens homeostasis, and members of the aquaporin (AQP) family have been found to mediate the water permeability of lens cells. In my thesis, I performed immunolabelling analysis of whole lens sections from three different species of mammalian lens to map the cellular and subcellular distribution of AQP5, a third water channel recently discovered in the lens. I found a common, yet species-dependent, subcellular redistribution of AQP5 from a cytoplasmic pool to the membranes of differentiating fibre cells that occurred in the outer cortex of mouse and bovine lenses, and in the transition zone between differentiating and mature fibre cells in the inner cortex of rat lenses. In addition to the programmed translocation of AQP5 that took place during fibre cell differentiation, I found that AQP5 translocation to the membrane of peripheral differentiating fibre cells can be induced by organ culturing of mouse lenses in solutions of varying extracellular osmolarity.

In addition, I compared the subcellular distribution of AQP5 with the well-characterised distribution of AQP0 throughout adult, embryonic and postnatal lenses and found that each AQP contributes differently to membrane water permeability in different lens regions. I found that, during embryonic development, AQP5 is predominantly in the cytoplasm while AQP0 remained membranous, and that these spatial differences in the subcellular distribution of AQP0 and AQP5 were maintained into the early postnatal period. The most profound changes in the distribution of AQP5 and AQP0 took place during the first and second week of postnatal development. AQP0 underwent an abrupt differentiation-dependent truncation of its C-terminus that began in the core of the lens which was complemented by the translocation of AQP5 from the cytoplasm to the membranes of first the lens core, and then subsequently differentiating fibre cells in the outer cortex without undergoing C-terminal truncation. These changes in protein distribution were established at a postnatal stage when the hyaloid vascular system feeding the lens regresses, transforming the lens into an avascular tissue solely dependent on the lens microcirculation system to maintain its transparency and homeostasis.

The existence of differences in the subcellular localisation of AQP5 in the outer cortex of mouse and rat lenses allowed me to propose the hypothesis that the relative functional contributions of AQP5 and AQP0 to cell water permeability in this peripheral region of the
lens are different. To test this hypothesis, I developed and optimised a novel fluorescence-based functional assay using mouse and rat lens fibre cell membrane vesicles derived from the outer cortex. To further delineate between the relative functional contributions that AQP5 and AQP0 make to water permeability, I used mercury as a specific pharmacological blocker of AQP5 since AQP0 is insensitive to mercury. I found that mouse vesicles displayed higher water permeability than rat vesicles and, in addition, showed sensitivity to mercury. Taken together my data suggest that AQP5 is a regulated water channel that can be inserted into lens fibre cell membranes under osmotic stress conditions to restore lens water homeostasis. Furthermore, since AQP5 is not truncated in the lens core, it is likely to remain functional in this lens region to facilitate the circulating water fluxes that are proposed to maintain adequate water and refractive index gradients in the lens.
Acknowledgements

I would like to express my special thanks to first and foremost my supervisor Prof. Paul Donaldson for his full support and encouragement throughout the course of my PhD. You have accepted me in your laboratory and guided me to accomplish so much. You have given me the freedom to carry on with my own ideas and experiments and have always been positive about my work and achievements. This journey has made me a confident researcher who is ready to take on new challenges and to continue to deliver first class research studies.

Next I would like to thank my co-supervisors Dr Gus Grey and Dr Kevin Webb. Gus you have been a great support for me by always encouraging me to keep going, ready to discuss and plan experiments to correct me when I am wrong, guide me when I write and finally for being such a nice and calm friend to whom I will endeavour to give lifts to work whenever this is possible. Kevin, you have been very encouraging and helped me a lot to establish the fluorescence assay. I could not have finished this project without your knowledge and guidance. I would like to say a big thank you for all the time you spent to teach me how to work the perfusion chamber, how to design a protocol to run the fluorescence based assay, how to analyse my data and finally for always encouraging me to keep thinking and improve on my work. You have been a grounded scientific pillar in this project using your sharp mind to critique my work and yet help me to make sense of it.

I would like to thank Prof. Kevin Schey who partly funded my project and for giving me this opportunity to work and discover the wonders of AQP5 expression and functional role in the lens. Thank you, Dr Ehsan Vaghefi for your consultation on the calculation of water permeability of epithelial cells and fibre cell vesicles your help is highly appreciated.

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I would like to thank my family, my mum Kristina and big sister Maryana, you have been the pillars of my life. I would not have achieved this if you didn’t teach me how to be a hard-working, consistent and ever curious person. Also to thank my son Stanley and husband Vlad for putting up with me when I was grumpy or tired, for loving me and for being there for me when I need you.

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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AQP</td>
<td>Aquaporin</td>
</tr>
<tr>
<td>HVS</td>
<td>Hyaloid Vascular System</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>Na+/K+ pump</td>
<td>Sodium-Potassium Pump</td>
</tr>
<tr>
<td>Na+/K+ ATPase</td>
<td>Sodium-potassium adenosine triphosphatase</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>Mercury ion</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>Mercury (II) Chloride</td>
</tr>
<tr>
<td>F</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>mOsmol/L</td>
<td>Milliosmoles/Litre</td>
</tr>
<tr>
<td>Cys or C</td>
<td>Cysteine amino acid</td>
</tr>
<tr>
<td>P_{H₂O}</td>
<td>Water Permeability</td>
</tr>
<tr>
<td>NPA</td>
<td>Asparagine-Proline-Alanine conserved motif found in the water pore of AQPs</td>
</tr>
<tr>
<td>MIP</td>
<td>Major Intrinsic Protein of the lens</td>
</tr>
<tr>
<td>Cat^{fr}</td>
<td>Cataract Fraser Mutation</td>
</tr>
<tr>
<td>Calcein AM</td>
<td>Calcein Acetoxyethyl</td>
</tr>
<tr>
<td>TRPV4</td>
<td>Transient Receptor Potential Vanilloid 4</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>TVL</td>
<td>Tunica Vaculosa Lentis</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous (flow regulator)</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>µl</td>
<td>Microlitre</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>τ</td>
<td>Time Constant, tau</td>
</tr>
<tr>
<td>EM-CCD</td>
<td>Electron Multiplying Charge Coupled Device (camera)</td>
</tr>
<tr>
<td>AAH</td>
<td>Artificial Aqueous Humour</td>
</tr>
<tr>
<td>ADI</td>
<td>Alpha Diagnostic International</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>BMPs</td>
<td>Bone Morphogenetic Proteins</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor Beta</td>
</tr>
<tr>
<td>MQ water</td>
<td>Milli-Q ultrapure water</td>
</tr>
<tr>
<td>r</td>
<td>Radius</td>
</tr>
<tr>
<td>V</td>
<td>Volume</td>
</tr>
<tr>
<td>osm</td>
<td>Osmolarity</td>
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<tr>
<td>mm</td>
<td>Millimetre</td>
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<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>µm</td>
<td>Micrometre</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet (light)</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat Germ Agglutinin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>NGS</td>
<td>Normal Goat Serum</td>
</tr>
<tr>
<td>g</td>
<td>g-force</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per Minute</td>
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Chapter 1. Introduction

Our sense of sight is one of our most precious sensory modalities and the quality of our vision is critically dependent on the ability of the transparent tissues in the front of the eye to correctly focus light onto the retina at the back of the eye. This is dramatically illustrated by the fact that loss of transparency of the ocular lens, commonly known as cataract, is the most common cause of blindness in the world today. Furthermore, age related changes to the structural properties of the lens have been implicated in the onset of presbyopia, the loss of the ability to focus on near objects that affects all people as they enter middle age. While it has long been known that the structural properties of the lens determines its transparent and refractive properties, more recent work has shown that the cellular structure of the lens needs to be actively maintained to preserve the optical properties of the lens. In this section, I will first briefly describe the overall anatomy of the eye before focusing on the unique structure and function of the lens. Central to lens function are ion and water fluxes that have been shown to circulate through the lens as part of a proposed internal microcirculation system that maintains the transparent and refractive properties of the avascular lens. This microcirculation is driven by spatial differences in the transport properties of cells in the different regions of the lens. This thesis focuses on the aquaporin family of water channels that mediate water flow in the different regions of the lens. Hence the last section of this chapter will provide a general introduction to the aquaporin family before concentrating on the three aquaporins now known to be expressed in the lens.

1.1. Visual system and structure of the eye

The visual system has evolved to become one of the most complex structures in our body. Our eyes transform light energy into electrical impulses, which when processed in the visual cortex of the brain produce detailed and precise images of the objects that surrounds us. Figure 1-1 shows the basic structure of the human eye.
Figure 1-1: Schematic representation of a cross section of the human eye. The black line represents the visual axis and path of light rays which enter the anterior side of the eye through the cornea, pass through the iris, lens and vitreous body and finally reach the photoreceptors cells of the retina at the back of the eye (adapted from the University of Florida internet site at: www.phys.ufl.edu).
The eye is positioned in the orbit and contains three flanking tissue layers, each of which demonstrate a specific function (Snell and Lemp, 1989). The outermost layer is the fibrous tunic, which consists of the cornea and sclera. The anteriorly positioned cornea is the principal refractive element of the eye and owes its transparency to the regular organization of its collagen fibres. At the limbus, the transparent cornea transitions to the opaque sclera. The sclera encompasses the eyeball and is composed of a collagen matrix that helps to maintain the shape of the eye and protect the more delicate inner layers from mechanical damage, while providing sites of anchorage for the extra ocular muscles that control the movement of the eye.

The middle layer, or uveal tract, is not structurally homogeneous and includes the iris, ciliary body and choroid. The iris is a pigmented tissue located behind the cornea, which by controlling the diameter of its central pupil acts as an aperture stop that controls the amount of light entering the eye. The ciliary body is adjacent to and continuous with the iris. In cross section the ciliary body has a triangular shape, the base of which extends from the iris root to the choroid. It is composed of a surface ciliary epithelium that secretes the aqueous humor and the ciliary muscle which is mechanically linked to the lens by the zonular fibres. The aqueous humor flows between the posterior surface of the iris and anterior surface of the lens, entering the anterior chamber through the pupil. The ciliary body continuously secretes the aqueous humour that fills the anterior chamber, and is removed from the chamber through the trabecular meshwork to enter the canal of Schlemm, a blood vessel encircling the anterior chamber (Kiel et al., 2011). Contraction of the ciliary muscle controls the tension on the zonules allowing the curvature and therefore the optical power of the lens to be altered during accommodation.

The choroid layer lines the posterior portion of the eye and extends forward of the equator into the anterior segment where it meets the ciliary body. It is highly vascularized and provides nutrients and oxygen to meet the high energy demands of the retina. The stroma of the choroid is densely packed with melanin pigment which absorbs excess light and eliminates light scatter within the eye. The posterior chamber of the eye contains the vitreous body, a gelatinous structure formed of collagen fibres intermixed with hyaluronic acid that has a tremendous affinity to attract water molecules. The vitreous interfaces with the innermost layer of the eye, which is the light sensitive retina. Retinal photoreceptors transform light energy into electrical impulses which are relayed via the optic nerve to the visual cortex for final processing and image formation.
Chapter 1 – Introduction

1.2. Optical properties of the normal and aging lens

The lens is the second and final focusing element in the eye and as such its optical properties are dependent on its transparency (the nett result of a unique cellular structure and function described in sections 1.3 & 1.4) and ability to refract light. The refractive properties of the lens are in turn the product of its surface curvature and the existence of a gradient of refractive index that corrects for spherical aberration. Spherical aberration is an optical error introduced into the ocular light pathway by the increased refraction of light rays that strike the periphery of the cornea relative to those that strike its centre (Garner and Smith, 1997, Smith, 2003). The lens compensates for this optical error by imposing and maintaining a negative spherical aberration through the establishment of an inherent gradient of refractive index (GRIN) (Pierscionek and Augusteyn, 1990, Smith et al., 2001). This gradient is generated by over expressing different subtypes of crystallin proteins with varying refractive indices (Smith, 2003, Augusteyn, 2010), thereby ensuring that incoming light is accurately focussed on the retina. The lens is an asymmetrical oblate spheroid with a flatter anterior surface relative to its posterior surface that faces the vitreous. During accommodation the lens changes its optical power by altering its surface curvatures.

1.2.1. Accommodation and presbyopia

During accommodation, contraction and relaxation of the ciliary muscles changes the tension on the zonular fibres that connect the equatorial rim of the lens to the ciliary body resulting in changes to the curvature of the lens that alter its optical power and therefore the point of focus of the whole eye (Figure 1-2). To focus on near objects, the ciliary muscle contracts releasing zonular tension causing the lens to become rounder, thereby increasing the optical power of the lens. To return to far vision, the ciliary muscle relaxes restoring tension to the zonules forcing the lens to assume a flatter curvature that reduces the optical power of the lens. Throughout life the lens grow continuously by the addition of new fibre cells. With aging, a change in the compressibility of the lens occurs which interferes with its ability to undergo accommodation in response to ciliary muscle contraction. These changes result in the loss of near vision and the onset of presbyopia. In addition, as we age there is a progressive increase in focal length of the unaccommodated lens that produces a gradual hyperopic shift which is believed to be caused by a change in the refractive index distribution and/or an increased thickening of the lens (Glasser and Campbell, 1998).
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Figure 1-2: Lens accommodation. (A) The lens is suspended in place by numerous zonular fibres (arrow) that extend from the equatorial rim of the lens, pass through the ciliary processes and attach to the pars plicata of the ciliary body. (B) Relaxation of the ciliary muscle increases zonular tension which leads to flattening of the lens surface curvature to allow the eye to focus on distant objects (left panel). During accommodation (right panel) the ciliary muscle contracts reducing the zonular tension, which allows the lens to assume a rounder shape with an increased optical power to bring near objects into focus. A, Albrecht von Graefes Archiv für Klinische und Experimentelle Ophthalmologie, 192, 2, 1974, 117-124, Scanning Electron Microscopy of the Zonule of Zinn, Bornfeld N, Spitznas M, Breipohl W, Bijvank GJ, Figure 4b, original copyright notice is given to the publication in which the material was originally published, with kind permission from Springer-Verlag; B, obtained with kind permission from Bianca Heilman’s PhD dissertation, published at Scholarly Depository, University of Miami at http://scholarlyrepository.miami.edu/oa_dissertations/1558/.
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1.2.2. Cataract: the loss of lens transparency

To fulfil its function as an optical element in the eye, the lens has to remain transparent through many decades of life. Lens cataracts interfere with the passage of light producing light scatter that initially reduces image quality, which if left untreated can ultimately result in blindness. While lens cataract is associated with a variety of pathologic (diabetes, metabolic changes), environmental (UV radiation, smoking) and hereditary factors (congenital cataract), by far the greatest risk factor for the onset of cataract is simply old age.

Cataracts can be classified into three main types, nuclear, cortical and subcapsular, based on their location in the lens (Thylefors et al., 2002). Nuclear cataract (Figure 1-3A, B) is the most prevalent type of cataract. It is strongly associated with aging of the lens and is characterised by accumulation of oxidised proteins (Truscott, 2005, Hains and Truscott, 2008), lens brunesence and nuclear hardening (Truscott, 2000). Posterior subcapsular cataract (Figure 1-3E, F) results from development of lens opacities beneath the lens capsule. It is common in individuals who suffer from myopia, retinitis pigmentosa, or who have taken steroid drugs. Glare sensitivity and problems with near vision are found in patients affected by this type of cataract (Hurst and Douthwaite, 1993). In cortical cataract (Figure 1-3C, D) the rupture of fibre cell membranes in the lens cortex lead to the formation of circumferential shades or spots which eventually extend radially to the nuclear region of the lens in a spike like fashion (Vrensen, 2009). With this cataract, there are problems associated with glare, loss of contrast and difficulty with near and distant vision.

The most common treatment of cataract is surgical replacement of the affected lens with an artificial intraocular lens. While cataract surgery is a safe and effective procedure, the sheer volume of medical intervention required to meet the demands of an aging population means that the cumulative costs to the health care system are staggering. Cataract affects approximately 20 million people in the United States and 150,000 in Australia (McCarty and Taylor, 2001, McCarty, 2002) requiring medical treatment estimated to be 3.4 billion dollars annually in the United States alone, consuming above 60% of the medical budget within the vision related conditions (Ellwein and Urato, 2002). As an alternative to surgical treatment, medical therapies to delay the onset and progression of cataract have been proposed (Varma, 1991, Kyselova et al., 2004). However, the design of effective therapies to target specific cataract sub-types will first require an improved understanding of the mechanisms underlying optimal physiological performance of the normal lens.
Figure 1-3: Major cataract subtypes. Slit lamp photographs (A,C,D,E & F) and dark field photograph of a donor lens (B) with nuclear cataract (A, B), cortical cataract (C,D) or posterior subcapsular cataract (E,F). Images A, C, D, E and F were obtained from the Digital Reference of Ophthalmology, property of the Edward S. Harkness Eye Institute, Department of Ophthalmology of Columbia University at http://dro.hs.columbia.edu/index.htm; Image B was obtained from Philosophical Transactions of the Royal Society B: Biological Sciences, 366, 2011, 1278-1292, The Ageing Lens and Cataract: A Model of Normal and Pathological Ageing, Michael R, Bron AJ, Figure 3e, original copyright notice is given to the publication in which the material was originally published, with kind permission from The Royal Society Publishing, (Michael and Bron, 2011).
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1.3. Lens structure

The schematic structure of the adult mammalian lens is shown in Figure 1-4. The axially orientated section taken through the long axis of the lens shows elongated fibre cells stretching along the anterior-posterior axis in concentric shells (Figure 1-4A). In an equatorial section, the fibre cells are arranged in radial shells of tightly packed columns and exhibit hexagonal cross-sectional profiles in the periphery which with maturation in the deeper cortex adopt a more rounded profile and then a web-like asymmetrical organisation in the nucleus or core of the lens (Figure 1-4B). In both orientations, the fibre cells are organised such that the oldest primary fibre cells, formed during embryonic development, are located in the nucleus of the lens, whereas the newly formed secondary fibre cells are found in close proximity to the capsule. Beneath the anterior capsule, the anterior surface of the lens is covered by an epithelial cell layer. This highly ordered tissue architecture is established during embryogenesis and is maintained throughout life.

Figure 1-4: Structure of the adult lens. (A) Axial view of the mammalian lens, showing the highly elongated fibre cells of the outer cortex (pink), inner cortex (magenta), and core (purple) in an anterior to posterior orientation. The anterior surface of the lens contains an epithelial monolayer (yellow), while the remainder of the lens is comprised of fibre cells which lose their cellular organelles and nuclei (grey) as they differentiate. (B) Equatorial section through the mammalian lens, illustrating the epithelial cell monolayer of the anterior pole around the outside and radial fibre cell columns of lens fibre cells showing a distinctive hexagonal profile (Illustration by H. Suzuki).
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1.3.1. Embryonic development of the lens

During embryogenesis, lens development begins around late gastrulation when the presumptive lens ectoderm (PLE) overlies the embryonic optic vesicle (OV) (Figure 1-5A) (Lovicu and McAvoy, 2005). After physical contact between the PLE and OV is established, the lens ectoderm thickens to form the lens placode (Figure 1-5B). The lens placode subsequently invaginates forming the lens pit while the OV invaginates to form the optic cup (Figure 1-5C). As the cells of the lens vesicle continue growing they eventually detach from the anterior overlying surface ectoderm that goes on to form the corneal epithelium (Figure 1-5D). The cells in the posterior half of the lens vesicle elongate towards the anterior side forming the primary fibre cells that eventually fill the lumen of the lens vesicle (Figure 1-5E and F). The cells lying in the anterior half of the lens vesicle form the lens epithelium. Initially all of the cells in the lens vesicle are capable of proliferation, but as differentiation progresses, the primary fibre cells lose this capacity and epithelial cell proliferation becomes restricted to a band of epithelial cells slightly anterior to the lens equator known as the germinative zone (Figure 1-5G) (Harding et al., 1971, McAvoy, 1978). As these cells proliferate, adjacent epithelial cells move closer to the lens equator where they withdraw from the cell cycle, elongate and differentiate into meridional rows to form secondary fibre cells. The post-mitotic region of epithelial cells is known as a transitional zone (Figure 1-5G), as these epithelial cells are in transition to form secondary fibre cells. In this way, the lens continues to grow throughout life by continuously adding new layers of secondary fibre cells around a lens nucleus composed of primary fibre cells.

Thus, the lens maintains its polarity having a monolayer of epithelial cells restricted to the anterior half of the lens, and a posterior portion composed of fibre cells exposed to the vitreous humour. Lens polarity is maintained throughout life and is tightly regulated by the ocular environment (Coulombre, 1969, Coulombre and Coulombre, 1969). The most important signalling pathway factor(s) that determine fibre cell differentiation have been identified by extensive work utilising a mammalian lens epithelial explant system (McAvoy, 1980). These studies showed that members of the fibroblast growth factor (FGF) family, in particular FGF-1 and FGF-2, can induce mammalian epithelial cells to undergo many of the fibre-specific morphologic (Lovicu and McAvoy, 1989) and molecular changes (Chamberlain and McAvoy, 1989, McAvoy and Chamberlain, 1989, Kok et al., 2002) including elongation, structural specialisation, and onset of specialised crystallin gene expression. It was found that FGF can induce different responses in lens epithelial cells with increased dosage. A low
concentration of FGF induces lens cell proliferation whereas sequentially higher doses induced epithelial cell migration and fibre cell differentiation (McAvoy and Chamberlain, 1989). This was supported by the identification of FGF-1 or FGF-2 in the vitreous (which bathes lens fibre cells in vivo) and a smaller amount found expressed in the aqueous, and that only the vitreous can induce fibre cell differentiation. Therefore it appears that a gradient of bioavailable FGF is required to maintain lens polarity. Later experiments with neutralising FGF antibodies showed that a small percentage of vitreous fractions was not blocked indicating the involvement of factors other than FGF-1 and FGF-2 such as FGF-23 which was found present in the vitreous of human patients (Nakanishi et al., 2002). Apart from the FGF signalling pathway, which can directly induce secondary fibre cell differentiation, there are other pathways that contribute to the regulation of differentiation of epithelial cells into fibre cells. In addition to FGF, other growth factors including bone morphogenic protein (BMPs), transforming growth factor –β (TGFβ) and their respective receptors have all been found to be expressed in the lens (de Iongh et al., 2001) and implicated in fibre cell differentiation. However, they all appear to be downstream from the FGF pathway and are not able to directly induce fibre cell differentiation, but are instead thought to modulate or enhance fibre cell differentiation (Lovicu and McAvoy, 2005) and therefore I have not reviewed them in as much depth.
Figure 1-5: Schematic diagram of the embryological development of the mouse lens. (A) Morphological development of the lens begins as the optic vesicle (OV) approaches the presumptive lens ectoderm (PLE). (B) Upon physical contact of the OV with the PLE, cells within the PLE elongate forming the lens placode. (C) The lens placode invaginates forming the lens pit and the OV invaginates forming the optic cup. (D) The lens pit deepens and the connection of the lens pit and overlying surface ectoderm is lost forming the lens vesicle. (E) The overlying surface ectoderm differentiates into the corneal epithelium and the cells at the posterior of the lens vesicle elongate forming the primary fibre cells. (F) The primary fibre cells fill the lumen of the lens vesicle as they reach the anterior lens cells making up the lens epithelium. The inner layer of the optic cup differentiates into the neural retina. (G) The mature lens consists of an anterior epithelial layer composed of non-proliferating central lens epithelial cells (cuboidal cells with white cytoplasm) and a narrow band of proliferating cells known as the germinative zone (pink cells). Just posterior to the germinative zone is the transitional zone (blue cells) where many genes important for fibre cell differentiation are initially expressed. Just posterior to the lens equator (dotted line) transitional zone epithelial cells begin elongating forming secondary fibre cells (green cells). As secondary fibre cells progress through later stages of differentiation, they lose their intracellular organelles (represented by the shrinkage and loss of red nuclei). The lens nucleus (yellow) is composed of fibre cells that were present in the embryonic lens. The mature lens is bathed on the anterior surface by the aqueous humor and on the posterior surface by the vitreous humor. Seminars in Cell & Developmental Biology, 17, 2006, 726-740, An Essential Role for FGF Receptor Signalling in Lens Development, Robinson ML, Figure 1, original copyright notice is given to the publication in which the material was originally published, with kind permission from Elsevier, (Robinson, 2006), adapted from (Lovicu and McAvoy, 2005).
1.3.2. Postnatal development and fibre cell differentiation

During postnatal development, the nourishment of the lens is provided by extensive branching of the hyaloid artery (HA) encompassing the posterior pole of the lens and forming the tunica vasculosa lentis (TVL). In humans, this system of blood vessels normally regresses before birth, but in many other species of mammals they remain for a certain period of time after birth. A high frequency Doppler study on the mouse eye vasculature found that this well-defined structure at birth, progressively degenerates during the second week postpartum (Brown et al., 2005). By P16, no evidence of the HA existed suggesting that from this time, the growing lens is avascular and can no longer rely on the systematic circulation system for nutrient delivery and removal of waste products.

The secondary fibre cells undergo a series of characteristic changes leading to their terminal differentiation. The first characteristic change is withdrawal from the cell cycle. Mitotic withdrawal is followed by morphological changes (Kuwabara, 1975, Lovicu and McAvoy, 1989, 1992) and molecular changes (Chamberlain and McAvoy, 1989, Kok et al., 2002) including elongation, structural specialization, crystallin accumulation and eventual degeneration of all intracellular organelles (Craig and Piatigorsky, 1973, Bassnett, 1992, Bassnett and Beebe, 1992, Wride, 2011). Therefore during the process of terminal differentiation, fibre cells pass through a specific course of “pre-programmed” changes. These changes are not always marked by gross or abrupt morphological alterations in fibre cell structure, but can be discerned as a gradual transition from fibre cell precursors, to elongating fibre cells, to maturating fibre cells and mature anucleated fibre cells (Lovicu and Robinson, 2004), (Figure 1-6).

The acquisition of crystallin gene expression in the differentiating fibre cells that are undergoing elongation is the most profound characteristic of the growing lens. Crystallins are water soluble proteins that compromise of up to 90% of the total proteins expressed in the lens fibre cells (Bloemendal, 1977). There are three different crystallin isoforms in mammals (α-crystallin, β-crystallin and γ-crystallin) that are related to other proteins in the body, such as enzymes and small heat shock proteins, but which are massively over-expressed in the lens. These high concentrations of crystallin proteins support lens transparency (Andley, 2009) due to short-range weak interactions between the crystallin proteins that is proposed to maintain a liquid-like order (Takemoto and Sorensen, 2008). Furthermore, since crystallin concentrations are highest in the lens core and lowest in the periphery, a gradient of refractive index is created that corrects for the inherent spherical aberration introduced to the optical
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pathway by the cornea, thereby improving overall vision quality. In addition to its structural role, \( \alpha \)-crystallin has been shown to function as molecular chaperone that maintains correct folding of \( \beta \)- and \( \gamma \)-crystallins, so as to prevent their aggregation and formation of light scattering elements (Horwitz, 1992).

**Figure 1-6: Schematic representation of the stages of lens fibre cell differentiation.** (A) Mitotically active cells near the equatorial margin of the lens epithelium. (B) Elongating fibre cells. (C) Maturing fibre cells. (D) Mature fibre cells. Development of the Ocular Lens, 2004, Lovicu FJ, Robinson ML, Figure 9.1, original copyright notice is given to the publication in which the material was originally published, with kind permission from Cambridge University Press, (Lovicu and Robinson, 2004).

Secondary lens fibre cells elongate along the apical-basal axis from the anterior to the posterior pole greatly enlarging their lateral membranes while retaining their apical and basal membrane domains (Figure 1-7). The apical surface of the elongating secondary lens fibre cells are directed toward the interior and form two distinct structures: the modiolus and the apical-apical interface. The modiolus is located close to the equator and is formed by the tapered apical domains of fibre cells as they first start to elongate. The basal domain of all differentiating fibre cells in the cortex are in direct contact with the capsule and are bathed from the aqueous and vitreous humor. As elongation and differentiation of the secondary fibre cell progress, their polarity remains unchanged and the apical membranes migrate toward the apical interface of the anterior cortex and the basal membrane moves toward the posterior pole.
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Upon internalisation of the fibre cells, their apical and basal membranes detach from the apical-apical interface and the posterior capsule, respectively, to fold inward and overlap with the tips of elongating cells from the opposing lens hemisphere forming the anterior and posterior sutures (Kuszak, 1995). The cells forming the sutures exhibit an omega shape and compose the core or nucleus of the lens. The sutures appear as extensive regions of membrane invagination containing a large percentage of the limited extracellular space in the lens, and as such represent an extracellular pathway that extends from the lens surface into the core of the lens (Zampighi et al., 1992).

Figure 1-7: Schematic representation of the structural interaction between epithelial and fibre cells in the lens. On the left, a schematic representation of the lens shows the apical interface between epithelial and differentiating fibre cells in the anterior pole, the tapered tips of elongating secondary fibres forming the modiolus and the suture lines composed by the overlapping of membrane tips of cells from opposing hemispheres in the anterior and posterior pole. Experimental Eye Research, 71, 2000, 415-435, Epithelial Organisation of the Mammalian Lens, Zampighi GA, Eskandari S, Kreman M, Figure 1, original copyright notice is given to the publication in which the material was originally published, with kind permission from Elsevier, (Zampighi et al., 2000). On the right, a confocal image of a mouse axial lens section labelled with WGA (membranes) in red and DAPI (nuclei) in blue shows the modiolus and partial segment of the apical interface. Scale bar 20µm.
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Fibre cells that have lost contact with the epithelial cells and the capsule stop elongation, and initiate the next phase of terminal differentiation that consists of degradation of cellular organelles and nuclei (Bassnett, 2002). The degradation of mitochondria, and ER is a rapid process whereas the degradation of the nuclei is thought to take a longer time and persists slightly deeper into the lens (Bassnett and Beebe, 1992). The terminal degradation of all internal organelles and nuclei shares common elements with apoptosis (Dahm, 1999). The process removes not only the light scattering organelles from the fibre cells, but also their ability to synthesise new proteins (Ozaki et al., 1985).

1.4. Lens physiology

The metabolic demand imposed on the lens to efficiently transport nutrients and remove waste from deeper lying cellular layers in the absence of an inherent blood supply have been a focus of extensive research to identify mechanisms for transportation of molecules, ions and fluid into and out of the lens. In this regard, Mathias et al. (Mathias et al., 1997, Mathias et al., 2007) have proposed the internal microcirculation model, in which ion fluxes directed through distinct extracellular and intracellular pathways generate an internal fluid circulation that delivers nutrients to and removes wastes from the lens core (Figure 1-8A). While not everyone agrees with this model (Beebe and Truscott, 2010), evidence in its favour is accumulating (Gao et al., 2000, Candia et al., 2012, Vaghefi et al., 2012, Gao et al., 2013)

The internal microcirculation model is based on the generation of a circulating flux of Na\textsuperscript{+} that enters the lens via the extracellular space between adjacent fibre cells. The Na\textsuperscript{+} eventually crosses the fibre cell membranes and then flows back to the surface of the lens via an intracellular pathway mediated by gap junction channels (Figure 1-8B). Because gap junctions in the outer cortex of the lens are primarily localised at the equator, the intracellular exit pathway is directed towards equatorial epithelial cells, where the highest densities of Na\textsuperscript{+}/K\textsuperscript{+} pumps (relative to the poles) are located, to complete the circulation of Na\textsuperscript{+} by actively transporting Na\textsuperscript{+} out of the lens (Gao et al., 2000, Tamiya et al., 2003). Thus at the equator, the Na\textsuperscript{+} current that is leaving the lens is highly concentrated causing the net current to be outward in this region of the lens. The generating power of the electromotive Na\textsuperscript{+} current is established and maintained by the expression of Na\textsuperscript{+}/K\textsuperscript{+} pumps localised in the epithelium and newly formed differentiating fibre cells. Deeper fibre cells lack functional Na\textsuperscript{+}/K\textsuperscript{+} pumps and K\textsuperscript{+} channels and a negative membrane potential is maintained in these
deeper cells by virtue of their coupling to the surface cells via gap junctions. This electrical connection together with the different expression of membrane proteins of the superficial and deeper fibre cells causes the standing current to flow. In this model, the circulating current generates an isotonic fluid flow that is also directed into the lens via the extracellular space and exits via an intracellular pathway. This extracellular flow of water convects nutrients to the deeper lying fibre cells, whereas the intracellular outflow removes waste, transforming the lens into a well stirred compartment. Thus, the electrochemical gradient of Na\(^+\) ions imposed by the surface cells is not only responsible for the transportation of nutrients, but also sets the negative membrane potential necessary to maintain the steady state volume of the inner fibre cells (Mathias et al., 2007, Donaldson et al., 2009).

The inclusion of water flow into the circulation model dramatically altered the model and led to the prediction that a large intracellular hydrostatic pressure gradient will be generated by the flow of water through gap junction channels (Figure 1-8C). This hydrostatic pressure gradient has been measured and is thought to drive intracellular flow of fluid from the lens core to the periphery (Gao et al., 2013). This hydrostatic pressure gradient of 0 mmHg in the periphery to 335 mmHg in the lens centre is remarkably preserved in several different species. This conservation of the pressure gradient among species has led to the suggestion that the gradient is actively modulated and controls the water content in the lens core and therefore sets the water/protein ratio that determines the gradient of refractive index that in turn contributes to the optical properties of the lens (Gao et al., 2013).

To achieve isotonic fluid flow, the membrane must be permeable to water and this water permeability is known to be mediated by the aquaporin (AQP) family of water channels. In the lens, it has been traditionally thought that water permeability in epithelial and fibre cells was mediated by AQP1 and AQP0, respectively (Varadaraj et al., 1999). However, recent research has shown that a third water channel, AQP5 is also expressed in the lens. In the remainder of this section, I will first give a general introduction to the AQP family of water channels before focussing on the AQPs expressed in the lens.
Figure 1-8: Microcirculation model of ion and water movement in the lens. (A) A net flux of Na⁺ and fluid enters the lens at both poles and exits at the equator to generate an internal micro-circulatory system that delivers nutrients to, and removes waste products from the avascular lens faster than would be achieved by passive diffusion alone (Mathias et al., 2007). (B) A more detailed view of Na⁺ and K⁺ fluxes. Na⁺ flows into the lens along the extracellular spaces between cells, and crosses fibre cells membranes by diffusing down its electrochemical gradient. Na⁺ then flows back to the lens surface via an intracellular pathway mediated by gap junctions, which direct Na⁺ to the equator where the Na⁺/K⁺ ATPase transports it out of the lens to complete the circulation. (C) It has been proposed that water fluxes in the lens are driven by the Na⁺ flux. Water enters each fibre cell through AQP0 water channels before flowing back to the surface via gap junctions and leaving the lens through AQP1 channels located in the epithelium. The movement of water through gap junctions generates a hydrostatic pressure gradient (\( p_i \) mmHg) that helps to drive the water from the core to the surface. The Journal of General Physiology, 137:507-520, doi:10.1085/jgp.201010538, Lens Intracellular Hydrostatic Pressure is Generated by the Circulation of Sodium and Modulated by Gap Junction Coupling, Gao G, Sun X, Moore LC, White TW, Brink PR, Mathias RT, Figure 1, original copyright notice is given to the publication in which the material was originally published, with kind permission from the Rockefeller University Press, (Gao et al., 2013).
1.5. The Aquaporin family of water channels

While water had long been known to diffuse through lipid bilayers, the observation that red blood cells had a basal water permeability which was much higher than that observed for artificial lipid bilayers, suggested that cells also contained an additional pathway for water diffusion that was mediated by some form of water channel (Borgnia et al., 1999). Furthermore, the low activation energy of water diffusion in red blood cells, plus its reversible blockage by mercury compounds indicated that this water channel was probably formed by an unknown membrane protein. A subsequent serendipitous finding suggested that CHIP 28, a 28kDa polypeptide isolated from red blood cells, might have been the sought after water channel. This was confirmed by a series of experiments, (Preston et al., 1992, Zeidel et al., 1992) for which Peter Agre was awarded a Nobel Prize in Chemistry in 2003. It is now known that the aquaporin (AQP) superfamily mediates the rapid diffusion of water across cell membranes which can be regulated independently of solute transport. Subsequent cloning of the amino acid sequence of CHIP 28 (renamed AQP1) revealed that it was related to a functionally undefined family of putative membrane channel proteins that included the Major Intrinsic Protein (MIP26) of the lens. Since the discovery of AQP1 some 23 years ago the structure and function of AQPs have been extensively studied (King et al., 2004).

Members of the AQP family share a common membrane topology consisting of six transmembrane $\alpha$-helices encoded by two tandem sequence repeats (Figure 1-9). The pore is formed by the association of two short loops B and E that contain the signature NPA motif, which fold inwardly from opposite sides of the bilayer to form a constricted water selectivity filter that resembles an hourglass structure (Figure 1-10A). This so-called hourglass model was subsequently confirmed by cryo-electron microscopy of AQP1 from human red blood cells (Murata et al., 2000) and X-ray studies of the crystal structure of bovine AQP1 and the glycerol transporter, GlpF (Sui et al., 2001). From these studies, we know that four aquaporin monomers associate as a tetramer in the membrane and that each subunit contains a functional water-selective channel (Figure 1-10B). Structural studies have also shown that the outside face of the tetramer is hydrophobic, whereas the internal portion facing towards the centre is hydrophilic which provides an explanation for the apparent requirement for tetramer formation (Smith and Agre, 1991).
The primary amino acid topology of AQP1 is depicted showing six transmembrane alpha helices (1 to 6) formed by two tandem/sequence repeats (grey). The short extracellular loop E and intracellular loop B are folded to form two hemipores connecting the second and third alpha helices at the signature Asp-Pro-Ala (NPA) motif. The N terminal and C-terminal domains are both situated in the intracellular side of the membrane so the repeats are orientated at 180° to each other. Sites of N-linked glycan, surface polymorphism and sensitivity to Hg are represented. Annual Review of Biochemistry, 68, 1999, 425-458, Cellular and Molecular Biology of the Aquaporin Water Channels, Borgnia M, Nielsen S, Engel A, Agre P. Figure 2, original copyright notice is given to the publication in which the material was originally published, with kind permission from Annual Reviews, (Borgnia et al., 1999).
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Figure 1-10: Three dimensional structure of AQP1. (A) The structure of the 28-kDa AQP1 monomer is shown, with membrane-spanning helices numbered 1–6 displayed as rods. The N-terminal half of the molecule is shown in purple and light blue, and the C-terminal half is shown in red and pink. Loops B and E, which fold into the membrane to form the pore are labelled, as are the conserved NPA motifs (shown in light grey). Portions of loops B and E form helices, and are therefore also shown as rods. The arrow highlights the route taken by water, which can move in both directions through the channel. (B) The AQP1 tetramer, as seen from above. Asterisks denote the location of the water pore in each subunit. Nature Reviews Molecular and Cell Biology, 5, 2004, 687-98, From Structure to Disease: the Evolving Tale of Aquaporin Biology, King LS, Kozono D, Agre P, Figure 1, original copyright notice is given to the publication in which the material was originally published, with kind permission from Nature Publishing Group (King et al., 2004).

Structural studies have also revealed the molecular basis for exclusive water selectivity of the AQP1s that results in the exclusion of other small solutes and ions, including the hydronium (H$_3$O$^+$) ion. Two principal mechanisms are involved (de Groot and Grubmüller, 2001, Kozono et al., 2002, Tajkhorshid et al., 2002). Firstly, the channel narrows to a diameter of 2.8Å, some 8Å above the centre of the bilayer, to create a physical limitation on the size of the molecules that can enter the channel. Highly conserved histidine and arginine residues in this region are important to the determination of the pore diameter. Substitution of the histidine residue in GlpF to a glycine residue results in a widening of the pore by approximately 1Å and allows larger polyols such as glycerol to permeate the channel, while the arginine residue bears a positive charge and serves to repel protonated water (H$_3$O$^+$). The second determinant of AQP selectivity involves the two hemipores that contain the highly conserved NPA motif. The partial charges of the asparagine residues in this region of the loops serve as a reorientation point of the traversing water molecules. The resultant dipole re-
orientation of the water molecules disrupts hydrogen bond formation with water molecules situated above and below the NPA motif serving as a barrier to proton conductance.

1.5.1. Aquaporin functional diversity

Thirteen AQP isoforms have been identified in humans and ongoing research has shown that differences in water permeability, transcriptional regulation, post-translational modification, protein stability and polarized distribution between the different AQP isoforms accounts for the functional differences in water permeability observed at the cellular level in different tissues. The AQP family of water channels in humans has been divided into two groups based on the relative sequence homologies of AQP isoforms to the water channel, AQPZ, and the glycerol transporter, GlpF, which are both expressed in the bacterium \textit{E. coli} (Figure 1-11). The first group consists of the “classical” aquaporins: AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8; that are deemed to only mediate water diffusion. However within this group, AQP6 also mediates ion permeation (Yasui et al., 1999) and AQP8 is thought to mediate both water and urea (Ishibashi et al., 1997). Their inclusion in this group is therefore based more on sequence homology to AQPZ than on functionality. More recently, AQP11 and AQP12 were also added to this group, but they are thought to be expressed in intracellular membranes in a similar fashion to AQP6 (Morishita et al., 2005, Ohta et al., 2009). Other members of the AQP superfamily have been placed into a second group called the aquaglyceroporins and have high homology to GlpF. This group includes AQP3, AQP7, AQP9 and AQP10 and these AQPs exhibit not only variable permeability to water, but also permeability to glycerol and other small solutes.
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Figure 1-11: The aquaporin family of proteins. This phylogenetic tree shows the relationship of the eleven human aquaporins (AQP0–AQP10) to the aquaporin homologues AqpZ and GlpF found in *Escherichia coli*. The assignments in the phylogenetic tree roughly correlate with permeability characteristics in that the aquaporins are generally permeated only by water, and the aquaglyceroporins are permeated by water and small solutes such as glycerol. The scale bar represents evolutionary distance: 0.1 equals 10 substitutions per 100 amino-acid residues (including reversions). So, the evolutionary distance, in terms of amino-acid substitutions, between two proteins is equal to the total distance along the path. The recently discovered AQP12 is not included in this tree. *Nature Reviews Molecular and Cell Biology*, 5, 2004, 687-98, From Structure to Disease: the Evolving Tale of Aquaporin Biology, King LS, Kozono D, Agre P, Figure 1, original copyright notice is given to the publication in which the material was originally published, with kind permission from Nature Publishing Group, (King et al., 2004).

While the different members of the aquaporin superfamily all contain similar structural motifs, the individual isoforms also possess unique structural features and exhibit differential expression patterns that allow cells to dynamically regulate their water permeability during different stages of development and differentiation. This regulation can occur at multiple levels (Table 1). AQPs resident in the plasma membrane can change their activity or gating in response to alterations in pH or Ca$^{2+}$ binding to modulate water permeability, while larger changes in water permeability can be induced by the shuttling of AQPs to and from the plasma membrane. An extensively studied example of this mechanism is the regulation of AQP2 expression in the cortical collecting duct cells in the kidney that involves the shuttling of AQP2 from a pool of intracellular vesicles to the apical membranes following vasopressin stimulation (Wade et al., 1981, Brown, 2003).
Table 1: Summary of mechanisms that regulate AQP mediated changes in permeability of membranes to water.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>AQP isoform</th>
<th>Activity/expression level</th>
<th>Signal</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gating</td>
<td>AQP0</td>
<td>Increased activity</td>
<td>Low pH or low Ca(^{2+})</td>
<td>Oocyte</td>
</tr>
<tr>
<td></td>
<td>AQP0</td>
<td>Reduced activity, pore closed</td>
<td>Location in junction plaques and single membranes</td>
<td>Lens fibre cells</td>
</tr>
<tr>
<td></td>
<td>AQP3</td>
<td>Reduced activity</td>
<td>Low pH</td>
<td>Oocyte</td>
</tr>
<tr>
<td></td>
<td>AQP3</td>
<td>Reduced activity</td>
<td>Low pH, nickel</td>
<td>Cultured lung epithelial cells</td>
</tr>
<tr>
<td></td>
<td>AQP4</td>
<td>Reduced activity</td>
<td>Activation by protein kinase C (PKC)</td>
<td>Oocytes, renal epithelial cells</td>
</tr>
<tr>
<td></td>
<td>AQP4</td>
<td>No effect</td>
<td>Phosphorylation by PKC</td>
<td>Proteoliposomes with reconstituted AQP4</td>
</tr>
<tr>
<td></td>
<td>AQP6</td>
<td>PH(_2)O/ion activation</td>
<td>Low pH, nitrate</td>
<td>Oocytes, cultured cells</td>
</tr>
<tr>
<td></td>
<td>AQP1</td>
<td>P(_i)on Increased activity</td>
<td>cGMP</td>
<td>Oocytes, undefined importance</td>
</tr>
<tr>
<td>Subcellular redeployment</td>
<td>AQP2</td>
<td>Insertion into the membranes</td>
<td>cAMP dependant vesicular trafficking</td>
<td>Kidney proximal cells</td>
</tr>
<tr>
<td></td>
<td>AQP5</td>
<td>Increased expression on mRNA and protein level</td>
<td>Hypertonic induction</td>
<td>Mouse lung epithelial cells (MLE-15)</td>
</tr>
<tr>
<td>Transcription/Protein level expression</td>
<td>AQP5</td>
<td>Reduce expression</td>
<td>Tumour necrosis factor-α</td>
<td>Cultured rat lung epithelial cells</td>
</tr>
<tr>
<td>Post-translational modification</td>
<td>AQP5</td>
<td>Increased abundance</td>
<td>cAMP</td>
<td>Cornea</td>
</tr>
<tr>
<td></td>
<td>AQP0</td>
<td>Increased activity</td>
<td>C-terminal phosphorylation</td>
<td>Lens fibre cells</td>
</tr>
<tr>
<td></td>
<td>AQP0</td>
<td>Unknown</td>
<td>C-terminal deamidation/ glycation</td>
<td>Lens fibre cells</td>
</tr>
<tr>
<td>Differential expression during development</td>
<td>AQP1</td>
<td>Low expression</td>
<td>Embryogenesis, shortly before birth</td>
<td>Lungs</td>
</tr>
<tr>
<td></td>
<td>AQP1</td>
<td>Increased expression</td>
<td>Postnatal development</td>
<td>Lungs</td>
</tr>
<tr>
<td></td>
<td>AQP3, 4 and 5</td>
<td>Increased expression</td>
<td>Postnatal development</td>
<td>Lungs</td>
</tr>
<tr>
<td></td>
<td>AQP1</td>
<td>Increased expression</td>
<td>Ubiquitilation</td>
<td>Lungs</td>
</tr>
<tr>
<td></td>
<td>AQP5</td>
<td>Insertion into membranes</td>
<td>Development and growth</td>
<td>Lens fibre cells</td>
</tr>
<tr>
<td></td>
<td>AQP2</td>
<td>Insertion into membranes</td>
<td>Hypertonic exposure</td>
<td>Kidney epithelial cells (MDCK)</td>
</tr>
</tbody>
</table>

This table was summarized from (Borgnia et al., 1999, King et al., 2004).
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Changes to the expression pattern of AQPs during embryological and postnatal development are also apparent that are regulated at both the transcriptional and posttranslational levels. This cellular specific expression of multiple AQP isoforms is apparent in a variety of tissues including the eye (Figure 1-12) (King et al., 2004, Verkman et al., 2008). In the adult cornea, AQP1 has been localised to the endothelium, while AQP3 and AQP5 are differentially expressed in the epithelium. AQP3 is expressed in the conjunctival epithelium, while in the ciliary epithelium, AQP1 and AQP4 are expressed. In the multi-cellular retina, AQP1 has been localised to retinal pigment epithelial cells, photoreceptors, and amacrine cells, while Müller cells have been shown to express both AQP1 and AQP5; and finally AQP0 has been detected at low levels in rat and mouse retinal bipolar cells. For the rest of this section, I will focus specifically on the properties of the AQPs expressed in the lens.
Figure 1-12: Differential expression of aquaporins in the eye. Traditionally AQP0 is thought to be present in the fibre cells of the lens. AQP1 is present in scleral fibroblasts (not shown), in the epithelial cells that cover the lens, and in the endothelial cells that line the blood vessels at the back of the cornea. AQP1 is also present in the epithelium of the anterior ciliary body and in the trabecular meshwork and canals of Schlemm, which all contribute to aqueous humor formation and removal from the anterior chamber of the eye. The conjunctival epithelium that covers the outer margins of the eye contains AQP3. AQP4 is present in the glial and Müller cells of the retina, as well as in the epithelium of the anterior ciliary body. AQP5 is present in the secretory cells of the tear-forming lacrimal glands (not shown), and also in the apical membrane of corneal epithelial cells. Nature Reviews Molecular and Cell Biology, 5, 2004, 687-98, From Structure to Disease: the Evolving Tale of Aquaporin Biology, King LS, Kozono D, Agre P, Figure 1, original copyright notice is given to the publication in which the material was originally published, with kind permission from Nature Publishing Group, (King et al., 2004).
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1.6. Aquaporins in the lens

Until recently it was believed that the lens only expressed two AQP5s: AQP0 in the fibre cells and AQP1 in the epithelium (Mathias et al., 2007). However more recently a number of genomic and proteomic studies (Wistow et al., 2002, Wang et al., 2008, Bassnett et al., 2009) have suggested that AQP5 is also expressed in the lens raising the possibility that the lens expresses a third water channel. In the remainder of this section, I will compare the properties of AQP0, AQP1 and AQP5, and review our current understanding of the roles they play in the lens.

1.6.1. AQP0 expression in the lens

Originally identified as MIP26, and subsequently renamed as AQP0 following the identification of the aquaporin family of water channels, AQP0 is almost exclusively expressed in the lens and has only recently been found to also be expressed in the retina. AQP0 is an enigmatic molecule which has been the centre of over 30 years of intense investigation to discover what role it plays in the lens. After a number of false starts, these studies have revealed that AQP0 may in fact have multiple functional roles in the lens. AQP0 is a 28kDa integral membrane protein which constitutes over 60% of the total membrane protein content of fibre cells (Bok et al., 1982). Early efforts to characterise the role of MIP26/AQP0 that utilised purified proteo-liposomes reconstituted into planar lipid bilayers indicated that the protein formed a large conductance ion channel with a weak selectivity for anions over cations, a finding that led investigators to believe that MIP26/AQP0 was in fact a gap junction protein. However the subsequent discovery of the connexin family of proteins and the identification of Cx46 and Cx50 (Paul et al., 1991, Yang and Louis, 1996) in the lens, plus the failure of electrophysiological experiments to detect electrical coupling in paired oocytes expressing AQP0 (Swenson et al., 1989) refuted the suggestion that MIP26/AQP0 acted as a gap junction protein. Instead, based on the localisation of MIP26/AQP0 to square arrays in thin and wavy junctions, it was proposed that the protein served as a junctional protein in the lens (Zampighi et al., 1989). However, the eventual identification of MIP26 as a member of the aquaporin family prompted researchers to test its water permeability to determine if MIP26 functions as a water channel in the lens. The subsequent exogenous expression of AQP0 in oocytes did indeed confirm AQP0 as being a weak water channel with a water permeability 30-fold lower than AQP1 and approximately 20-fold lower than AQP5 (Kushmerick et al., 1995, Mulders et al., 1995, Chandy et al., 1997, Yang and Verkman, 1997). Unlike AQP1 and AQP5, AQP0 is not sensitive to mercury compounds.
(Mulders et al., 1995). In other AQPs, water permeability can be blocked with mercury compounds due to the presence of a cysteine residue contained within the pore which is sensitive to mercurial inhibition (Krane et al., 2001, Savage and Stroud, 2007). Interestingly, AQP0 does not contain an equivalent cysteine residue in its pore, which is the structural difference that explains its lack of inhibition by mercury (Mulders et al., 1995).

Confirmation that AQP0 forms water channels in the lens was obtained from experiments performed on native membrane vesicles spontaneously formed from lens fibre cells isolated from either a normal mouse or a mouse with the cataract Fraser mutation (CatFr) (Varadaraj et al., 1999). The water permeability of CatFr/- membrane vesicles dropped to about half of the control vesicles due to lack of transportation of mutated AQP0 protein to the cellular membrane. Further studies have elucidated the sites of regulation of AQP0 water permeability. For example, the water permeability of AQP0 can be increased 2-fold by incubating vesicles in high Ca\(^{2+}\) or low pH (Varadaraj et al., 2005). The sensitivity to external pH is confined to a histidine residue (His 40) localised in the first extracellular loop, whereas sensitivity to Ca\(^{2+}\) is determined by the interaction of the C-terminal domain of AQP0 with calmodulin. Combining high Ca\(^{2+}\) and low pH did not have a cumulative effect on water permeability, suggesting that the two regulatory sites worked through a common mechanism, either increasing the open probability of the water channel or the open-channel permeability (Varadaraj et al., 2005). These findings were generally consistent with results obtained after using exogenously expressed bovine AQP0 in oocytes to study the regulation of AQP0 water permeability (Németh-Cahalan and Hall, 2000). However, the effect of elevated Ca\(^{2+}\) had opposite effects in the two experimental systems. That is, in oocytes, elevation of Ca\(^{2+}\) in the medium resulted in a decrease of membrane water permeability. This difference is thought to be due to both the different membrane composition of oocytes and lens fibre cell vesicles, and the differences in the affinity of apo-calmodulin and Ca\(^{2+}\)-calmodulin to the C-terminus domain of AQP0 in the native (lens) and exogenous (oocytes) systems.

As the lens grows and ages, AQP0 undergoes differentiation dependent post-translational modification to its C-terminal domain such as phosphorylation, truncation, glycation and deamidation (Ball et al., 2004). While the effect of glycation and deamidation on AQP0 water permeability is unclear, the most abundant modifications, phosphorylation and truncation, are thought to alter the regulation of its water permeability. Several sites of phosphorylation have been identified (Rose et al., 2008), while extensive C-terminal truncation in the core of the
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lens has been well characterised (Wang et al., 2008, Grey et al., 2009). Phosphorylation of AQP0 at Ser235, the most abundant phosphorylation site, is via PKA and is mediated by AKAP2 (Gold et al, 2011). The phosphorylation of the C-terminal tail has been shown to disrupt calmodulin binding (Rose et al., 2008) and therefore change the regulation of AQP0 activity in the presence of elevated Ca^{2+}.

It has been also shown that AQP0 exists in two forms/configurations: a pore in the periphery and adhesion protein in deeper cellular layers (Gonen et al., 2004, Harries et al., 2004). Electron crystallography data suggest that AQP0 acts as an adhesion molecule due to its identification in 11-13nm “thin lens junctions” (Gonen et al., 2005). Evidence for the dual function of AQP0 in the lens is compelling. This dual role of AQP0 is exemplified in zebrafish in which an evolutionary duplication of the AQP0 gene resulted in sub-functionalisation into two isoforms: AQP0a functions as a water channel while AQP0b is not a water channel but has a potential adhesive role, although this has yet to be confirmed. Deletion of either isoform, results in the formation of cataract, demonstrating that both isoforms and therefore both functions are required for the normal development and transparency of the lens (Froger et al., 2008, Froger et al., 2010, Clemens et al., 2012). Mutations in AQP0 are manifested in development of cataract in humans and mice (Shiels et al., 2001), since mutated AQP0 molecules were not functional due to lack of transportation to the plasma membrane. The phenotypic abnormality may result from disruption of the structural connections between fibre cells rather than a loss of water permeability function. This is further supported by studies on AQP0 knock mice where replacement of AQP0 with a better water channel, AQP1, did not restore lens morphology and transparency suggesting that AQP0 has additional functionality in the lens (Varadaraj et al., 2010). AQP1 replacement also failed in zebrafish lens (Clemens et al., 2013).

1.6.2. AQP1 expression in the lens

AQP1 (Agre et al., 1993) is abundantly expressed in a variety of tissues, and in the eye it is found in the corneal endothelium and lens epithelium (Bondy et al., 1993). During development, AQP1 is firstly expressed in anteriorly positioned epithelial cells at E17 in the mouse lens. AQP1 expression progressively increases during postnatal development which coincides with an increase in the size of the lens (Varadaraj et al., 2007). This increased expression of AQP1 translates into an increase in the mercury sensitive water permeability of
isolated lens epithelial cells (Chandy et al., 1997, Varadaraj et al., 2007). Deletion of AQP1 in the lens epithelium resulted in an approximately threefold reduction of the water permeability of AQP1 null mice lenses (Ruiz-Ederra and Verkman, 2006). Although lack of AQP1 expression did not affect lens morphology and transparency, it emerged that incubation of lenses from AQP1 knockout mice in high glucose solutions resulted in accelerated loss of lens transparency relative to that seen in wild type lenses. Therefore, AQP1 is required to maintain the transparency of the lens, especially following exposure to stress conditions such as hyperglycaemia and osmotic imbalance. Finally, as outlined above the swapping of AQP1 for AQP0 was unable to rescue cataract formation from an AQP0 knockout mouse despite the similarities between the structure and function of the two AQPs (Varadaraj et al., 2010), reinforcing the point that the differential expression of the two functionally distinct AQP isoforms is critical for overall lens function.

### 1.6.3. AQP5 expression in the lens

Indications that the lens may express a third water channel surfaced when AQP5mRNA was detected in rat lenses (Patil et al., 1997) and in adult human lenses (Wistow et al., 2002). Later proteomic approaches identified AQP5 at the protein level in fibre cells from both the mouse and bovine lens (Wang et al., 2008, Bassnett et al., 2009). In the time since I began my PhD studies, the expression of AQP5 in the adult lens was subsequently confirmed not only in our laboratory (Grey et al., 2013), but also by other researchers (Kumari et al., 2012). Using Western blotting and mass spectrometry, our laboratory showed that not only is AQP5 expressed in lenses of several species, but also that AQP5 is expressed throughout all regions of the mouse, rat, bovine and human lenses. Interestingly, immunomapping of the distribution of AQP5 across the lens (Grey et al., 2013), showed that there was a differentiation dependent change in the sub-cellular localisation of AQP5 from a cytoplasmic to membraneous labelling pattern at discrete stages of fibre cell differentiation. The labelling pattern observed for AQP5 was very different to that observed for AQP0 (Grey et al., 2009) and raises questions about the relative functional roles of the two AQPs in the different regions of the lens, especially since AQP5 has a ~20 fold higher water permeability than AQP0 (Yang and Verkman, 1997).

Due to the recent identification of AQP5 expression in the lens, its function and regulation has yet to be extensively studied. However initial characterisation of lenses from AQP5 knockout animals have recently been reported (Kumari and Varadaraj, 2013). In this study, it was found that in comparison to wild type lenses, organ cultured knockout lenses developed
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cataract after 60 hours incubation in high glucose (55.6mM) media. In addition, the wet weight of the hyperglycaemia treated KO lenses increased and there was an observable release of cellular contents in the incubation media. Although these observations corroborated a functional contribution of AQP5 in maintaining the transparency of lenses exposed to hyperglycaemia, there were no experiments conducted to measure the actual water permeability of AQP5 in fibre cells.
1.7. Thesis objectives

The goal of my thesis was to build on preliminary studies conducted in our laboratory that verified the expression of AQP5 in the lens in an effort to understand how AQP0 and AQP5 contribute to fibre cell function in different regions in the lens, and how the relative contributions of the two proteins may change as a function of lens development and growth. To achieve this I have used immunohistochemistry to map and systematically compare the cellular and subcellular distribution of AQP5 and AQP0 across the adult and developing lens (Chapters 3-4) and have in parallel developed a functional assay to measure the water permeability of epithelial cells and fibre cell membrane vesicles isolated from the outer cortex of the rat and mouse lens (Chapters 5-6).

The specific objectives of each chapter of this project are outlined below:

Chapter 3: Identification and localisation of AQP5 in the adult lens

The primary aim of this chapter was:

- To obtain a high resolution map of the subcellular distribution of AQP5 throughout the adult lens that can be compared the previous maps generated for AQP0 distribution.

These experiments identified a cytoplasmic pool of AQP5 water channels that translocated to the plasma membrane in a differentiation and species-specific manner and led to a secondary aim:

- To identify stimuli that could induce the dynamic insertion of AQP5 from the cytoplasmic pool to the plasma membrane of differentiating fibre cells

Chapter 4: Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

Knowledge of the spatially distinct subcellular distribution patterns of AQP5 and AQP0 in the adult lens prompted identification of how the adult pattern of expression is set up during the embryonic and postnatal development in the mouse lens with the aim to:

- Identify the specific stages during embryonic and postnatal development when the subcellular distribution AQP5 and AQP0 observed in the adult are first established, and when does post translational truncation of the C-terminal tail of AQP0 first occur.
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The results from these immunohistochemical studies allowed me to formulate a series of hypotheses on the contributions of AQP5 and AQP0 to fibre cell function in different regions of the lens.

Chapter 5: Development and optimisation of a fluorescence-based assay to measure water permeability in lens cells

To test these hypotheses functional assays that can quantify the relative contributions of AQP0 and AQP5 to water permeability in the different regions of the lens are required. The aim of this chapter was therefore:

- To develop and optimise a fluorescence dye dilution assay to measure the water permeability of epithelial cells and fibre cell membrane vesicles isolated from the outer cortex of the lens

Chapter 6: Water permeability of lens cells

Using this assay water permeability measurements were then made on epithelial cells and fibre cell membrane vesicles in the absence and presence of mercury compounds with the aim to:

- Compare the relative water permeability’s of epithelial and fibre cells derived from rat and mouse lenses
- Determine whether the Hg$^{2+}$-sensitive AQP5 water channel contributes to the water permeability of fibre cell membrane vesicles derived from the outer cortex of either the rat or mouse lens.

This approach has provided invaluable new information on the global expression of AQP5 and AQP0 in different regions of the lens during the continuum that is lens embryonic development and postnatal growth and has highlighted the need to consider the contributions that both AQP5 and AQP0 make to the maintenance of the homeostasis and therefore transparency of the lens which are discussed in the final chapter of this thesis.
Chapter 2. Materials and Methods

2.1. Introduction to general laboratory procedures

All experimental procedures presented in this chapter represent the final optimised protocols developed during the course of this thesis. Details on the optimisation of a specific protocol, if relevant to the interpretation of the subsequent data, are given in the associated results chapter.

All experimental procedures were performed with extreme care to maximise safety and to minimise any contamination. Laboratory coats and disposable gloves were worn at all times, and gloves were changed frequently to preserve sterility. Work space and equipment were regularly cleaned with 70% ethanol. In addition, glassware and plastic materials were autoclaved, where appropriate.

The handling and use of animals for experimental procedures were performed in accordance with the University of Auckland Animal Ethics Committee (R867 and R1303) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
2.2. General buffers and solutions

Phosphate buffered saline (PBS) 2.7mM KCl, 10mM phosphate buffer, 137mM NaCl in distilled water, pH 7.4 (Phosphate saline tablets, Sigma-Aldrich)

Artificial Aqueous Humour (AAH) 125mM NaCl, 0.5mM MgCl₂, 4.5mM KCl, 10mM NaHCO₃, 2mM CaCl₂, 5mM glucose, 10mM sucrose, 10mM HEPES, pH 7.4, 300mOsmol/L

Osmotically modified AAH solutions contain the same chemicals as Isotonic AAH with varying concentrations of NaCl as follows:
270mOsmol/L Hypotonic AAH (-10%) 109mM NaCl
290mOsmol/L Hypotonic AAH (-3%) 119mM NaCl
310mOsmol/L Hypertonic AAH (+3%) 129mM NaCl
405mOsmol/L Hypertonic AAH (+30%) 176mM NaCl

300mOsmol/L Isotonic saline 139mM NaCl, 4.7mM KCl, 1mM MgCl₂, 5mM glucose, 5mM HEPES pH 7.4

The anisosmotic saline solutions below were made by the addition or removal of NaCl from isotonic saline:
390mOsmol/L Hypertonic saline (+30%) 184mM NaCl, 4.7mM KCl, 1mM MgCl₂, 5mM glucose, 5mM HEPES pH 7.4
210mOsmol/L Hypotonic saline (-30%) 94mM NaCl, 4.7mM KCl, 1mM MgCl₂, 5mM glucose, 5mM HEPES pH 7.4
260mOsmol/L Hypotonic saline (-13%) 119mM NaCl, 4.7mM KCl, 1mM MgCl₂, 5mM glucose, 5mM HEPES pH 7.4

Physiological saline containing Ca²⁺ 150mM NaCl, 4.7mM KCl, 1mM MgCl₂, 5mM glucose, 5mM HEPES pH 7.4, 5mM CaCl₂

0.01M Citrate buffer, pH 6 Tri-sodium citrate (dehydrate) 2.94g to 1L dH₂O, adjust pH to 6.0 with 1N HCl

0.75% Paraformaldehyde 0.75% w/v paraformaldehyde in 1x PBS, pH 7.0–7.5 (adjusted with 2mM NaOH)

50% Sucrose stock solution 50% w/v sucrose in 1x PBS
Blocking solution 3% w/v bovine serum albumin (BSA), 3% v/v normal goat serum (NGS) in 1x PBS

Collagenase dissociation buffer 150mM Na-glucuronate, 4.7mM KCl, 5mM Glucose, 5mM HEPES, pH 7.4, 0.125% Collagenase (Type IV, Sigma Aldrich, Cat. # C5138)

All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

2.3. Animals

For this study 21-23 day-old Wistar rats, young (postnatal day P3, P6, P15, P22) and adult (6-8 week-old and 3 to 8 month-old) C57BL/6 mice were supplied by the Vernon Jansen Unit (VJU), located in the Faculty of Medical and Health Sciences at the University of Auckland. Mouse embryonic tissue (E10, E11, E14, E16, E17.5 and E18.5) and postnatal day P0 was kindly gifted by Professor Frank Lovicu (The University of Sydney) and Associate Professor Robb de Jongh (The University of Melbourne). Bovine eyes were obtained from Auckland Meat Processors Ltd. (851 Great South Road, Otahuhu Auckland 1062).

2.4. Lens dissection

After CO₂ asphyxiation the eyeballs of postnatal rats or mice were immediately removed from the eye sockets using curved dissection scissors and placed in PBS pre-warmed to 37°C. Using a dissection microscope, lenses were removed from eyes in warm, sterile AAH by making three incisions on the posterior surfaces of the eye extending from the optic nerve opening to the borders of the ciliary body, and then gently pressing down on the cornea to extract the lens. To avoid damage to the ocular lens, direct contact between dissection tools and the ocular lens was minimised. Any adherent retina and ciliary body was carefully removed. Dissected lenses were either, immediately fixed and used for immunohistochemistry (Section 2.5.1), or placed in organ culture to induce osmotic stress. This was achieved by in vitro incubation in AAH containing different concentration of NaCl to vary the osmolarity of the solution ranging from 290mOsmol/L to 405mOsmol/L (Section 2.2). Lenses were transferred into individual wells of a 24-well plate containing 2ml of pre-warmed AHH of varying osmolarity and incubated at 37°C for 2 hours in a tissue culture
incubator supplied with 5% CO₂ (HERAcell 150i, Thermo Scientific INC, USA). At the end of the incubation period, lenses were immediately fixed in 0.75% paraformaldehyde and processed for immunohistochemistry (see Section 2.5.1).

Bovine lenses were dissected from the eyeball by removing the posterior chamber and vitreous body using a surgical blade and scissors. The lens remained attached to the zonules in the anterior chamber and was extracted by carefully cutting the zonules with surgical grade scissors. Dissected lenses were immediately used for experiments.

2.5. Immunohistochemistry and immunocytochemistry

2.5.1. Fixation, cryoprotection and cryosectioning of mouse and rat lenses

Mouse and rat lenses were fixed in freshly prepared 0.75% paraformaldehyde for 24 hours at room temperature (RT), following protocols developed in the Molecular Vision Laboratory (Jacobs et al., 2004a). Lenses were washed 3x10min in PBS. 10, 20 and 30% sucrose solutions for cryoprotection were prepared by diluting 50% sucrose stock solution with PBS, and fixed lenses were cryoprotected by sequential immersion in 10% and 20% sucrose solutions for one hour at room temperature (RT), followed by 30% sucrose incubation overnight at 4°C. Lenses were stored in 30% sucrose at 4°C for up to two weeks before cryosectioning.

Chucks were covered with two layers of Tissue-Tek Optimal Cutting Temperature (OCT, Sakura Finetek USA Inc.) and were pre-frozen in a -20°C freezer. Excess sucrose was removed from cryoprotected lenses with filter paper and lenses were mounted onto chucks in either an equatorial or axial orientation. Lenses were coated with OCT and snap-frozen in liquid nitrogen for 10-20s. Lenses were cryosectioned at -20°C (CM3050, Leica Microsystems, Germany) with double-edged disposable blades (S-35 blades; Feather Safety Razor Co., Japan). Equatorial sections were obtained by sectioning the lens parallel to the equator, and selecting the sections near the equator region (Figure 2-1). To achieve this, lenses were mounted with their anterior pole up and the top 600-800μm removed by cryosectioning. Sections were collected and examined under a microscope to ensure they were obtained from the lens equator. The presence of a single layered epithelium and nucleated fibre cells in the lens cortex were used as indicators of an equatorial location. To obtain axial sections, lenses were mounted so that the equator was perpendicular to the plane
of section (Figure 2-1). Again the first 600-800µm of lens was removed and then cryosections were collected that contained both anterior and posterior sutures, with visible bow regions. Sections 12-14µm thick were collected with a drop of PBS onto plain microscope slides and kept moist with PBS at all times. Cryosections were washed three times with PBS to remove excess OCT and were stored in a humidity box at 4°C to prevent drying of sections.

2.5.2. Fixation, cryoprotection and cryosectioning of bovine lenses

Bovine lenses were fixed in freshly prepared 2% paraformaldehyde and 0.01% glutaraldehyde in PBS at RT for 48 hours. After fixation the lenses were washed 3x10min with PBS to remove the fixative and cryoprotected by sequentially incubating in 10% sucrose and 20% sucrose for one hour at RT and then 30% sucrose overnight at 4°C. Cryoprotected lenses were mounted on chucks covered with layers of Tissue-Tek OCT in an equatorial orientation, embedded in Tissue-Tek OCT and snap-frozen in liquid nitrogen as described above. 12-14µm thick cryosections were collected on plain microscope slides in a drop of PBS and kept moist with PBS. Sections were washed three times with PBS to remove excess OCT and stored in a humidity box at 4°C for up to 1-2 weeks until further use.

2.5.3. Fixation of fibre cell vesicles

Rat and mouse fibre cell vesicles (see Section 2.6.2 on fibre cell preparation) were labelled to confirm the presence of AQP5 and AQP0 water channels. The vesicles were spread on poly-L-lysine coated glass slides (1% w/v in MQ water) and allowed to adhere for 30min at RT. Samples were then fixed in freshly-made ice-cold 2%PFA/0.1% Triton X-100 in PBS, pH 7.4 for 30min. The slides were then washed 3x5min in PBS following by immediate immunolabelling.
Figure 2-1: Schematic diagram showing axial and equatorial sectioning protocol. A.) Equatorial sectioning of the lens. The lower diagrams show representative sections from the lens epithelium, E1; lens sutures, E2/12; and from the equator where fibre cells are seen in cross-section, E7. (B) Axial sectioning of the lens. The lower diagrams represent sections from the fibre cell/epithelium interface, A1; from young fibre cells, A5; and through the centre of the lens, A8 (Bond et al., 1996)
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2.5.4. Fixation and sectioning of mouse embryonic tissue

Whole mouse embryos or heads were obtained from superovulated FVB/N mice (E10 and E11), or wild type C57BL/6 black mice (E14, E15, E16, E17.5, E18.5). Embryonic tissue was fixed in 10% neutral buffered formalin (RT, 2 hours) then washed three times in 70% ethanol. The tissue was then dehydrated in a series of graded ethanol washes, prior to clearing in xylene. Finally, tissue was embedded in paraffin wax, and 5μm thick paraffin sections collected using a microtome (Ultracut UCT, Leica Microsystems, Germany). Sections were deparaffinised by washing with xylene followed by a wash in 100% ethanol and then water. An antigen retrieval step, using treatment in 0.01M Citrate buffer (pH 6) in a pressure cooker for 1 hour followed by 20min in PBS, was applied prior to immunolabelling.

2.5.5. Immunolabelling

2.5.5.1. Immunolabelling of lens and embryonic tissue

To assess lens fibre cell morphology, lens cryosections and mouse embryo sections were labelled with the selective membrane marker, wheat germ agglutinin (WGA) conjugated with Alexa Fluor 594 which binds to N-acetyl-D-glucosaminyl sugar and sialic acid residues found on the surface of membrane glycoproteins and glycolipids. Cell nuclei were stained with DAPI. Antibodies (Ab) and fluorophores used for immunohistochemical labelling experiments, together with their working concentrations and commercial sources, are summarised in Table 2. A schematic diagram of AQP5 and AQP0 primary amino acid structures is provided to show the relationship of the different antibody epitopes used in this study (Figure 2-2).

For immunohistochemistry, pre-incubation with blocking solution followed by primary and secondary Ab incubations were carried out prior to labelling with WGA. Sections were initially incubated in 100µl blocking solution for one hour at RT. Primary Abs were diluted in 100µl blocking solution using optimised concentrations (Table 2) and incubated overnight at 4°C. To test the specificity of the AQP5 C-terminus Ab, it was pre-incubated in the presence of a 50-fold excess (i.e. 1µg Ab was reacted with 50µg peptide) of its antigenic (neutralising) peptide. Both primary Ab and primary Ab/antigenic peptide solutions were incubated for 24 hours at 4°C to facilitate comparison between the two conditions. Both solutions were then centrifuged at 14,000rpm in 4°C, 15min and the supernatants applied to lens tissue sections and incubated overnight at 4°C.
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Following overnight incubation, sections were washed 3x5min in PBS. Secondary Ab (goat anti-rabbit IgG conjugated with Alexa 488) was diluted to 1:200 in blocking solution containing DAPI at a 1:10,000 final concentration and applied to sections for 2 hours at RT. Following 3x5min PBS washes the sections were incubated with WGA (1:100 in PBS) for one hour at RT. Finally, sections were washed 3x5min in PBS then mounted using Vectashield hard set mounting medium (In Vitro Technologies, Cat. # H-1400). Cover-slips were placed over the sections by gently lowering them to prevent air bubble formation and then sealed with nail polish. Slides were kept in the dark at 4°C until analysed by confocal microscopy.
### Table 2: List of reagents used in immunohistochemistry.

<table>
<thead>
<tr>
<th>Ab Target</th>
<th>Supplier</th>
<th>Concentration</th>
<th>Antigen/Target</th>
<th>Ab Host</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP0 whole protein</td>
<td>Developed and gifted by Prof James Hall</td>
<td>1:100</td>
<td>AQP0 whole protein, serum</td>
<td>Rb</td>
</tr>
<tr>
<td>AQP0 229-239 C-terminus</td>
<td>Developed and gifted by Prof Kevin Schey</td>
<td>1:200</td>
<td>AQP0 10 amino acid synthetic peptide, affinity purified</td>
<td>Rb</td>
</tr>
<tr>
<td>AQP0 C-terminus</td>
<td>Alpha Diagnostic International (Cat.#AQP0-1a)</td>
<td>1:200</td>
<td>Human AQP0 247-263 (17 amino acid) C-terminus synthetic peptide, affinity purified</td>
<td>Rb</td>
</tr>
<tr>
<td>AQP5 C-terminus</td>
<td>Millipore (Cat.# AB15858)</td>
<td>1:200</td>
<td>Rat AQP5 248-265 (17 amino acid) C-terminus synthetic peptide, affinity purified</td>
<td>Rb</td>
</tr>
<tr>
<td>AQP5 C-terminus</td>
<td>MyBioSource (Cat.#MBS175122)</td>
<td>1:100</td>
<td>Mouse AQP5 (19 amino acid) C-terminus peptide affinity purified</td>
<td>Rb</td>
</tr>
<tr>
<td>Goat anti rabbit IgG Alexa Fluor 488 conjugate</td>
<td>Life Technologies (A11034)</td>
<td>1:100</td>
<td>Anti-rabbit IgG</td>
<td>Gt</td>
</tr>
<tr>
<td>WGA Alexa Fluor 594 conjugate</td>
<td>Molecular probes (W11262)</td>
<td>1:100</td>
<td>Cell membrane</td>
<td>-</td>
</tr>
<tr>
<td>DAPI</td>
<td>Sigma Aldrich (Cat.#D9542)</td>
<td>1.25mg/ml PBS stock diluted to 1:10,000 final concentration</td>
<td>Nuclei</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 2-2: Epitopes of antibodies directed against AQP0 and AQP5 proteins. (A) A schematic diagram of rat AQP0 membrane topology shows the epitope for the 229-239 Ab (yellow amino acids), the epitope for the Ab supplied by Alpha Diagnostic Int. (green amino acids), and the region containing the epitope for the whole protein Ab (blue line). (B) A schematic diagram of the putative human AQP5 membrane topology showing the epitope (green amino acids) of the Millipore C-terminus Ab. H1-H6-transmembrane α-helices.
2.5.6. Immunolabelling of mouse and rat fibre cell vesicles

For single immunolabelling of vesicles an overnight incubation with AQP5 C-terminus Ab diluted in blocking solution was applied. After the overnight incubation, slides were washed twice for 5 min in PBS and labelled with a goat anti-rabbit Alexa 594 IgG Ab diluted in blocking solution for 1 hour at RT. They were then washed twice for 5 min with PBS, and cover slipped. For double immunolabelling the same protocol was applied with an additional overnight incubation with Atto 488 conjugated AQP0 229-239 Ab (see Section 1.5.6.1) at 1:50 dilution following application of the secondary antibody. Immunolabeled vesicles were imaged using confocal microscopy.

2.5.7. Direct conjugation of primary antibodies

All primary Ab used in this study were raised in rabbit which did not allow double immunolabelling experiments to be performed. To overcome this, direct conjugation of AQP5 and AQP0 Abs to fluorophores using two commercially available conjugation kits was trialled. The two conjugation protocols are outlined below.

2.5.7.1. Lightning Link (LL) Atto 488 conjugation kit

The Atto 488 conjugation kit, Novus Biological (Cat.# 733-0010) was used to conjugate AQP0 229-239 Ab to the Atto 488 label as per the manufacturer’s instructions. Briefly, the Ab was dialysed in 10mM HEPES buffer (pH 7.4) to achieve an optimal pH range between 7.3 and 7.6. 100µl of the antibody at concentration 1mg/ml was added to 10µl of LL-modifier reagent and gently mixed. The combined Ab and LL-modifier was then added to the LL-mix by direct pipetting and then re-suspended by gentle mixing. The solution was incubated overnight at 4°C. After completion of the incubation, 10µl of LL-quencher reagent was added and incubated at RT for 30 min, after which the conjugated Ab was ready to be used without the need for additional purification steps. The conjugated Ab was stored in 0.02% Na Azide at 4°C in the short term, or frozen at -80°C for longer term storage.

2.5.7.2. Apex Alexa Fluor 594 antibody labelling kit

The kit was obtained from Invitrogen (Cat. # A10474) and used to conjugate AQP5 (Millipore) or AQP0 (ADI) C-terminus Ab’s to the fluorophore Alexa 594, as per the manufacturer’s instructions. Briefly, the Apex column was hydrated with 100µl of wash buffer and 12µl of each Ab (1mg/ml) was added. 2µl of DMSO and 18µl of labelling buffer
was added to the reactive dye and dissolved by pipetting. 15µl of reactive dye was then added to the apex column and incubated overnight at 4°C. On the next day the column was washed twice with wash buffer. 10µl of neutralising buffer was added to a clean microfuge tube. The Ab was eluted into the microfuge tube containing the neutralising buffer by applying 40µl of elution buffer. Following elution, the antibody was dialysed in PBS containing 0.05% Na Azide and stored at 4°C.

2.5.8. Confocal microscopy and image processing

Lens sections, embryonic tissue and fibre cell vesicles were visualised using either a Leica TCS SP2 Laser Scanning Confocal Microscope (Leica, Heidelberg, Germany) or an Olympus FV1000 Confocal Microscope (Olympus Corporation, Tokyo, Japan) located in the Biomedical Imaging Research Unit (BIRU) at the University of Auckland. Specific emission filters were used to distinguish secondary Ab signals, selective membrane label or cell nuclei stain. Images were obtained at a resolution of 0.19-0.39µm/pixel for low magnification images and at 0.078µm/pixel for high magnification images. Raw images obtained from separate channels were combined and adjusted to an optimal intensity colour level using Adobe Photoshop CS6.

To investigate immunolabelling over large distances in the lens, image tiling was performed. Adjacent images of each fluorophore labelling pattern that overlapped by ~20% were obtained from flat tissue sections. Individual channels were stitched together, pseudo-coloured if necessary and overlaid using Adobe Photoshop CS6 software. Gain and offset were adjusted prior to image tile collection to ensure the whole dynamic range of each fluorophore labelling pattern was captured. In this way monitoring changes in intensity and spatial distribution of Ab labelling patterns from the edge to the centre of lens sections were obtained.

2.6. Imaging changes in epithelial cell and fibre cell vesicle volume

2.6.1. Preparation and calcein AM loading of lens epithelial cells

Following dissection, lens capsules were collected and incubated in collagenase dissociation buffer for 30min at 37°C. After the first 15min of incubation, 4µM calcein AM (Invitrogen, Cat. # C3099) was added to the dissociation buffer to load cells during the remaining 15min
of the incubation. Following incubation, cells were pelleted at 150g for 3-5min using a bench top microcentrifuge, and washed twice with physiological saline without Ca²⁺. Isolated epithelial cells loaded with calcein were used immediately in the swelling assay.

2.6.2. Preparation and calcein AM loading of lens fibre cell vesicles

Following lens dissection in warm PBS, pH 7.4 the lens capsule was discarded and fibre cells were dissociated from the outer cortex with tweezers. The collected clumps of cells were then transferred to Ca²⁺-containing physiological saline and incubated for 30min at RT to induce spontaneous vesicle formation. To dye-load the vesicles, 6µM calcein AM was added to the Ca²⁺ containing physiological saline for the duration of the 30min incubation time. Vesicles were pelleted at 150g, for 3-5min and washed twice in Ca²⁺ free physiological saline, and then stored in Ca²⁺ free physiological saline until further use.

2.6.3. Perfusion system

Epithelial cells and fibre cell vesicles were placed into a custom designed recording chamber that consisted of a perfusion bath and suction reservoir. Solutions entered the chamber via a narrow 1mm diameter perfusion port and exited the bath via a narrow recessed channel to the suction reservoir. Both the width and depth of the perfusion bath was designed to match the diameter of the perfusion inlet port in order to provide both fast solution exchange and laminar flow through the perfusion bath. Epithelial cells and fibre cell vesicles were attached to the bottom of the recording chamber using Cell-Tak tissue adhesive (Cell-Tak and tissue adhesive, BD Biosciences, Cat# 354240). Briefly, the chamber was coated by premixing 2µl Cell-Tak at 1.47µg/ml with 8µl of 0.1M Sodium bicarbonate buffer, pH 8.0 and spreading it into a thin liquid film on the surface of the coverslip that formed the bottom of the chamber using a micropipette tip. The coating was left for 30min at RT or until the mixture had evaporated. The coverslip was then gently rinsed once with MQ water. 80µl of the prepared lens epithelial cells or fibre cell vesicles were then pipetted into the chamber in 4 x 20µl portions to ensure gentle deployment of the sample. Adherence of the sample took place over the following 30min at RT.

The chamber was perfused using a gravity feed perfusion system (Figure 2-3) that consisted of 1L perfusate reservoirs, to prevent loss of pressure into the head solution, connected to IV flow regulators (Wolf medical supplies, Cat. # RF2500) supplied with gravity flow
controllers which could be adjusted to fine–tune the flow rate through the chamber. A one-way stopcock positioned at the exit of the solution reservoirs was used to switch between solutions. A 4-way manifold (Warner Instrument, Cat. #64-0208, MP series) was used to deliver the solutions into a common output tubing (PE-160 1.14mm ID, 1.57mm OD, Warner instruments, Cat. #64-0755), that was connected to the recording chamber. This allowed delivery of up to 4 different solutions and application of pharmacological mercury blocking agent used to inhibit the water permeability of AQP1 and AQP5. Unused input lines were blocked with pin plugs. The common output tubing had a short length of 3cm to minimise solution exchange times.

The perfusate was removed to waste by vacuum suction using a custom-made metal suction tube with an orifice resembling the number ‘8’. It was positioned at 90° to the solution surface in the middle of the suction reservoir of the recording chamber to ensure precise control of the suction. The suction device was anchored using a magnetic clamp (Magnetic clam kit, Warner Instruments, Cat. # 64-0357).
Figure 2-3: Schematic representation of the gravity feed perfusion system. Gravity feed reservoirs (1L glass bottles) containing solutions with different osmolarity were positioned 1 meter above the perfusion chamber. Switching between solutions was achieved by manually controlling a one-way stopcock fitted to each solution line. To adjust the flow rate solutions were delivered via IV Flow regulators containing a gravity flow controller for precise flow regulation. Solution lines converged into a common 4-way manifold which allowed feeding of up to 4 different solutions. Excess amount of fluid was suctioned into an Erlenmeyer waste fluid container.
2.6.4. Real time imaging of cell volume

A Nikon Eclipse TE 300 inverted epi-fluorescence microscope equipped with a 40x CFI Plan Fluor DLL 40x NA 0.75 objective suitable for Differential Interference Contrast (DIC) and epi-fluorescence imaging was used to monitor the rate of change in volume of epithelial cells and fibre cell vesicles in real time. First DIC microscopy was used to obtain high quality contrast images from epithelial cells and fibre cell vesicles to measure their initial/resting area. Then total fluorescence from a small region of calcein AM loaded epithelial cells or fibre cell vesicles was collected every 2s to record the time course of the change in fluorescence in response to hypertonic challenge in the presence or absence of mercury. Figure 2-4 shows a schematic diagram of the light path and microscope set up used for this study. A Polychrome II (TILL Photonics) illumination system consisting of a 75W xenon arc-lamp light source, monochromator, galvanometric scanner and mirror optics was used to illuminate the sample. The monochromatic light was coupled to epi-fluorescent condensers via a 1.25x1.8mm solid quartz fibre to deliver a narrow bandwidth of evenly illuminated field at the objective. To reduce photobleaching and photo-damage of the cells the output light exposure time was limited to 200ms in duration by a computer controlled shutter. To further improve the quality of excitation light and select for longer wavelength emission light emitted from the sample a filter cube (Nikon, B-2A) consisting of excitation filter (EX450-490), dichroic mirror (DM510) and barrier filter (BA590) appropriate for excitation of calcein AM at its peak excitation was used. DIC images and light emitted from the fluorescent sample was detected with an Electron Multiplying CCD camera (Cascade: II 512B EM CCD, Photometrics, Tucson, USA), suitable for imaging of low-light applications and fast frame rates. In principle during data acquisition, the CCD array is exposed to a source light and charge accumulates in the pixels. After the defined exposure time, the accumulated signal is read out of the array, digitized, and then transferred to the host computer. Digitized data was displayed or stored via Imaging Workbench 5.2 application (Indec BioSystem, Santa Clara, CA 95054, USA) and analysed offline.
Figure 2-4: Optical path and microscope set up used for epi-fluorescence imaging. (A.) The optical pathway of output and input light traveling from the fluorescent light source to the sample and then to the detector camera. (B) Microscope setup used to collect and measure fluorescence emitted from calcein AM loaded epithelial cells and fibre cell vesicles.
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2.7. Analysis of results

2.7.1. Calculation of apparent water permeability

Apparent water permeability was calculated by analysing the time course of change in fluorescence after hyperosmotic challenge in the presence or absence of mercury, a known blocker of AQP1 and AQP5 water channels but not AQP0. Briefly the timecourse of change in fluorescence trace was fitted with an exponential fit to the first flat region using Clampfit 9.2 software (Molecular Devices, Sunnyville, CA) to extract the time constant ($\tau$), magnitude ($A$) and constant term ($C$) of the slope which was then subtracted point by point from the rest of the trace to produce bleach subtracted response trace (Figure 5-9). To the peak of the bleach subtracted timecourse of fluorescence change a simple exponential was fitted and the time constant ($\tau$) extracted (Figure 5-10) which along with the initial rate of fluorescence change, external osmotic gradient and initial resting area were fitted into a modified equation adopted from (Varadaraj et al., 1999) to calculate the apparent water permeability of epithelial cells and fibre cell vesicles expressed in arbitrary units (AU):

$$P_{H2O} = \frac{1}{S_m \frac{C_{H2O}}{C_i(0)} - C_0} \frac{dF_i(0)}{dt}$$

where, $C_{H2O} \approx 55M$ is the concentration of water, $C_i(0)=300mOsmol/L$ is the initial bathing solution equal to the initial intracellular osmolarity and $C_0=390mOsmol/L$; $S_m$ is the initial surface area determined by tracing and counting pixels from a digitized DIC image of the cell (Figure 5-9A) and the rate of change of fluorescence is determined from equation:

$$\frac{dF_i(0)}{dt} = \frac{F_2 - F_1}{\tau}$$

where $F_1$ and $F_2$ are the best fit values for the initial and final fluorescence, respectively; and $\tau$ is the time constant extracted from the rate of change of fluorescence induced by hyperosmotic challenge. For a detailed account on the measurement of water permeability of lens cells see chapter 5.

2.7.2. Statistical analysis

Mean data of experiments are given $\pm$ standard error of the mean (SEM). Statistical significance was tested with Mann-Whitney U test using GraphPad Prism software (La Jolla, California, USA). * indicates a significant difference of compared values ($P \leq 0.05$).
Chapter 3. Identification and localisation of AQP5 in the adult lens

As indicated in the introduction the current dogma that water permeability in lens fibre cells is mediated exclusively by AQP0 has been challenged by the discovery that lens fibre cells also express AQP5. In this chapter I extend work conducted in the Molecular Vision Laboratory (MVL) that used Western blotting, immunohistochemistry and mass spectrometry to verify that AQP5 is expressed in adult lenses of a variety of species. This work has subsequently been published (Grey et al., 2013) and my contribution to this study was to perform high resolution confocal imaging of the subcellular distribution of AQP5 in the mouse lens, in order to compare it to distribution patterns for AQP5 in the rat lens obtained by Ms Kerry Walker. In addition to this comparison of AQP5 distribution in rodent lenses, I have also mapped the distribution of AQP5 in larger bovine lenses. This detailed mapping of the expression of AQP5 in multiple species confirmed that AQP5 is expressed throughout all regions of the lens and revealed that in differentiating fibre cells AQP5 is initially located in a cytoplasmic pool, but with distance into the lens there is an insertion of AQP5 into fibre cell membranes. Interestingly, the relative locations of this membrane insertion varied among the three species of lens I have studied. In the mouse and bovine lenses the membrane insertion was very abrupt and was localised to the equatorial region of superficial fibre cell layers, while in the rat lens the cytoplasmic pool of AQP5 extended much deeper into the lens cortex, with membrane insertion of AQP5 occurring at the transition between differentiating and mature fibre cells. In this chapter I also show that organ culturing mouse lenses in the presence of solutions of differing osmolality can induce AQP5 translocation from the cytoplasmic pool to the plasma membrane of differentiating fibre cells. This result suggests that lenses can dynamically alter their water permeability in response to osmotic stress by inserting AQP5 into the membranes of differentiating fibre cells. However, before presenting the results of my localisation studies I will first summarise the studies performed by others in the MVL that confirmed the expression of AQP5 in lens fibre cells to help facilitate the interpretation of my immunolabelling patterns presented later in this chapter.
3.1. Verification of AQP5 expression in the lens

As indicated in the introduction, data extracted from genomic (Patil et al., 1997, Wistow et al., 2002) and proteomic (Wang et al., 2008, Bassnett et al., 2009) screens of rodent and human lenses had suggested that in addition to AQP0 and AQP1, the lens also expresses AQP5. To verify these reports members of the MVL performed Western blotting on membrane homogenates obtained from whole rat, mouse, bovine and human lenses using an AQP5 antibody raised against the C-terminus of the protein (Figure 3-1). In all species of lens a 23kDa band was labelled, which matched the size of the band obtained in the rat cornea, a tissue known to abundantly express AQP5. Interestingly, a second, higher molecular weight band was observed in the human lens which may represent a splice variant of AQP5, or a post-translational modification to the AQP5 protein. To identify where in the lens AQP5 is expressed, Western blotting was performed on fractions obtained from different regions of the lens in all four species (Figure 3-2). Lenses were micro-dissected into outer cortex, inner cortex, and core regions in the rat, bovine and human lenses. Due to the small size of mouse lenses, lenses from this species were only segregated into cortex and core regions. In all species of lens AQP5 labelling was strongest in the outer cortex (rat, bovine and human) and cortex (mouse) and was greatly diminished in the inner cortex and core fractions with no labelling being observed for AQP5 in the core of the rat lens (Figure 3-2B). Interestingly, in the human lens the doublet for AQP5 was only present in the outer cortex with only the high molecular weight band being detected in the inner cortex and core albeit at a lower intensity to that found in the outer cortex (Figure 3-2D). Taken together these results validate the previously published genomic and proteomic studies suggesting that AQP5 is expressed in the lens, and show that AQP5 expression is conserved across lenses of all four mammalian species. Furthermore, they show that AQP5 can be detected throughout the lens with a C-terminal antibody. This is in contrast to AQP0 which is known to have its C-terminal tail cleaved in the core of the lens and suggests that the structure and potentially function of AQP5 is preserved in the deeper regions of the lens.
Figure 3-1: AQP5 is expressed in the lens of several mammalian species. Western blot for AQP5 in total fibre cell preparations obtained from mouse (Lane 1), rat (Lane 2), bovine (Lane 3) and human (Lane 4) lenses. The ~23kDa band labelled in all species of lens also matches that obtained from the rat cornea (Lane 5). (Adapted from (Grey et al., 2013)).
Figure 3-2: Regional expression of AQP5 in the mammalian lens. Western blots of crude membrane fractions isolated by micro-dissection from the outer cortex (Lane 1), inner cortex (Lane 2) and lens core (Lane 3) of four species of lens that have been probed with an antibody raised against the C-terminus of AQP5. (A) Mouse, (B) Rat, (C) Bovine and (D) Human lenses. Note: an AQP5 doublet is apparent in the human lens outer cortex, but only the higher band is present in the inner cortex and core regions.
3.2. High resolution mapping and localisation of AQP5 in the adult mouse, rat and bovine lenses

Using the Western blot results obtained by others in the laboratory as a guide, the first goal of my PhD thesis was to map the subcellular distribution of AQP5 in the lens so that I could compare it to high resolution maps of AQP0 distribution obtained previously. To achieve this I performed immunohistochemistry on lens cryosections that were double labelled with an AQP5 antibody that recognised the C-terminus of the AQP5 protein, and WGA to highlight cell membranes. In addition, to distinguish between differentiating and mature fibres cells, some sections were labelled with the nuclear stain DAPI, which served as a marker of fibre cell differentiation. To map the distribution of AQP5 from periphery to core, overlapping high resolution images were collected using confocal microscopy and the adjacent images subsequently stitched together to form a seamless image montage (Chapter 2, 2.5.8). In this way AQP5 localisation could be captured over long distances at high resolution, thereby allowing changes to the subcellular distribution of AQP5 across the lens to be identified. Protocols for the fixation and sectioning of rat lenses have been optimised previously in our laboratory (Jacobs et al., 2003), and in the current study I adapted these methods to the smaller mouse and larger bovine lenses. In all species I detected AQP5 labelling in all regions of the lens, but the pattern of labelling varied slightly between the three species of lens. Hence, I will first present the results from the different species separately, before comparing them to highlight the observed differences in expression patterns.

3.2.1. Cellular and subcellular localisation of AQP5 in the mouse lens

Construction of an image montage from equatorial lens sections that captured the morphology (Figure 3-3A) and distribution of AQP5 (Figure 3-3B) across the mouse lens revealed that although AQP5 labelling is present from the periphery to the core of the lens, its intensity varied with distance into the lens. This labelling was deemed to be specific for AQP5 since pre-incubating the AQP5 antibody with a control antigenic peptide eliminated labelling in all regions of the lens (Figure 3-3A). Strong labelling for AQP5 was initially evident in the epithelium and outer cortical regions of the mouse lens, but the labelling intensity dropped in a discrete zone in the inner cortex located ~300 to 600μm in from the epithelium, before increasing in intensity again in the core of the lens. The drop in AQP5 labelling intensity in the inner cortex did not appear to be due to tissue folding and suggests that AQP5 in this region of the lens may either be expressed at a lower abundance or
alternatively that masking of the antibody epitope is present that attenuates antibody labelling.

Figure 3-3: High resolution mapping of the radial subcellular distribution of AQP5 in the adult mouse lens. Equatorial cyrosection from 6 week old mouse lens labelled with an Abcam antibody raised against the C-terminus of AQP5 (green) plus WGA (red) and DAPI (blue) to detect cell membranes and nuclei, respectively. (A) Image montage double labelled with AQP5 antibody pre-incubated with its antigenic peptide and WGA. (B) Image montage triple labelled for AQP5, membranes and cell nuclei. The white boxes indicate regions from which high magnification images (C-L) were obtained. (C-D) AQP5 labelling is strongly cytoplasmic in differentiating fibre cells of the outer cortex localised next to the epithelium. (E-F) Differentiating fibre cells localised in the nuclear bow region of the outer cortex showed uniform membrane labelling along with reduced intensity and dispersed punctate cytoplasmic labelling of AQP5. The labelling in the inner cortex was membranous and punctate (G-H) although with reduced intensity in an adjacent region (I-J). In the core the intensity of AQP5 labelling increased and was found around the entire membranes of mature fibre cells. For clarity only AQP5 labelling is presented in the lower panels (D, F, H, J, L).
Chapter 3 – Identification and localisation of AQP5 in the adult lens

The subcellular localisation of AQP5 was further examined by taking high magnification confocal images from the different regions of the lens (Figure 3-3C-L). In the fibre cells adjacent to the capsule I detected dense cytoplasmic punctate AQP5 labelling, which did not co-localise with the membrane marker, WGA (Figure 3-3C, D). Still in the outer cortex but slightly further in from the capsule the labelling of AQP5 became abruptly associated with the membranes of differentiating fibre cells, although some cytoplasmic labelling was still present (Figure 3-3E, F).

In the inner cortex (Figure 3-3G-J) and lens core (Figure 3-3K-L) AQP5 labelling remained membranous revealing a punctate pattern of membrane distribution and an absence of AQP5 labelling in the cytoplasm. Images obtained from the deeper inner cortex where AQP5 labelling intensity decreased, revealed that AQP5 labelling, while present, was less abundant possibly due to a masking of the antibody epitope or reduced expression of AQP5 in this region (Figure 3-3I-J). Previously, our laboratory has distinguished between these possibilities by utilising a second antibody directed against a different epitope (Jacobs et al., 2004b). Unfortunately, in the case of AQP5 the only other antibodies I could obtain were also directed against the C-terminus of the AQP5 protein and therefore could not be used to distinguish between these possibilities. I have however included the labelling pattern obtained using an additional AQP5 antibody directed against a different C-terminus epitope sourced from MyBiosource (Figure 3-4) and compared it to the overall labelling pattern obtained using the Abcam antibody that produced the labelling patterns shown in Figure 3-3.

Figure 3-4: Comparison of labelling patterns obtained using a different AQP5 antibody. Overviews of equatorial cryosections labelled antibodies raised against the C-terminus of AQP5 obtained from either MyBiosource (A) or Abcam (B) double labelled with DAPI to show cell nuclei. With both antibodies AQP5 labelling was detected in all regions of the lens starting initially as a cytoplasmic pool in peripheral fibre cells before translocation to the membranes of differentiating fibre cells in deeper lens regions. Both antibody labelling patterns revealed a zone (dashed lines) in the inner cortex of the lens where AQP5 labelling has a lower intensity suggesting that the reduction of signal intensity of AQP5 is specific to this region. Green - AQP5; blue - DAPI.
Although the quality of AQP5 labelling obtained with the MyBiosource antibody was lower than the Abcam antibody, I still observed AQP5 labelling in all regions of the lens which was initially cytoplasmic in peripheral fibre cells before becoming membranous in the deeper regions of the lens. Both antibodies produced a similar drop in labelling intensity in the inner cortex, suggesting that the reduction in labelling is specific to this region and not an artefact of antibody labelling.

While equatorial sections are ideal for determining how the subcellular distribution of a membrane protein changes in a radial direction from the periphery to the lens core, this orientation of section cannot be used to determine if the protein distribution differs as the fibre cells elongate towards both poles in an axial direction. To investigate this I have mapped the distribution of AQP5 in cryosections cut in an axial orientation in order to visualise the subcellular localisation of AQP5 at the apical and basal ends of the elongating differentiating fibre cells (Figure 3-5). To obtain an overview of AQP5 expression along the anterior-posterior axis, adjacent images were stitched together to form a seamless montage of the subcellular distribution of AQP5 (Figure 3-5A). In contrast to the labelling observed in equatorially orientated overviews, no changes in AQP5 signal intensity were observed in the axial orientation. However, it was evident that changes in the subcellular distribution of AQP5 were occurring as epithelial cells differentiated into fibre cells and underwent the massive elongation required to reach the anterior and posterior poles. To capture how this process of differentiation and elongation affected the subcellular location of AQP5, high magnification images were extracted from the image montage at critical stages of the continuum that is fibre cell differentiation. At the equator, epithelial cells initially elongate forming the modiolus, a structure consisting of the tapered apical membranes of fibre cells that are preferentially migrating along the capsule towards the posterior pole (Figure 3-5H, I). In this region, AQP5 labelling is cytoplasmic as would be expected since equatorial sections are typically taken through this region. Moving away from the equator towards either the anterior (Figure 3-5D-G) or posterior (Figure 3-5J-M) poles, AQP5 labelling of the cytoplasm was progressively reduced as the association of the AQP5 with the membrane increased. While at the anterior (Figure 3-5B, C) and posterior (Figure 3-5N, O) poles AQP5 labelling was only associated with the membranes. The observation of progressive insertion of AQP5 from an initial cytoplasmic pool into the membrane shows that the subcellular localisation of AQP5 changes within the individual fibre cells as they elongate from the equator towards both poles. Furthermore, the punctate labelling in the cytoplasm of elongating fibre cells suggests that AQP5 is being transported to the growing tips of the fibre
cells from the bow region of the lens which contains the nuclei and presumably the protein synthesis machinery.

Figure 3-5: High resolution mapping of the subcellular distribution of AQP5 along the anterior-posterior axis of the adult mouse lens. Axial cryosection from a 6-week-old mouse lens labelled with an Abcam antibody raised against the C-terminus of AQP5 (green) plus WGA (red) and DAPI (blue) to detect cell membranes and nuclei, respectively. (A) Image montage triple labelled for AQP5, membranes and cell nuclei spanning from the anterior to posterior pole. High magnification images (B-O) were taken from the areas indicated by the white boxes. (B,C) Differentiating fibre cells located at the anterior pole show exclusive association of AQP5 labelling with the membranes. (D,E) Extending tips of differentiating fibre cells located anterior to the modiolus show strong association of AQP5 labelling with the membranes although cytoplasmic labelling is still present. (F,G) At a region above the modiolus an accumulation of AQP5 expression is detected at the lateral membranes of the elongating fibre cells (arrows) with a punctate cytoplasmic labelling. (H,I) At the modiolus the labelling of AQP5 is completely cytoplasmic. (J,K) At a posterior region, below the modiolus, elongating fibre cells show accumulation of AQP5 along their lateral membranes (arrows) although cytoplasmic labelling is still present. (L,M) The membranous labelling of AQP5 is further increased and cytoplasmic labelling decreased in a deeper posterior region. (N,O) At the posterior pole the elongated fibre cells show only membrane labelling. For clarity, images without WGA labelling are presented in the bottom of the figure (C, E, G, I, K, M, O).
Because I detected labelling of AQP5 in the epithelium I further examined AQP5 expression in high magnification images obtained from axial sections of the mouse lens. Due to the quiescent nature of central epithelial cells and mitotically active nature of equatorial cells I compared the expression of AQP5 from these regions to identify differences in the labelling pattern (Figure 3-6). Generally the labelling showed no differences in the expression of AQP5 from both the anterior (Figure 3-6A) and equatorial regions (Figure 3-6B). The labelling was very strong and cytoplasmic, suggesting expression of AQP5 in storage vesicles of the ER or Golgi apparatus. Interestingly, strong labelling of AQP5 was observed not only in the nuclear membrane (Figure 3-6A), but also inside the nucleus (Figure 3-6A&B).

Figure 3-6: Expression of AQP5 in the epithelial cells of the mouse lens. (A,B) Expression of AQP5 in a region above the equator shows a cytoplasmic and dispersed localisation of AQP5 labelling with occasional association with the membranes. In epithelial cells at the equator (C,D) where subtle changes to the morphology of the cells is evident, the labelling of AQP5 remains mostly cytoplasmic with a stronger aggregation of AQP5 vesicles around the lateral and apical membranes. For clarity the WGA (red) signal is removed from panels B and D. Labelling of AQP5 is also present around the nuclear membrane visible for one cell in (A,B) at the top right corner. Labelling: red-WGA, blue- DAPI and green-AQP5.
3.2.2. Cellular and subcellular localisation of AQP5 in the rat lens

The distribution pattern of AQP5 across the rat lens was generally similar to that found for AQP5 in the mouse lens, but subtle differences in the extent of cytoplasmic labelling were apparent. An image montage taken through the equator of a rat lens shows that, like the mouse, the intensity of AQP5 labelling is very strong in the epithelium and outer cortex but it decreases in the inner cortex at ~600um from the capsule before increasing again in the lens core (Figure 3-7A). This finding of AQP5 labelling in the core of the rat lens obtained with immunohistochemistry is at odds with the Western blot results that did not detect AQP5 in the core (Figure 3-2B). This observed loss in AQP5 immunoreactivity in the deeper regions of the lens could be due to masking of the antibody epitope by an unknown protein that interacts with AQP5. However, since SDS-PAGE was run under denaturing and reducing conditions, masking of AQP5 immunoreactivity is unlikely. It is more probable that the loss of AQP5 immunoreactivity in the core of the rat lens is due to a degradation of AQP5 protein during the preparation of crude membrane fractions which may lead to loss of the antibody epitope binding site on the C-terminal tail of AQP5.

In terms of the subcellular distribution of AQP5 in different regions of the rat lens, dense punctate cytoplasmic labelling with minimal association to the membranes was found in the peripheral elongating fibre cells (Figure 3-7B, C), the deeper cortex (Figure 3-7D, E) and the early inner cortex (Figure 3-7F,G) in which fibre cells have lost their nuclei. It was not until the deeper inner cortex that association of the AQP5 labelling with the membranes of mature fibre cells was definitively detected (Figure 3-7H, I) and this association became very strong in the core of the lens (Figure 3-7J, K).

Thus while the pattern of initial cytoplasmic labelling followed by membrane insertion during fibre cell differentiation was similar between the mouse and the rat lenses, the zone of translocation occurred at a much later stage of differentiation in the rat lens, suggesting that the phenomenon is species dependent. To investigate this further I chose to characterise the subcellular distribution of AQP5 in a third species, the bovine lens.
Figure 3-7: High resolution mapping of the radial subcellular distribution of AQP5 in the rat lens. Equatorial cryosection from a 3-week-old rat lens labelled with an Abcam antibody raised against the C-terminus of AQP5 (green) and WGA (red) to detect cell membranes. (A) Image montage extending from the outer cortex to the lens core double-labelled for AQP5 and WGA. Boxes in the montage indicate regions from which high magnification images were obtained from the outer cortex (B-E), inner cortex (F-I) and core (J, K). For clarity only AQP5 labelling is presented in the images at the bottom of the figure (C, E, G, I, K). n-Nuclei.
Chapter 3 – Identification and localisation of AQP5 in the adult lens

3.2.3. Cellular and subcellular localisation of AQP5 in the bovine lens

Equatorial cryosections obtained from the bovine lens were triple labelled with the same AQP5 antibody used previously to map the subcellular distribution of AQP5 in the mouse and rat lenses, plus WGA to visualise cellular morphology and DAPI to stain the nuclei which acts as a marker of fibre cell differentiation. Because of the large size of the bovine lens (radius = 8000µm), obtaining a high resolution image montage to provide an overview of AQP5 labelling intensity across the whole lens was technically difficult. Instead I have taken high magnification images from representative areas in the outer cortex, adult nucleus, juvenile nucleus and embryonic nucleus of the bovine lens that have been previously defined on the basis of their morphological differences (Al-Ghoul and Costello, 1997). During the collection of these images the gain and offset settings of the confocal microscope were kept constant, thereby allowing any fluctuations in the intensity of AQP5 labelling to be detected. Using this approach I did not observe any noticeable reduction in the intensity of AQP5 labelling throughout the entire section (Figure 3-8). In the bovine lens the subcellular distribution of AQP5 was more similar to the mouse than the rat lens, being mostly punctate and cytoplasmic in the peripheral newly elongated differentiating fibre cells before transitioning into the membrane. (Figure 3-8A, A’). In the deeper cortex (Figure 3-8B), adult nucleus (Figure 3-8C), juvenile nucleus (Figure 3-8C, D) and embryonic nucleus (Figure 3-8E-H) AQP5 labelling was strongly associated with the membranes. Thus it appears that the pattern of labelling for AQP5 in the bovine lens is more similar to the mouse than the rat. This comparison is studied in more detail in the next section.
**Figure 3-8: Mapping the cellular and subcellular distribution of AQP5 in the bovine lens.** An equatorial bovine lens cryosection was labelled with AQP5 (green), the membrane marker WGA (red) and nuclei stain DAPI (blue). (A,A’) outer cortical staining, (B) adult nucleus, (C,D) juvenile nucleus, (E,F,G and H) embryonic nucleus. To highlight AQP5 labelling, WGA membrane labelling is omitted in the top half panel and present in the lower half panel of the images. (See text for details)
3.3. Species differences in the differentiation-dependent membrane insertion of AQP5

The translocation of AQP5 from a cytoplasmic pool to the membranes of fibre cells appears to be a common feature of fibre cell differentiation in all three species of lens I have studied. To facilitate comparison between lenses of vastly different sizes, the distance from the lens centre \((a)\) can be expressed as a fraction of the lens radius \((r)\), where \(r/a=1\) and \(r/a=0\) correspond to the lens periphery and central core, respectively. Using this scale, an abrupt translocation of AQP5 labelling from cytoplasmic to membranous was observed at similar positions of \(r/a = 0.95\) in the mouse lens (Figure 3-9A) and \(r/a=0.98\) in the bovine lens (Figure 3-9C). In contrast, the translocation of AQP5 into the membranes of mature rat lens fibre cells in the deeper inner cortex was not as abrupt and occurred much deeper at \(r/a=0.60\) (Figure 3-9C). These observations show a species- and differentiation-dependent insertion of AQP5 from a cytoplasmic pool that is probably inactive, to the plasma membrane of deeper fibre cells to presumably increase the water permeability above these cells whose basal permeability is set by AQP0.
Figure 3-9: Differentiation-dependent translocation of AQP5 protein from cytoplasm to membrane in fibre cells of the mouse, rat and bovine lenses. Translocation of AQP5 labelling occurs in peripheral fibre cells in the outer cortex of the mouse (A) and bovine (B) lenses and in mature fibre cells in the deeper inner cortex of the rat lens (C).
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The subcellular changes of the localisation of AQP5 across the adult mouse, rat and bovine lenses are summarised in below (Table 3).

Table 3: Summary of the subcellular distribution of AQP5 across the mouse, rat and bovine lenses.

<table>
<thead>
<tr>
<th>Lens species</th>
<th>Outer cortex</th>
<th>Inner Cortex</th>
<th>Core</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>C/M</td>
<td>M reduced intensity</td>
<td>M</td>
</tr>
<tr>
<td>Rat</td>
<td>C</td>
<td>C/M reduced intensity</td>
<td>M</td>
</tr>
<tr>
<td>Bovine</td>
<td>C/M</td>
<td>M</td>
<td>M</td>
</tr>
</tbody>
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Abbreviations: C-Cytoplasmic, M-Membranous

The observation that AQP5 resides in a cytoplasmic pool in the OC of the three species of lens suggests that at this stage of cellular differentiation it is predominantly synthesised and deposited in storage vesicle and thus does not contribute to the water permeability of the plasma membrane. Therefore, I hypothesised that the predominantly cytoplasmic localisation of AQP5 in the lens represents a pool of water channels that are capable of both programmed and dynamic membrane insertion to increase the water permeability of fibre cell membranes.
3.4. Osmotic stress increases AQP5 membrane insertion in the outer cortex of the mouse lens

To partially test the above hypothesis that cytoplasmic AQP5 can be induced to insert in the membranes of differentiating fibre cells in response to external stimuli, mouse lenses were organ cultured in the presence of artificial aqueous humour (AAH) of varying osmolarity for 2 hours to determine whether osmotic stress can alter the subcellular localisation of AQP5 in the outer cortex of the mouse lenses (Figure 3-10). In contrast, to the cytoplasmic location of AQP5 observed in peripheral fibre cells in lenses not subjected to organ culturing (Figure 3-10B), organ culturing lenses in Hypotonic AAH (Figure 3-10C, D) induced an insertion of AQP5 into the membranes of differentiating fibre cells in this region, although cytoplasmic labelling was still present. Culturing lenses in Hypertonic AAH had a similar effect, although the level of association of AQP5 with the membrane increased further with a concomitant decrease in the extent of cytoplasmic labelling (Figure 3-10F). This shift in membrane labelling was specific to peripheral cells in the outer cortex since no additional changes in the localisation of AQP5 were observed in response to changes in the osmolarity of the bathing media in differentiating fibre cells located in the deeper outer cortex (Figure 3-10G-K).

Thus it appears that culturing lenses in solutions of different osmolarity can induce a dynamic increase in the translocation of AQP5 from a cytoplasmic pool to the plasma membrane specifically in peripheral fibre cells at the mouse lens equator. Interestingly, lenses cultured in Isotonic AAH also triggered a marked membrane translocation of AQP5 labelling (Figure 3-10E) indicating that in addition to responding to changes in osmolarity, additional factors simply associated with the process of organ culturing lens may be triggering the dynamic insertion of AQP5 in this region of the lens.
Figure 3-10: Organ culturing mouse lenses in the presence of osmotic stress increases the membrane insertion of AQP5. A) An overview of an equatorial section of a 6 week-old mouse lens labelled with WGA (red), DAPI (blue) used to indicate where higher magnification images of AQP5 labelling in fibre cells located peripheral (B-F) and deeper (G-K) outer cortex were taken from. AQP5 (green) is localised to the cytoplasm of peripheral differentiating fibre cells in non-organ cultured lenses (B) but becomes progressively inserted in the membranes of differentiating fibre cells in this same region by organ culturing lenses in 270mOsm Hypotonic AAH (C), 290mOsm Hypotonic AAH (D), 300mOsm Isotonic AAH (E) or 405mOsm Hypertonic AAH (F) for 2 hours. No additional differences in the immunolabelling pattern of AQP5 are observed in the deeper cortex of either non-organ cultured lenses (G) or lenses organ cultured in 270mOsm AAH (H), 290mOsm AAH (I), 300mOsm AAH (J) and 405mOsm AAH (K).
3.5. Conclusions

In this chapter I have utilised immunohistochemistry to map the subcellular distribution of AQP5 in different regions of the adult mouse, rat and bovine lens. I have found that although some subtle and important differences exist, the three species have a similar pattern of AQP5 labelling. The following features are common to all species:

- AQP5 is expressed throughout all regions of the mammalian lens
- The C-terminal tail of AQP5 does not undergo extensive C-terminal truncation in the core of the lens
- In equatorial sections, AQP5 was initially found in a cytoplasmic pool in peripheral differentiating fibre cells of all lens species, but with distance into the lens AQP5 undergoes a differentiation-dependent translocation into the plasma membrane, the location of which was species dependent.

While specifically in the mouse lens I have shown that:

- In axial sections translocation of AQP5 to the plasma membranes of differentiating elongating fibre cells increases towards both anterior and posterior poles.
- The cytoplasmic pool of AQP5 in peripheral fibre cells can be induced to insert into the plasma membrane by organ culturing lenses in different extracellular osmolarities.

Based on these observations, it is possible to suggest some preliminary functional roles for AQP5 in the lens. It appears that in differentiating fibre cells, AQP5 is first expressed as a cytoplasmic pool of presumably inactive water channels that, at a later stage of fibre cell differentiation, are translocated to the plasma membrane where they can then contribute to water permeability of the membrane. This phenomenon of differentiation-dependent membrane insertion has also been shown for a variety of other membrane proteins expressed in the lens (Grey et al., 2003, Lim et al., 2006, Lim et al., 2007). Interestingly, it appears that the differentiation event that triggers AQP5 translocation is similar for the mouse and bovine lenses, but different for rat lenses, suggesting that the potential change in water permeability induced by AQP5 membrane insertion is required at different stages of fibre cell differentiation.
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Support for this notion that the cytoplasmic labelling of AQP5 in peripheral fibre cells represents an inactive pool of water channels that undergo translocation to the plasma membrane comes from the experiments performed on organ cultured mouse lenses. Culturing these lenses in the presence of an osmotic challenge produced a dynamic insertion of AQP5 into the plasma membrane and a corresponding drop in cytoplasmic labelling for AQP5. This observation suggests that the inactive pool of cytoplasmic AQP5 water channels may be recruited to the plasma membrane on demand to increase water permeability, thereby alleviating local volume changes in response to osmotic imbalance. Interestingly, AQP5 membrane insertion was also observed after incubating lenses in isotonic conditions, suggesting that other external factors such as membrane stretch or hydrostatic pressure may also contribute to the regulation of the subcellular distribution of AQP5.

Finally, the observation that an antibody directed against the C-terminus of AQP5 labelled all regions of the lens including the core, shows that unlike AQP0, AQP5 is not being extensively truncated in the core of the lens. This result suggests that the expression of AQP5 in the lens core may compensate for the loss of or change to the water permeability of AQP0. Since AQP5 has a water permeability that is ~20 times higher that AQP0, it is interesting to speculate that the expression of uncleaved AQP5 in the lens core may compensate for the loss of AQP0 functionality caused by its truncation in this region of the lens.

To further study these potential roles for AQP5 in the lens I have, in the next section, compared the relative expression patterns of AQP5 and AQP0 throughout embryonic development and postnatal growth.
Chapter 4. Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

In the previous chapter I established the subcellular expression pattern of AQP5 in adult rodent and bovine lenses using immunohistochemistry. In this chapter I compare the expression pattern of AQP5 to the well characterised pattern of AQP0 expression in the adult (Grey et al., 2009) and embryonic lens (Varadaraj et al., 2007), in order to gain potential insights into how the relative contributions of the two AQPs to fibre cell function may change throughout lens development and growth. This comparison revealed changes to the distribution of both AQPs over the course of lens development and postnatal growth that were specific to different regions of the lens. Using antibodies directed against the C-terminus of each AQP, I found AQP5 was abundantly expressed early in development, being found in the cytoplasm of cells of the lens vesicle and surrounding tissues (E10), while AQP0 was detected later (E11), and only in the membranes of elongating primary fibre cells. During the course of subsequent embryonic and postnatal development the pattern of cytoplasmic AQP5 and membranous AQP0 labelling was maintained until postnatal day 6 (P6). From P6, AQP5 labelling became progressively more membranous, initially in the lens core and then later in all regions of the lens, while AQP0 labelling was abruptly lost in the lens core due to C-terminal truncation. My results show that the spatial distribution patterns of AQP0 and AQP5 observed in the adult lens are established during a narrow window of postnatal development (P6-P15) that precedes eye opening and coincides with regression of the hyaloid vascular system. From these results I hypothesize that, in the older fibre cells, insertion of AQP5 into the fibre cell membrane compensates for any change in the functionality of AQP0 induced by truncation of its C-terminal tail.
4.1. AQP0 and AQP5 expression patterns in the adult mouse lens

Our laboratory has previously shown in the rat lens that the subcellular distribution of AQP0 changes with fibre cell differentiation (Figure 4-1), with the protein undergoing post-translational truncation in the lens core (Grey et al. 2009). In cortical fibre cells, antibodies directed against the AQP0 C-terminus label the cell membrane. In differentiating fibre cells undergoing nuclear degradation, AQP0 coalesces into large plaque-like structures. As cell nuclei degrade, a loss of labelling with this antibody is evident, possibly due to epitope masking. In mature fibre cells of the inner cortex the AQP0 C-terminus antibody labels cell membranes strongly, however, in the lens core the signal is abruptly lost, indicating truncation of the AQP0 C-terminus, a finding which has been confirmed by mass spectrometry (Grey et al. 2009).

Figure 4-1: Expression of AQP0 in the rat lens. Equatorial cryosection of rat lens labelled with AQP0 C-terminus antibody showing expression of AQP0 protein throughout the lens and cleavage in the core region (*). Adapted from (Grey et al., 2009).

Since it has been shown by others that C-terminal truncation removes the ability of AQP0 to interact with cytoskeletal proteins (Rose et al., 2006, Wang and Schey, 2011), and functional modulators such as AKAP2 (Gold et al., 2012), and calmodulin (Németh-Cahalan and Hall, 2000) we have speculated that C-terminal truncation must change AQP0 functionality in the lens core relative to the cortex. Having chosen the mouse lens to perform high resolution mapping of AQP5, in this section I have first performed a similar mapping of AQP0 in the adult mouse lens to enable the relative subcellular distribution patterns of the two AQPs to be compared.

4.1.1. Mapping the AQP0 subcellular distribution pattern in the adult mouse lens

To examine the subcellular distribution of AQP0 in the adult mouse lens I performed immunomapping experiments on equatorial mouse cryosections labelled with either an AQP0 C-terminus antibody or an antibody raised against the whole AQP0 protein (Figure 4-2). The
immunomapping analysis showed that AQP0 labelling in the mouse lens was similar to that of the rat lens, with AQP0 labelling being detected in the outer cortex and inner cortex but not the lens core (Figure 4-2B). Mass spectrometry showed that the loss of AQP0 labelling in the mouse lens was also due to a C-terminal truncation of AQP0 protein (Petrova et al., 2015), and this loss of the C-terminal epitope was confirmed by utilising a different antibody raised against the whole AQP0 protein that showed positive staining of AQP0 not only in the periphery, but also in the core of the lens (Figure 4-2A).

Figure 4-2: The subcellular distribution of AQP0 in different regions of the mouse lens. Image montages of equatorial cryosections of mouse lenses labelled with antibodies raised against either the whole protein (A) that demonstrates AQP0 labelling was present throughout the lens, or an epitope in the C-terminus (B) showing AQP0 labelling in the outer and inner cortex but not in the lens core due to truncation of the C-terminus. C-L) High magnification images taken from the regions designated by the boxes in B, displayed in the presence (C-G) and absence (H-L) of the membrane marker WGA. In the lens periphery (C,H) AQP0 labelling was absent from the epithelium but strongly labelled the membranes of differentiating fibre cells. In deeper regions of the outer cortex (D,I, E&J) and inner cortex (F,K) AQP0 remained membranous before the labelling for AQP0 was lost in the lens core (H,L). Labelling: AQP0-green, membrane marker WGA-red, nuclei marker DAPI-blue.
To further examine the subcellular distribution of AQP0 as a function of fibre cell differentiation I collected high magnification images using the C-terminus AQP0 Ab (Figure 4-2C-I). At the lens surface, AQP0 was absent from the epithelial cells, but was localised around the entire membrane of newly differentiating fibre cells (Figure 4-2C,H). In deeper differentiating fibre cells in the outer cortex AQP0 labelling became progressively less homogenous (Figure 4-2D&I, E&J), before reverting to a more continuous labelling of the membranes of mature fibre cells located in the inner cortex (Figure 4-2F,K). In contrast, no AQP0 labelling with the C-terminal antibody was detected in the lens core (Figure 4-2H,L).

The subcellular distribution of AQP0 in the mouse lens was similar to the that observed in the in rat lens (Grey et al., 2009), although the differentiation-dependent rearrangement of AQP0 labelling into plaque-like structures observed in the outer cortex of the rat lens was not as evident in the mouse lens. The loss in AQP0 labelling in the core was however, identical in both the rat and mouse lens and indicates that the C-terminal cleavage of AQP0 is common phenomenon in both species of lens. Having established the subcellular distribution of AQP0 in the mouse lens I am now in a position to compare it to the pattern for AQP5 established in Chapter III.

4.1.2. Comparison of subcellular distribution patterns of AQP0 and AQP5 in the adult mouse lens

The most direct method to compare subcellular distributions of the two AQPs would be to double label lens sections with the AQP0 and AQP5 primary antibodies. Unfortunately, the best performing antibodies used in this study, that target the C-termini of AQP0 or AQP5 proteins, were both raised in rabbit. In an attempt to circumvent this technical difficulty I initially chose to investigate whether direct conjugation of AQP0 antibodies to a fluorophore would allow reliable double labelling experiments to be performed (Figure 4-3). Unfortunately, the conjugation of AQP0 antibodies (Figure 4-3F-I) did not give the same labelling patterns to those obtained by the non-conjugated antibody (Figure 4-3B-E). Direct conjugation of the ADI antibody raised against the C-terminus of AQP0 with an Alexa 594 fluorophore produced labelling similar to its conjugated counterpart in the outer and inner cortex of the lens, but produced robust labelling in the lens core, where it is known from mass spectrometry that the epitope is lost due to C-terminal truncation. This comparison between the conjugated and non-conjugated AQP0 antibodies means that the conjugated antibody
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does not faithfully report the subcellular distribution of AQP0 in the lens core. However, to test the utility of the conjugated AQP0 antibody to compare relative subcellular locations of AQP0 and AQP5 in outer regions of the lens, double labelling experiments with conjugated AQP0 and non-conjugated AQP5 were trialled (Figure 4-3N-Q).

**Figure 4-3**: Characterisation of antibody conjugation protocols to visualise AQP0 and AQP5 double labelling in different region of the mouse lens. (A) An overview image of an equatorial section from a 6 week-old adult mouse lens labelled with WGA to define the regions (boxes) from which high magnification images were obtained. (B-I). High power images showing AQP0 labelling (red) with either the normal C-terminal antibody detected by a secondary antibody (B-E), or following the direct conjugation of the Alexa 594 fluorophore to the same antibody (F-I) in the lens peripheral (F), deeper outer cortex (G), inner cortex (H) and lens core (E). (J-M) High power images showing the subcellular distribution of AQP5 labelling (green) in peripheral fibre cells (J), deeper outer cortex (K), inner cortex (L) and core of the lens (M). (N-Q). Double labelling with conjugated-AQP0 (red) and AQP5 (green) antibodies showed a the conservation of subcellular labelling patterns in the outer (N,O) and inner cortex (P) but not the lens core (Q), where non-specific membrane binding of direct conjugated AQP0 was observed.
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The double labelled section that, utilised the C-terminal AQP0 antibody directly conjugated to the fluorophore, showed the expected membrane labelling for AQP0 in the outer (Figure 4-3N,O) and inner cortex of the lens (Figure 4-3O), but also in the lens core (Figure 4-3Q) which deviated from the labelling obtained with the non-conjugated AQP0 antibody (Figure 4-3E). In the same section AQP5 was cytoplasmic in the peripheral fibre cells (Figure 4-3N) and membranous in the deeper outer cortex (Figure 4-3O), inner cortex (Figure 4-3P) and core (Figure 4-3Q), a pattern identical to that seen in the absence of double labelling. This result suggests that although AQP5 labelling was preserved in both single and double labelled sections, the conjugated C-terminal AQP0 antibody was binding to a non-specific epitope since we know that the C-terminus of AQP0 is missing in the core of the mouse lens. Due to this potential non-specific binding of the directly conjugated AQP0 antibody in the core of the lens we could not verify the specificity of the antibody in other regions of the lens. Thus I decide not to utilise this double labelling approach to map the distribution of AQP5 and AQP0.

Instead I chose to use single serial sections labelled with epitope specific antibodies raised against the C-termini of either AQP0 (Figure 4-3B-E) or AQP5 (Figure 4-3J-M), which showed differences in the expression patterns of AQP0 and AQP5 in the different regions of the lens. AQP0 was membranous through all regions of the lens and its C-terminus was cleaved in the lens core while AQP5 was initially cytoplasmic, and inserted in the membranes in the OC and remained membranous in the IC and C of the lens without undergoing C-terminal truncation. The identification of regional differences in the subcellular distributions of AQP0 and AQP5 in the adult mouse lens raises questions about how these observed differences are initially established. To address this question, in the next section I have compared AQP5 and AQP0 in serial sections taken from lenses at different stages of embryonic and postnatal development.

4.2. Expression and localisation patterns of AQP5 and AQP0 during lens embryological development

To determine how the differential distribution patterns of the two AQPs are observed in the adult mouse lens are original established, I compared the labelling patterns for AQP0 to AQP5 during embryological development by performing immunohistochemistry on paraffin
embedded sections taken through the mouse eye. At embryonic stage E10 (the earliest stage studied), the developing mouse eye contains the lens vesicle. While AQP0 labelling was not detected in the lens vesicle at E10 (Figure 4-4A, B), it was detected in the cell membranes of elongating primary fibre cells at E11 (Figure 4-4C), indicating that this is a significant lens developmental event to potentially enhance membrane water permeability and/or cell adhesion in primary fibre cells. This onset of AQP0 protein expression is in agreement with that found in a previous investigation (Varadaraj et al., 2007). In contrast, AQP5 labelling was detected in the lens vesicle and optic cup at E10 (Figure 4-4D), indicating that AQP5 protein expression occurs earlier in mouse lens development than AQP0. Interestingly, the subcellular distribution of AQP5 in the lens vesicle cells was predominately cytoplasmic at E10 and E11 (Figure 4-4E, F).

Figure 4-4: Distribution of AQP0 and AQP5 in the lens vesicle. Axial embryonic paraffin sections through the lens vesicle labelled with AQP specific antibodies (green) and the cell nuclei marker DAPI (blue). At developmental stage E10 AQP0 protein expression is not yet present (A, B), but AQP5 is present and is localised predominantly to the cytoplasm of cells in the lens vesicle (D, E). By E11, AQP0 protein expression is evident in the membrane of elongating primary fibre cells (C), while AQP5 continues to be expressed in the cytoplasm of primary fibre cells (F). Boxes in A and D show locations of zoomed up images at E10 (B, E) and E11 (C, F).
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

At embryonic stage E14, AQP0 was localised to the membranes of all fibre cells in the bulk of the lens, but not the overlying anterior epithelium (Figure 4-5A). Higher magnification images confirmed its membranous localisation in differentiating fibre cells in the outer cortex (Figure 4-5B), secondary fibre cells (Figure 4-5C) and primary fibre cells (Figure 4-5D) that comprise the lens core. In contrast, AQP5 was localised to both epithelial and fibre cells and was predominantly cytoplasmic (Figure 4-5E). The highest signal intensity of this cytoplasmic labelling for AQP5 was detected in differentiating fibre cells located in the lens cortex (Figure 4-5F). While lower signal intensities were observed in deeper regions of the lens that contained mature fibre cells (Figure 4-5G) and primary fibre cells (Figure 4-5H), AQP5 labelling in the lens core remained cytoplasmic.

Figure 4-5: Distribution of AQP0 and AQP5 at embryonic stage E14. Axial paraffin sections of whole embryos at developmental stage E14 show that AQP0 is expressed exclusively in fibre cells in all regions of the lens, but is not detected in the anterior epithelium (A). Furthermore, it is localised to the cell membrane of differentiating fibre cells in the periphery (B), cortex (C) and primary fibre cells in the lens core (D). In contrast, AQP5 is localised to the cytoplasm of lens epithelial cells and fibre cells in all regions of the lens (E). This distribution continues in differentiating fibre cells in the periphery (F), cortex (G), and primary fibre cells in the lens core (H), where cell membranes have been labelled with WGA (red) for clarity. AQP proteins were labelled with specific antibodies (green) and cell nuclei were labelled with DAPI (blue).
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Similar to E14, at E16 (Figure 4-6), E17.5 (Figure 4-7) and the embryonic stage before birth E18.5 (Figure 4-8), AQP0 remained membranous in the bulk of the lens and was excluded from the epithelium, while AQP5 was predominantly cytoplasmic in both fibre and epithelial cells. No additional changes in the localisation and post-translational truncation of AQP0 or AQP5 were observed across the entire lens throughout these developmental stages.

In summary, AQP0 was first expressed at E11 and its distribution throughout the embryonic mouse lens was characterised by an abundant membranous signal for AQP0 in lens fibre cells, which indicates that during embryonic development there is no truncation of AQP0. In contrast, AQP5 was detected in epithelial and fibre cells prior to the onset of AQP0 expression, but was predominantly cytoplasmic in all regions of the embryonic lens, although it was also found sporadically to be associated with the membranes of elongating differentiating fibre cells in the outer cortex and, like AQP0, it was not truncated. These results showed that the translocation of AQP5 to the membranes of primary fibres/mature fibres and C-terminus truncation of AQP0 did not happen during the embryonic stages of development, suggesting that these changes are happening after birth. To investigate this I therefore examined AQP0 and AQP5 expression post-partum.
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Figure 4-6: Distribution of AQP0 and AQP5 at embryonic stage E16. Axial sections of paraffin embedded embryos at developmental stage E16 show that AQP0 is expressed exclusively in fibre cells in all regions of the lens, but is not detected in the anterior epithelium (A). Furthermore, it is localised to the cell membrane of differentiating fibre cells in the periphery (B), cortex (C) and primary fibre cells in the lens core (D). In contrast, AQP5 is localised to the cytoplasm of lens epithelial cells and fibre cells in all regions of the lens (E). The cytoplasmic localisation is evident in magnified images of the differentiating fibre cells in the periphery (F), predominantly cytoplasmic and sporadic punctate labelling associated with the membranes of mature fibre cells in the cortex (G), and diminished cytoplasmic signal in the primary fibre cells in the lens core (H), where cell membranes have been labelled with WGA (red) for clarity. AQP proteins were labelled with specific antibodies (green) and cell nuclei were labelled with DAPI (blue).
Figure 4-7: Distribution of AQP0 and AQP5 at embryonic stage E17.5. Axial paraffin sections at developmental stage E17.5 show that AQP0 is expressed exclusively in fibre cells in all regions of the lens, but is not detected in the anterior epithelium (A). Furthermore, it is localised to the cell membrane of differentiating fibre cells in the periphery (B), cortex (C) and primary fibre cells in the lens core (D). In contrast, AQP5 is localised to the cytoplasm of lens epithelial cells and fibre cells in all regions of the lens (E). This distribution continues in differentiating fibre cells in the periphery (F), cortex (G), and primary fibre cells in the lens core (H), where cell membranes have been labelled with WGA (red) for clarity. AQP proteins were labelled with specific antibodies (green) and cell nuclei were labelled with DAPI (blue).
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Figure 4-8: Distribution of AQP0 and AQP5 at embryonic stage E18.5. Axial paraffin sections at developmental stage E18.5 show that AQP0 is expressed exclusively in fibre cells in all regions of the lens, but is not detected in the anterior epithelium (A). Furthermore, it is localised to the cell membrane of differentiating fibre cells in the periphery (B), cortex (C) and primary fibre cells in the lens core (D). In contrast, AQP5 is localised to the cytoplasm of lens epithelial cells and fibre cells in all regions of the lens (E). This distribution continues in differentiating fibre cells in the periphery (F), cortex (G), and primary fibre cells in the lens core (H), where cell membranes have been labelled with WGA (red) for clarity. AQP proteins were labelled with specific antibodies (green) and cell nuclei were labelled with DAPI (blue).
4.3. Comparison of the expression of AQP0 and AQP5 during lens postnatal development

Similar distributions for each AQP to those observed in late embryonic development were detected in equatorial paraffin sections from early postnatal (P) lenses. P0 lenses (Figure 4-9) labelled with an antibody directed against the AQP0 C terminus showed AQP0 signal in the membrane of differentiating secondary fibre cells (Figure 4-9B, C), and primary fibre cells (Figure 4-9D), with no evidence of loss of signal in the lens core which showsAQP0 C terminus truncation had not yet commenced. AQP5 immunolabelling was localised predominantly to the cytoplasm of differentiating fibre cells in the outer cortex and deeper cortex (Figure 4-9F, G). Interestingly, plaque-like immunolabelling of AQP5 was associated with the membranes of differentiating fibre cells in the mid cortex (Figure 4-9G), in addition to the cytoplasmic labelling characteristic of the outer cortex and primary fibre cells (Figure 4-9H). The signal intensity for AQP5 was strongest in the periphery and decreased in the deeper cortex and core and stayed predominantly cytoplasmic.

Figure 4-9: Distribution of AQP0 and AQP5 at postnatal stage P0. Axial paraffin sections at postnatal stage P0 show that AQP0 is expressed exclusively in fibre cells in all regions of the lens, but is not detected in the anterior epithelium (A). Furthermore, it is localised to the cell membrane of differentiating fibre cells in the periphery (B), cortex (C) and primary fibre cells in the lens core (D). In contrast, AQP5 is localised to the cytoplasm of lens epithelial cells and fibre cells in all regions of the lens (E). This distribution continues in differentiating fibre cells in the periphery (F). In the cortex, in addition to the cytoplasmic labelling, some plaque-like AQP5 labelling is found associated with the membranes (G). Labelling in primary fibre cells in the core is predominantly cytoplasmic (H). Cell membranes have been labelled with WGA (red) for clarity, AQP proteins were labelled with isoform specific antibodies raised against the C-terminus (green) and cell nuclei were labelled with DAPI (blue).
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

Figure 4-10: Comparison of tissue preparation methods for AQP0 immunolabelling in the postnatal lens. Images taken from either paraffin embedded sections treated for antigen retrieval (A-D) or cryosections (E-G) from a P3 mouse lens labelled with AQP0 (green) and DAPI (blue). (A) An overview of a paraffin embedded section showing continuous membrane localisation of AQP0 from periphery to core with no signal fluctuations. White boxes indicate regions from which high magnification images were collected. (B-D) Subcellular localisation of AQP0 in a paraffin embedded section from the outer cortex (B), inner cortex (C) and lens core (D), and equivalent regions from a cryosection (E-G) showing no observable difference in the membrane labelling for AQP0.

All subsequent postnatal tissue sections used to investigate AQP spatial distributions in postnatal development were prepared from cryosections. This was done to avoid tissue collapsing and the formation of multiple internal micro-folds and cracks which were observed in the mid cortical lens region after several trials to section paraffin embedded adult lenses, which was probably due to over-hardening of the processed tissue. In contrast, all embryonic tissue and very young postnatal stages were suitable for paraffin embedding. To test whether the different tissue preparation strategies might alter immunolabelling patterns, lenses from P3 were prepared using both protocols and labelled with AQP0 antibodies (Figure 4-10). No differences between the immunolabelling patterns of AQP0 obtained using paraffin and cryosections were observed, confirming that the different tissue fixation and preparation methods did not affect AQP immunolabelling.
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

The AQP immunolabelling of equatorial cryosections from P3 lenses revealed similar distribution patterns to those seen in P0 and embryonic lenses. Using the same AQP0 C-terminus antibody, AQP0 signal was detected in the membranes of differentiating fibre cells (Figure 4-11B, C), mature fibre cells (Figure 4-11D), and primary fibre cells (Figure 4-11E), with no evidence of signal decrease or loss in the lens core. AQP5 was localised predominantly to the fibre cell cytoplasm in the epithelium and differentiating fibre cells (Figure 4-11F, G), mature fibre cells (Figure 4-11H) and primary fibre cells (Figure 4-11I).

Figure 4-11: Distribution of AQP0 and AQP5 at postnatal stage P3. An overview of an equatorial cryosection from a P3 lens labelled with the membrane marker WGA (A, red) to indicate where higher magnification images for AQP0 (B-E, green) and AQP5 (F-I green) were obtained. AQP0 is detected in fibre cell membranes of all lens regions, while AQP5 is detected predominantly in the fibre cell cytoplasm in all lens regions. Cell nuclei are labelled with DAPI in blue.
Towards the end of the first week of postnatal development (P6), changes in to AQP labelling patterns started to appear (Figure 4-12). Using an antibody directed against the AQP0 C-terminus, AQP0 was detected in the fibre cell membranes in all lens regions (Figure 4-12B-E, F-I). Interestingly, the signal intensity for AQP0 membrane labelling decreased in fibre cells located in the lens core (Figure 4-12E, I) relative to more peripheral lens fibre cells using identical imaging parameters, which suggested that the AQP0 C-terminus was starting to undergo truncation in the lens core. In contrast to AQP0, AQP5 labelling localised predominantly to the cytoplasm of lens epithelial cells (Figure 4-12J, N) and fibre cells in all regions of the lens (Figure 4-12K-M, O-Q), although the AQP5 labelling became less diffuse and more punctate in the cytoplasm and began to associate with the cell membranes of differentiating fibre cells (Figure 4-12K-L, O-P).

These changes in the relative distributions of AQP0 and AQP5, which were initiated at P6, continued throughout the period of postnatal development. Representative immunolabelling patterns of lens fibre cell AQPs during this period are shown for P15 (Figure 4-13). Labelling for AQP0 remained localised to fibre cell membranes in peripheral and cortical lens regions (Figure 4-13B-E, G-J), but now with significant loss of signal intensity in the lens core indicating extensive truncation of the C-terminal tail of AQP0 (Figure 4-13F,K). While AQP5 remained localised predominantly to the cytoplasm of the most superficial differentiating fibre cells in the lens outer cortex (Figure 4-13L-M, Q-R), its distribution in the deeper cortex and core was markedly different. Signal for AQP5 was localised to both the cell membrane and cytoplasm in the cortex (Figure 4-13N, S), and exclusively to the fibre cell membrane in discrete puncta in the deeper cortex (Figure 4-13O,T), and in more continuous membrane regions in the lens core (Figure 4-13P,U). This indicates a translocation of the AQP5 protein from the cytoplasm to the membrane in the anucleate mature fibre and primary fibre cells that make up the lens core has occurred during postnatal development.
Figure 4-12: Distribution of AQP0 and AQP5 at postnatal stage P6. An overview of an equatorial cryosection from a P6 lens labelled with the membrane marker WGA (A, red) to indicate where higher magnification images for AQP0 (B-E in greyscale, F-I in green) and AQP5 (J-M in greyscale, N-Q in green) were obtained. AQP0 localises to the fibre cell membrane in all lens regions. In the lens core, using an antibody to the AQP0 C-terminus (E,I) the initial truncation of the AQP0 C-terminus is evident due to the lower signal intensity observed in the region. In contrast, AQP5 is predominantly cytoplasmic in all lens regions, although some labelling associated with the membranes of differentiating fibre cells in the lens cortex is starting to become evident (K,L,O,P). Cell nuclei (blue) are labelled with DAPI.
Figure 4-13: Distribution of AQP0 and AQP5 at postnatal stage P15. An overview of an equatorial cryosection from a P15 lens labelled with the membrane marker WGA (A, red) to indicate the location of higher magnification images for AQP0 (B-F in greyscale, G-K in green) and AQP5 (L-P in greyscale, Q-U in green). Full-length AQP0 is absent in the epithelium (B,G), and is associated with the fibre cell membrane in the lens cortex (C-E&H-J). A loss of antibody labelling in the lens core is indicative of AQP0 C-terminal truncation (F,K). AQP5 is cytoplasmic in lens epithelium (L,Q) and differentiating fibre cells in the lens periphery (M,R). In the cortex (N,S), AQP5 is localised to both the cytoplasm and cell membrane, while it is associated exclusively with the cell membrane in the inner cortex (O,T) and lens core (P/U) regions. Cell nuclei (blue) are labelled with DAPI.
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

The spatial distributions of AQP0 and AQP5 in juvenile mice (P22), which have fully functional eyes is shown in Figure 4-14. AQP0 immunolabelling has no additional changes and remains membranous throughout the lens (Figure 4-14C, D) but its C-terminus is truncated in the core (Figure 4-14E). In contrast AQP5 membrane insertion continues progressively and is observed not only in the inner cortex (Figure 4-14H) and lens core (Figure 4-14I), but also shows a mixed cytoplasmic and membrane localisation in the differentiating fibre cells of deeper outer cortex (Figure 4-14G) and remains strongly cytoplasmic only in the very peripheral fibre cells in the outer cortex (Figure 4-14F). No signs of C-terminal truncation of AQP5 are observed in these young lenses.

Figure 4-14: Distribution of AQP0 and AQP5 at postnatal stage P22. An overview of an equatorial cryosection from a P22 mouse lens labelled with the membrane marker WGA (A, red) to indicate where higher magnification images for AQP0 (B-E, green) and AQP5 (F-I green) were obtained. AQP0 is detected in fibre cell membranes of the outer cortex (B,C), inner cortex (D) and becomes truncated in the lens core (E). AQP5 is detected predominantly in the cytoplasm of fibre cells in peripheral outer cortex (F), while deeper differentiating fibre cells show mixed cytoplasmic and membrane localisation (G) and exclusively membrane labelling in the inner cortex (H) and lens core (I).
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

It would appear that the development-dependent insertion of AQP5 observed during postnatal development is not restricted to the lens. The corneal epithelium is another ocular tissue known to express AQP5 (Funaki et al. 1998, Kumari et al. 2012, Raina et al. 1995), and I confirmed the strong membrane labelling for AQP5 observed by others in the central corneal epithelium of an 8 month-old adult mouse (Figure 4-15A). However, in sections from embryonic (Figure 4-15C) and postnatal eyes (Figure 4-15B), the labelling for AQP5 was predominantly cytoplasmic. These results from the cornea not only confirm the specificity of the AQP5 antibody (Kumari et al. 2012), but show that in the cornea, another transparent avascular tissue like the lens, AQP5 undergoes a translocation from a cytoplasmic pool to the cell membrane during tissue development that preceeds eye opening at P12/P15.

Figure 4-15: Age dependent membrane insertion of AQP5 in the mouse cornea. Immunolabelling of AQP5 (green) from mouse cornea taken from 8 month (A), postnatal day P0 (B) and embryologic stage E16 (C) animals. AQP5 was localised to the membranes of epithelial cells of the adult cornea (A, inset) while cytoplasmic localisation was evident in the epithelium from embryonic (C, inset) and early postnatal (B, inset) tissue. Nuclei (blue) were stained with DAPI.
4.5. Comparison of the subcellular localisation of AQP0 and AQP5 in the adult lens

After 6 weeks of postnatal development, when mice are considered young adults, both AQP0 C-terminal truncation in the lens core and AQP5 translocation to the fibre cell membrane had continued (Figure 4-16). C-terminal labelling for AQP0 was decreased in the inner cortex (Figure 4-16E) and was absent from the lens core (Figure 4-16F), indicating further C-terminal truncation had occurred with advancing age. In contrast, AQP5 translocation to fibre cell membranes, and a lack of cytoplasmic signal, was evident in the outer cortex (Figure 4-16I), as well as in the inner cortex and lens core (Figure 4-16J, K).

Figure 4-16: Distribution of AQP0 and AQP5 in the adult mouse at 6 weeks. An overview of an equatorial cryosection of a 6 week-old mouse lens labelled with WGA (red) and DAPI (blue) to indicate where higher magnification images of AQP0 (B-F, green) and AQP5 (G-K, green) are taken from. AQP0 is localised to the fibre cell membrane in peripheral, cortical and inner cortical fibre cells. Signal for AQP0 is absent in the core indicating C-terminal truncation. In contrast, AQP5 labelling is cytoplasmic in peripheral fibre cells, but is localised to the fibre cell membrane in all other lens regions.
Since ongoing changes in the immunolabelling patterns of the two fibre cell AQPs in postnatal lens development were observed, the distributions of these two proteins were investigated in lenses taken from mouse at 8 months of age (Figure 4-17). The spatial distribution of AQP0 in the aged mouse lens appeared similar to the younger adult lens in that AQP0 was localised to the membrane of peripheral and cortical lens fibre cells (Figure 4-17B-D). A low level of signal was detected in the inner cortex (Figure 4-17E), and no signal was detected in fibre cells of the lens core (Figure 4-17F). In contrast, AQP5 was detected predominantly in the membranes of fibre cells in all lens regions, while cytoplasmic labelling was also detected in the lens epithelium and peripheral fibre cells (Figure 4-17G). Since no evidence of signal loss that could be attributed to AQP5 C-terminal truncation in the lens core was detected (Figure 4-17K), this would suggest that the function of AQP5 is maintained in this region of the lens.

Figure 4-17: Distribution of AQP0 and AQP5 in the adult mouse at 8 months. An overview of an equatorial lens cryosection from an 8 month-old mouse lens labelled with WGA (red) and DAPI (blue) to indicate where higher magnification images of AQP0 (B-F, green) and AQP5 (G-K, green) are taken from. AQP0 is localised to fibre cell membranes in peripheral and cortical lens fibres. Weaker signal in the inner cortex may indicate truncation of the AQP0 C-terminus (E). In the core AQP0 is truncated, as indicated by a lack of signal (F). In contrast, AQP5 is localised to the fibre cell membranes throughout the lens (G-K). Some cytoplasmic labelling is maintained in epithelial and peripheral lens fibres (G).
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

However, other post-translational modifications that alter AQP5 function cannot be ruled out and will require further investigation. Regardless of the functional implications, my data show that while AQP5 translocation to the fibre cell membrane initially coincides with truncation of the C-terminus of AQP0 in the postnatal lens core, in adult lenses AQP5 continues to translocate to the membranes of fibre cells throughout lens aging in the absence of further AQP0 truncation in the lens cortex.

4.6. Verification of the C-terminus truncation of AQP0 in the core of the mouse lens

To confirm that signal loss using the antibody directed against the AQP0 C-terminus was due to truncation, immunolabelling patterns in the cortex and core of P6, P15, and 6-week-old mouse lenses using two AQP0 antibodies directed against different epitopes were compared (Figure 4-18). Using the AQP0 C-terminal antibody, labelling was detected in the mouse cortex as expected at P6 (Figure 4-18A), P15 (Figure 4-18E) and 6 weeks (Figure 4-18I), and was decreased in the lens core at P6 (Figure 4-18B and Figure 4-18E,I) and absent at P15 (Figure 4-18F) and 6 weeks (Figure 4-18J). Using a polyclonal antibody directed against the whole AQP0 protein, membrane labelling was detected in the cortex (Figure 4-18C, G, K) and lens core (Figure 4-18D, H, L) at each stage of post-natal development analysed. In addition these results were confirmed by MALDI-TOF mass spectrometry of lens membrane protein preparations from the cortex and core of adult mouse lenses (Petrova et al., 2015)
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens

Figure 4-18: AQP0 is truncated in the postnatal mouse lens core. High magnification confocal microscopy of equatorial cryosections of P6 (A-D), P15 (E-H), and 6 week-old (I-L) mouse lenses show AQP0 (green) and cell membrane marker WGA (red) in differentiating fibre cells (A,C; E,G; I,K) and mature fibre cells in the lens core (B,D; F,H; J,L). Using an antibody to the AQP0 C-terminus, signal is detected in differentiating cells in each age (A, E, I), while signal is reduced in the core of the P6 lens (B) and is lost in the lens core of P15 (F) and 6 week-old mice (J). Using a polyclonal antibody to the whole protein, signal for AQP0 in the cortex (C, G, K) and core (D, H, L) is maintained in each age, indicating that the reduction/loss of signal observed in the lens core using the AQP0 C-terminus antibody is due to truncation.
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4.7. Conclusion

In this chapter I have shown how the spatial differences in the expression patterns of AQP5 observed in adult lens fibre cells are initially established by comparing the AQP5 patterns to established AQP0 expression patterns. My results have shown that the expression patterns, subcellular distributions and processing of the two AQPs change during lens development, differentiation and growth.

During embryonic development I found that:

- AQP0 expression was first detected at E11 in the membranes of primary fibre cells that had started to elongate to fill the lens vesicle
- AQP5 expression preceded the expression of AQP0 expression being detected at E10, but was only expressed in the cell cytoplasm
- The pattern of membranous AQP0, and cytoplasmic AQP5 labelling was maintained throughout embryonic development

During the period of postnatal development I found that:

- The initial embryonic pattern of membranous AQP0, and cytoplasmic AQP5 labelling was maintained into the early postnatal period (P1-P6)
- From P6 to P15, loss of AQP0 labelling by the C-terminal antibody in the core occurred, which was mirrored by an increase in the association of AQP5 with the membranes of primary fibre cells and mature fibre cells in the core

Following eye opening in the adult lens I showed that:

- AQP5 continues to translocate to the membranes of fibre cells throughout lens aging in the absence of further AQP0 truncation in the lens cortex.
- The loss of AQP0 signal in the lens core is due to truncation of the C-terminus since a polyclonal antibody raised against the whole protein labelled AQP0 in the core of the mouse lens.

From these observations it is evident that both AQPs are subjected to distinctly different posttranslational modifications that are abruptly initiated during the period of postnatal development.
Chapter 4 – Comparison of the expression patterns of AQP0 and AQP5 in embryonic, postnatal and adult mouse lens development that proceeds eye opening. These changes in the observed subcellular distribution patterns for AQP5 and AQP0 are summarised in Table 4 and correlated with key milestones in lens development.

Table 4: Observed AQP0 and AQP5 subcellular distribution changes correlated to major milestones in lens development.

<table>
<thead>
<tr>
<th>Age</th>
<th>Milestone</th>
<th>Protein Expression Patterns</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>AQP0</td>
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<tr>
<td></td>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>E11.5</td>
<td>Lens Vesicle Formation</td>
<td>M</td>
</tr>
<tr>
<td>E13.5</td>
<td>Vesicle Lumen Disappears</td>
<td>M</td>
</tr>
<tr>
<td>E17.5</td>
<td>AQP1 protein expression *</td>
<td>M</td>
</tr>
<tr>
<td>P0</td>
<td>Birth</td>
<td>M</td>
</tr>
<tr>
<td>P14</td>
<td>Eye Opening</td>
<td>M</td>
</tr>
<tr>
<td>P21</td>
<td>Weaning</td>
<td>M</td>
</tr>
<tr>
<td>P30</td>
<td>Maximal AQP1 expression</td>
<td>M</td>
</tr>
<tr>
<td>P42</td>
<td>Animal reaches adulthood</td>
<td>M</td>
</tr>
</tbody>
</table>

* data from (Varadaraj et al., 2007); C - cytoplasmic, M - membranous, T – truncated

In conclusion, my comparative mapping of the spatial distributions of AQP5 and AQP0 at subcellular resolution in embryonic and postnatal lenses by immunohistochemistry has revealed that the distinct spatial distributions of the two AQPs observed in the adult lens are abruptly established during a narrow window of postnatal development that occurs prior to eye opening. Taken together our results suggest that the truncation of AQP0 and the translocation of AQP5 in mature fibre cells act to increase membrane water permeability in the core of the adult mouse lens, and that with advancing age the contribution of AQP5 to overall water permeability in the lens increases. To test these hypotheses, novel assays to measure the water permeability of differentiating fibre and mature fibre cells are required. In the next sections I detail my efforts to develop and optimise a fluorescence-based assay to measure water permeability in rodent lenses.
Chapter 5. Development and optimisation of a fluorescence-based assay to measure water permeability in lens cells

Having used localisation data derived from immunohistochemistry to develop hypotheses on the relative functional roles of AQP0 and AQP5 in different regions of the lens, I will now describe in this chapter the development of an assay designed to test these hypotheses by measuring the water permeability in lens cells. Since this fluorescence assay was new to our laboratory, I initially invested considerable time into the development and optimisation of the technique, and then into the analysis and validation of the data produced by the assay. Critical technical parameters for the success of the assay included the design of a recording chamber and perfusion system that delivered laminar flow and fast solution exchange, and the use of Cell-Tak™ to enhance the adherence of lens cells to the bottom of the recording chamber. Having optimised these technical issues for both epithelial and fibre cell preparations, I then established how acknowledged limitations of the fluorescence assay approach (dye leakage, changes in cell shape and fluorescent probe compartmentalisation) potentially affect the measurement of cell volume in lens cells. Finally, having optimised the system and established its limitations, I then utilised data obtained from rat epithelial cells to illustrate how the data obtained from the assay was analysed to extract time constants that enabled the calculation of both absolute and apparent water permeability. I will begin this section by briefly introducing the methods used by others to measure water permeability in a variety of cell types, including the lens, before focusing on the fluorescence assay used in this project.
Chapter 5 – Development and optimisation of a fluorescence-based assay to measure water permeability in lens cells

5.1. Methods to measure the water permeability of cell membranes

The water permeability of cell membranes is determined by both the diffusion of water through the lipid bilayer and the membrane proteins of the aquaporin family of water channels. A direct approach to measure water permeability is to measure the rate of change in cell volume in response to osmotic challenge. A number of methods that use different principles to measure rapid changes in cell volume have been developed which are summarised in Table 5.

Table 5: Summary of methods used to measure cell water volume changes

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle used to measure cell volume</th>
<th>Technical Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dye dilution</td>
<td>Measures the change in concentration of a fluorescent volume marker</td>
<td>Dye photobleaching, photodamage of cells, requirement of confocal microscope for optical sectioning or use of high NA objectives</td>
</tr>
<tr>
<td>Light scattering</td>
<td>Measures reflected light intensity</td>
<td>Needs to be corroborated with measurements by additional methods to determine cell height</td>
</tr>
<tr>
<td>Dynamic fluorescence quenching</td>
<td>Measure the collisional quenching of chemical compounds sensitive to changes in the concentration of intracellular ions</td>
<td>Only performed in Cl− free solutions</td>
</tr>
<tr>
<td>Fluorescence self-quenching</td>
<td>Loading of a fluorophore marker to measure its self-quenching after hypertonic cell shrinkage</td>
<td>Only suitable for selected cells as the required high fluorophore concentration can be toxic to the cells</td>
</tr>
<tr>
<td>Cell height</td>
<td>Measures cell height by attaching fluorescent microbeads at the basal and apical side</td>
<td>Not useful for measuring rapid changes in cell volume</td>
</tr>
<tr>
<td>Morphometric</td>
<td>Uses optical images to measure the change in cell size</td>
<td>Assuming isomorphic change of shape through the whole cell and limited to cells with simple spherical geometry</td>
</tr>
<tr>
<td>Laser scanning reflection microscopy</td>
<td>Recording of vertical-reflection mode x-z-scan section areas after hypotonic swelling of unstained cells</td>
<td>Use of expensive equipment (Confocal laser scanning microscopes)</td>
</tr>
<tr>
<td>Ion-sensitive electrodes</td>
<td>Use of microelectrodes to measure changes in the concentration of impermeant ions loaded in the cell</td>
<td>Invasive, requires membrane permeabilisation and use of specialised microelectrodes</td>
</tr>
<tr>
<td>Electrical impedance</td>
<td>Measures extracellular electrical resistance</td>
<td>Requires specialised equipment such as gold covered, low-impedance inert electrodes</td>
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</table>

Information in this table was obtained from (Johnson, 1998, Srinivas and Bonanno, 2003, Srinivas et al., 2003). NA-numerical aperture

5.1.1. Methods used to measure water permeability in the lens

The water permeability of lens epithelial and fibre cells has been measured using morphometric methods which monitored changes in the cross sectional cell shape and calculated changes in cell volume in response to exposure to a hypertonic challenge (Varadaraj et al., 1999, Varadaraj et al., 2005). In these studies, fibre cell membrane vesicles,
formed by isolating the long and thin fibre cells in the presence of extracellular Ca\(^{2+}\) to induce spontaneous vesiculation, were used to measure water permeability (Wang et al., 2001). These membrane vesicles have a relatively simple spherical morphology, similar to the spherical shape of dissociated lens epithelial cells, and were therefore a suitable preparation to compare the water permeability of lens epithelial and fibre cells. Using a morphological approach to measure changes in cell volume, Varadaraj et al showed that lens rabbit epithelial cells exhibit a Hg\(^{2+}\)-sensitive water permeability (Control \(P_{H2O}\) 136 ±17\(\mu m/s\); 1mM Hg\(^{2+}\) 10 ± 6\(\mu m/s\)), that was ~4 fold higher than the Hg\(^{2+}\)-insensitive water permeability (Control \(P_{H2O}\) 32 ± 7\(\mu m/s\);) observed in lens fibre vesicles (Varadaraj et al., 2005). To determine the contribution of AQP0 to the water permeability of lens fibre cell membranes, these researchers utilised Cataract Fraser (CatFr) mouse lenses, which express a mutant form of AQP0 that does not traffic to the plasma membrane. They found that the water permeability of mutant fibre cell vesicles (\(P_{H2O}\) = 7\(\mu m/s\)) was 20% of wild type vesicles (\(P_{H2O}\) = 35\(\mu m/s\)). Furthermore, increasing either the Ca\(^{2+}\) concentration or reducing pH increased water permeability of wild type fibre cell vesicles by 2.2 fold and 2.1 fold, respectively, but had no effect on fibre vesicles derived from CatFr lenses, suggesting that the changes in water permeability induced by Ca\(^{2+}\) and pH are mediated by AQP0.

While these measurements have shown that AQP0 forms a Hg\(^{2+}\)-insensitive water channel, the water permeability of which is increased by increasing Ca\(^{2+}\) and reducing pH, the morphometric approach used to measure the rate in change in cell volume used in these studies suffers from a number of inherent limitations (Table 1). These measurements rely on the assumption that in response to osmotic challenge cells symmetrically change their volume so that the change in volume can then be simply calculated from knowledge of the change in cross sectional area. Furthermore, the accuracy of these measurements is limited by difficulties in identifying the precise membrane outline of cells. Due to these limitations I have chosen to use the fluorescent dye dilution method to measure relative changes in cell volume. Of course this method has its own inherent limitations (Table 1) which I will discuss in general terms in the next section, before describing how the assay was optimised for use on lens cells.

5.1.2. Use of fluorescent probes to measure membrane water permeability

Fluorescent dye dilution is an optical method that measures how the concentration of a fluorescent dye loaded in a cell changes in response to an applied osmotic challenge. If the
intracellular content of the probe is constant, changes in its concentration reflect changes in cell water volume and hence changes in fluorescence intensity are inversely proportional to changes in cell volume (Figure 5-1).

![Diagram showing the principle of the fluorescent dye dilution technique to measure changes in cell volume.](image)

**Figure 5-1: The principle of the fluorescent dye dilution technique to measure changes in cell volume.** Each large circle represents a cell, loaded with fluorescent dye as a marker of cell volume (●), that has been placed in solutions of differing extracellular tonicity. Since the intracellular content of the dye remains constant and its concentration is inversely proportional to cellular water content, changes in cell volume in response to an applied extracellular osmotic challenge can be monitored in real time as a change in fluorescence intensity and used to calculate the water permeability of the cell membrane.

The technique initially utilised fluorescent dyes such as 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF), or the aminopolycarboxylic acid derivative FURA-2, both of which were primarily designed for use as ion indicators to measure changes in intracellular pH and Ca\(^{2+}\), respectively (Miyata et al., 1989, Muallem et al., 1992). These dyes were subsequently replaced with calcein acetomethyl (AM) which is more suited to measurements of cell volume due to its brightness, stability and commercial availability (Alvarez-Leefmans et al., 1995). The inherent chemical composition of calcein AM allows it to be non-invasively loaded into the cells without compromising the cell membrane. This is achieved by chemical shielding of the charges on the carboxyl groups with AM groups which transform the probe into a non-fluorescent, electroneutral and hydrophobic molecule that readily diffuses across cell membranes. Once inside the cell the dye undergoes hydrolysis of the AM groups by endogenous cellular esterases. This hydrolysed form of calcein is not only highly fluorescent, but also hydrophilic and therefore membrane impermeant, a change which effectively traps the dye within the cell (De Clerck et al., 1994).
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Not only is calcein well internalised and retained (Johnson, 1998), but its fluorescence intensity is independent of changes of native intracellular ion concentration, rendering it insensitive to changes in intracellular pH, Ca\(^{2+}\) and Mg\(^{2+}\) levels, but sensitive to changes in cell water and therefore volume. In addition, calcein is some two to three times more fluorescent than other fluorescent probes with well separated excitation (497nm) and emission (516nm) peaks (Figure 5-2) that can be detected by standard FITC filter sets, and which provide a higher signal to noise ratio and baseline stability relative to other dyes (Crowe et al., 1995). Finally, using the calcein AM dye as a selective volume reporter has the additional advantage that the hydrolysis of the dye by intracellular esterases acts as a reporter of cell viability (Callewaert et al., 1991). However, this method also has its own limitations, some of which are addressed below in the context of optimising the assay system to enabling the real time monitoring of changes in cell volume in epithelial cell and fibre cell vesicles.

Figure 5-2: Spectral and structural properties of the fluorescent reporter dye calcein. Calcein is a highly fluorescent dye with excitation (blue) at 497nm and emission (red) at 516nm. Insert: Chemical structure of calcein. Image obtained from ThermoFisher Scientific at www.thermofisher.com/nz.
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5.2. Optimisation of the fluorescence-based assay

Before measuring the water permeability in lens cells I spent considerable time optimising several critical parameters required for successful performance of the fluorescence-based assay. This involved designing an appropriate perfusion chamber that permitted fast solution exchange with a time constant significantly faster than the water permeation rates, generated laminar flow, and minimised unstirred layer effects. I further tested and optimised the delivery of external solutions to the recording chamber and methods that allowed good retention of cells/vesicles to the bottom of the recording chamber. My efforts to optimise these critical parameters are detailed below.

5.2.1. Chamber design and perfusion rate

To illustrate how critical the chamber design is to the success of these experiments, I have included an example of my initial attempts to monitor changes in cell volume using a commercial diamond-shaped chamber (Figure 5-3A), which the manufacturers claimed to produce a laminar flow across the bath at flow rates of less than 1ml/min\(^1\) (RC-25F, Series 20 Chambers, Warner Instruments; Model No. 64-0233). Despite the claims of the manufacturer, the shape of the bath proved unsuitable for generation of laminar fluid flow. This was visualised by the addition of Toluidine Blue dye to perfusion buffers, and revealed that this shape of chamber induced vigorous fluid turbulences along the edges of the bath that caused cell and vesicle movement and often detachment from the chamber bottom. Furthermore, the large volume (500µl) of the bath prevented the rapid and homogenous solution exchange critically required to produce the necessary step change in solution osmolarity. Finally, efforts to increase the delivery of solution to this chamber, via the use of a peristaltic pump, produced solution switching artefacts due to changes in the depth of solution in the recording chamber that interfered with the analysis of the rate of volume change in the cells that remained attached to the recording chamber (Figure 5-3B).
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Figure 5-3: Characterisation of the Warner Instruments recording chamber. (A) Schematic of the chamber obtained from Warner Instruments showing the dimensions of the diamond-shaped perfusion chamber. (B) Rate of change in calcein fluorescence in a fibre cell vesicle in response to delivery of a hypertonic solution (390mOsmol/L) delivered to the Warner Instruments chamber by a peristaltic pump showing artefactual changes in fluorescence associated with the change in solution.
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The poor performance of the Warner Instruments chamber prompted me to construct an alternative chamber to fulfil the crucial requirements of rapid exchange of bath solutions and minimal focal displacement of sample during solution changes. The final design is shown in Figure 5-4A and consists of a simple slot (2mm wide x 1.5mm deep x 21mm long) machined on to a rectangular piece of plexiglass into which a glass cover slip (22 x 40mm, size #1) could be sealed with Vaseline to form the bottom of the recording chamber. The slot is divided into two sections: the perfusion bath with a volume of 150µl, and the suction reservoir. The solutions enter the perfusion bath from a four way manifold fitted through a short section of tubing that has an internal diameter of 1mm. Since the tubing diameter and depth of perfusion bath are of a similar size, turbulent flow was virtually eliminated and a laminar flow was achieved. In addition, by coupling this re-designed chamber to elevated solution reservoirs (Figure 2-3), I could simply switch between reservoirs and utilise gravity feed to rapidly change the osmolarity of the solution entering the chamber. Cells in the chamber were initially perfused with isotonic (300mOsmol/L) solution at a flow rate of 5ml.min⁻¹ before changing to a hypertonic (390mOsmol/L) solution and perfused at a slower rate of 3.5ml.min⁻¹ to account for the increased density of the hypertonic solution relative to the isotonic solution. At these flow rates the chamber volume was completely exchanged with a time constant of 1.5-2s (Figure 5-4B). Unfortunately, at flow rates of this magnitude, I found that occasionally epithelial cells and, more frequently, fibre cell membrane vesicles had a poor adhesion to the glass coverslip, prompting me to develop ways of increasing the adhesion of cells to the glass coverslip that comprised the bottom of the chamber.
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Figure 5-4: Characterisation of flow rates in a custom designed perfusion chamber. (A) Schematic diagram of the custom designed chamber showing the ports for perfusion and bath dimensions. (B) Time course of solution exchange measured by monitoring the rate of change in fluorescence intensity as hypertonic solution containing Fluorescein is washed into the bath.
5.2.2. Adhesion of epithelial cells and fibre cell vesicles to the recording chamber

Despite achieving laminar flow conditions, the high flow rates required to rapidly exchange the bath volume meant that I experienced considerable loss of epithelial cells and fibre cell membrane vesicles. Vesicles are essentially membranous globular sacks that are formed from swollen isolated fibre cells (Wang et al., 2001, Charras, 2008), and hence contain disrupted cytoskeleton, lack internal cellular organelles, and are therefore not as robust as epithelial cells. They were therefore more likely to be either “battered” or displaced by rapid perfusion. To reduce these adverse effects I trialled a series of immobilisation agents, such as Poly-L-Lysine and Cell-Tak™, in an effort to immobilise the cells to the chamber bottom. I found that Cell-Tak™ was the superior adhesive agent, producing an effective and gentle immobilisation of both epithelial cells and fibre cell vesicles onto the coverslip.

5.3. Considerations in using fluorescence dilution for measuring water permeability

Having discussed the optimisation of the chamber design, perfusion system and attachment of the cells to the bottom of the recording chamber, I will now turn my attention to the optimisation of the fluorescence assay used to measure the rate of change in cell volume. In doing so, I will point out the inherent limitations of the technique that need to be taken into consideration when using this approach to calculate water permeability.

5.3.1. Photobleaching and Photodamage

Photobleaching is the irreversible reduction in light output of an indicator as a result of exposure to strong light. The mechanisms of photobleaching are not completely understood, but involve destruction of the fluorophore or changes in its quantum yield, which is defined as the number of emitted photons per absorbed photon. The rate of photobleaching depends on the chemical environment and the intensity of the excitation light. With respect to photobleaching and light intensity, it is still controversial whether exposure to more intense light for shorter times produces more bleaching than exposure to less intense light over a longer time period. In addition, a fluorescently loaded cell is susceptible to damage by light through a process which is not well understood, but may involve the production of free radicals within the cell. Both processes depend on the intensity and the duration of exposure to the excitation light and the concentration of intracellular entrapped dye, which can vary
between cells within the sample being analysed. In an effort to reduce photobleaching and photodamage, I used low levels of excitation light and minimised the exposure times to duration of 0.2s. My data collection protocol was designed to collect a continuous series of time points at 0.5s intervals using an electronic shutter which additionally minimised the time of light exposure. Damaging UV radiation was eliminated by the use of a monochromator. Despite my best efforts, some photobleaching was observed which was corrected for during analysis and is described in Section 5.4 of this chapter.

5.3.2. Dye leakage

Dye leakage may produce an undesirable drift in the baseline fluorescence level since many cells are capable of removing loaded dyes via multidrug resistance transporter proteins (Homolya et al., 1993). This problem is not serious in the case of calcein, which seems to be well retained due to its highly charged nature. Dye leakage was estimated from calcein loaded rat fibre cell membrane vesicles (n=7) by monitoring the fluorescence level for 30min. Data was collected at 30s intervals at a shutter opening of 0.2s duration to minimise bleaching of the probe. After 30min, a 5 to 10% reduction in the fluorescence level was observed (Figure 5-5) indicating a good retention of the calcein probe.
Figure 5-5: Estimation of dye leakage in rat fibre cell membrane vesicles. Passive dye leakage and active membrane extrusion was estimated in mouse fibre cell vesicles loaded with calcein AM by monitoring the fluorescence intensity levels over a 30min period. To eliminate the difference in the level of fluorescence staining between vesicles, relative fluorescence was used to monitor the leakage and was expressed as $F_t/F_0$ where $F_0$ is the baseline fluorescence level and $F_t$ is the change in fluorescence. A 5-10% dye loss was detected over the time period, indicating a good retention of the dye. Scale bar 10µm.
5.3.3. Vesicle movement and shape change

The fluorescence dilution assay monitors the change in signal intensity of a fixed region of increase in a cell, and since the cell changes shape in response to an osmotic challenge the focal volume being recorded from could also change during the recording. To minimize this potential problem, the objective of the epi-fluorescent microscope was focused at a focal plane where the fluorescence intensity is maximal, which I predicted to be near or at the axial center of the cell/vesicle. This assumption was tested by comparing the pixel intensity profiles of a collection of serial optical slices (z-stacks) obtained from calcein-loaded vesicles imaged through the z-axis on a confocal microscope (Crowe et al. 1995), as shown in Figure 5-6. Because the volume of a spherical vesicle changes with the cube of the vesicle radius, changes in the axial path length due to changes in vesicle volume are negligible, or at least minimized. For example, for a spherical vesicle or cell having a radius of 15 µm, an increase (or decrease) in volume as large as 50% would produce an increase (or decrease) in the axial path length of about 2 µm. The pixel intensities of a spatial area within the vesicle (Figure 5-6B) defined by optical slices 25 to 45, exhibit maximal fluorescence intensity which corresponds to an optical segment with a 4 µm thickness located at the axial center of the vesicle. Any changes (decrease or increase) in the axial path length will therefore be contained within this optical volume and will therefore have a minimal effect on the collected signal intensity. In addition, movements along the x-axis are tolerable within ±2 µm from the axial center. Thus it appears that focusing at the axial centre of a cell/vesicle does maximise the fluorescent signal and that changes in the path length will have a minimal effect on fluorescence intensity.
Figure 5-6: Effect of changes in the path length on fluorescence intensity. Distribution of fluorescence intensities of a fibre cell membrane vesicle loaded with calcein AM that was optically sectioned in 0.2µm steps by a confocal microscope. (A) Representative images (18 to 48) of a single vesicle extracted from a z-series of 59 optical sections. The white rectangular box represents a region selected within each of the ten optical sections which was used to plot individual pixel intensity profiles shown in B. (B) Pixel intensity profiles obtained from selected images from the z series. The maximal pixel intensity is contained within slices 26 to 41, located in the axial center, whereas slices 18, 21 and 48 fall below and above the axial center and display lower pixel intensity. The optical slices 25 to 45 are located in close proximity to the axial center of the vesicle and compose an optical segment within the vesicle with a thickness of around 4µm.
5.3.4. Investigation of the osmotic relationship and dye binding in rat epithelial cells and fibre cell vesicles

Although the change in fluorescence of calcein AM is inversely proportional to changes in cell volume, the change in signal intensity cannot be directly converted into an absolute change in cell volume due to differences in the concentration of the dye loaded into individual cells and differences in the size of the cells being measured. Changes in the fluorescence signal can in principle be converted to an absolute change in cell volume by a calibration procedure that involves characterising the relationship between the relative fluorescence and the external osmolarity of the bathing solution (Crowe et al., 1995). I have attempted to study this relationship by measuring the changes in relative fluorescence intensity that occurred when rat epithelial cells were briefly exposed to 390mOsmol/L hypertonic or 210mOsmol/L hypotonic saline (a ±30% change in osmolarity relative to isotonic saline) (Figure 5-7A&B).
Figure 5-7: Osmotic response of rat epithelial cells to solutions with varying osmolarity. Representative time course of the change in normalized fluorescence intensity recorded from rat lens epithelial cells loaded with calcein dye and exposed to either Hypotonic (A) or Hypertonic (B) challenge. (C) The relationship between changes of the external osmolarity $Osm_0/Osm_t$ and the reciprocal of relative fluorescence $F_0/F_t$ of five rat epithelial cells was fitted with a regression line to examine the slope and ordinate interception. The red line represents a hypothetical cell with a perfect osmometric behavior. Solutions used: Isotonic – isotonic saline, Hypertonic – hypertonic saline, Hypotonic – hypotonic saline.
Exposure of rat epithelial cells to either hypotonic or hypertonic solutions resulted in rapid changes to the relative fluorescence intensities that reached an apparent steady state within ~30s after the onset of osmotic challenge. Using these particular exposure times and osmolarities tested, the cells returned to their initial baseline fluorescence levels upon reintroduction of isotonic saline. Under these conditions the cells did not appear to activate any regulatory volume responses to combat the sudden change in osmolarity, indicating that the change in fluorescence intensity is faithfully reporting the actual change in cell volume, and both hypo- and hypertonic challenges can be potentially used as experimental perturbations to determine water permeability. However, exposure of the epithelial cells to 210mOsmol/L hypotonic saline resulted in an increased incidence of cell membrane rupture. This effect of hypotonic challenge was more pronounced in rat fibre cell vesicles with ~80% of vesicles exhibiting membrane rupture that was manifested as a sudden, irreversible decrease in the fluorescence signal. These observations led me to adopt 390mOsmol/L saline as the preferred solution to investigate the water permeability of epithelial cells and fibre cell membrane vesicles, since both cell types appeared to tolerate this intervention better and it produced an increase in signal intensity which was easier to detect than the signal decrease initiated by exposure to hypotonic solutions.

From these observations I further plotted the relationship between the reciprocal of the apparent steady state changes in fluorescence ($F_0/F_l$) as a function of the reciprocal of the relative osmotic pressure of the medium ($osm_0/osm_t$) (Figure 5-7C). I found that the relationship between these two variables is linear, suggesting that changes in fluorescence intensity reflect changes in the concentration of calcein and therefore changes in cell volume. However, calculation of the slope of the line was 0.24 instead of 1, which is the gradient expected for ideal osmometric behaviour. The gradient of one is anticipated in the ideal situation where the change in fluorescence is simply proportional to change in calcein concentration and all dye molecules are expected to be freely distributed within the cell cytoplasm and are hence osmotically active. This departure from the ideal osmometric behaviour suggests that not all of the fluorescence signal originates from osmotically responsive dye molecules, and that a substantial fraction of dye is unresponsive due to either being trapped in internal cellular compartments or is directly bound to cellular proteins or lipids. To quantify the fraction of dye that was unresponsive to osmotic challenge, I fitted my data with a linear relationship that resulted in an intercept on the Y-axis of 0.73, that suggests ~73% of the calcein probe is not responding to the change in external osmolarity.
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Although high, this value is consistent with observations in other cell types (McManus et al., 1993).

I have further attempted to assess the fraction of bound or compartmentalised dye by permeabilising epithelial cells or fibre cell vesicles with digitonin (Crowe et al., 1995). It is known that membrane permeable dyes such as calcein-AM not only permeate the plasma membrane, but also the membranes of intracellular organelles. Once reporter dyes are taken up into these intracellular compartments the cell effectively contains two pools of dye: one associated with intracellular organelles (compartmentalised dye) and another associated with the cytoplasm (free dye). This compartmentalization, and in addition any binding of dye to proteins or lipids within the cell, causes errors in the quantitative assessment of the actual change in concentration of the free dye within the cytosol of the cell. To estimate the amount of releasable dye (cytosolic plus intracellular organelle pools) and the amount of non-releasable bound dye, cells have been exposed to various detergents that differentially permeabilise the plasma membrane and membranes of intracellular organelles (Crowe et al., 1995). Using this approaches it has been shown for a variety of different cell types that the amount of non-responsive dye is between 60-68% (Blatter and Wier, 1990, Roe et al., 1990, Crowe et al., 1995).

In the current study I have utilised a low concentration of digitonin in an attempt to differentiate between the two pools of releasable dye and the bound fraction of calcein in loaded epithelial and fibre cell membranes vesicles (Figure 5-8). For the epithelial cells, digitonin induced two different modes of calcein release that based on the literature; I have interpreted as being indicative of the differential permeation of the plasma and intracellular membranes. In a small number of cells, digitonin induced a partial release of the dye (~40%), which I interpreted as being the free cytoplasmic pool of calcein that is responsive to changes in cell volume (Figure 5-8A). However, in most cells digitonin produced a rapid and large release of dye (~80%) which is consistent with release from both the cytoplasmic and intracellular organelle pools. This shows that approximately 20% of the dye is bound within the cell. This interpretation was supported by the fluorescence images taken after cell permeabilisation that showed epithelial cells that exhibited large calcein release contained microdomains of residual labelling (Figure 5-8B, b), while those in which a slow release was observed had a more homogenous and bright staining, which was consistent with the fact that the cell retained most of the compartmentalised calcein dye. (Figure 5-8B, a).
In contrast, treating rat fibre cell vesicles with digitonin produced a rapid and extensive release of the dye (~90%, Figure 5-8A), which was expected since the membrane vesicles that are formed from the breakup of the elongated fibre cells most probably do not contain any significant intracellular organelles that could accumulate the dye. This assumption is supported by the observation of an even distribution of the residual bound calcein dye followed digitonin release (Figure 5-8B, c). Together, these experiments confirm that in rat epithelial cells, up to 60% of the calcein dye is either compartmentalised or bound, while in fibre cell membrane vesicles, only ~10% of the dye is bound and unresponsive.

**Figure 5-8: Dye compartmentalisation and binding in rat epithelial cells and fibre cell membrane vesicles.**

(A) Membrane permeabilisation of rat epithelial cells with 1 µM digitonin resulted in either a slow or rapid loss of fluorescence intensity, whereas membrane vesicles only showed a rapid and large loss of dye. (B) Images of calcein loading before (left) and after (right) digitonin permeabilisation of rat epithelial cells that show slow (a) or rapid (b) dye release or fibre cell vesicles (c) that only showed rapid release. Note the area of residual calcein staining (arrow) in the cell that exhibited large dye release suggests that dye is trapped in residual organelles and is not released after permeabilisation with digitonin. Scale bar 5 µm.
This assessment of the bound fraction of calcein dye in rat epithelial cells can in principle be used to calibrate the change in fluorescence to produce a quantification of the actual change in cell volume and the calculation of water permeability. I have not attempted this in rat lens epithelial cells since I have primarily utilised them as a cell system to develop and test the fluorescence dye dilution assay before applying it to fibre cell membrane vesicles that express AQP5 and AQP0. Thus the observed change in the volume of epithelial cells, and therefore their calculated water permeability is probably underestimated relative to fibre cell vesicles due to the higher percentage of compartmentalised dye in epithelial cells relative to membrane vesicles. A detailed account on the calculation of this relative water permeability is presented in the next section.

5.4. Analysis of water permeability in rat epithelial cells

Having optimised the recording system and the data collection protocols, and investigated the inherent limitations of the assay in both epithelial cells and fibre cell membrane vesicles, in this section I will illustrate how water permeability is calculated using a representative response of rat lens epithelial cells to hyperosmotic challenge. Using a morphologic method to measure the change in cell volume Varadaraj et al., have utilised equation (1) to calculate absolute water permeability (Varadaraj et al., 1999):

\[ P_{H2O} = \frac{1}{S_m c_i(0) - c_0} \frac{dV_i(0)}{dt} \]  

This equation is based on the assumption that water flows in and out of the cell driven by the gradient in osmolarity established across the membrane at a rate determined by the surface area \((S_m)\) and rate of change volume of the cell \(\frac{dV_i(0)}{dt}\).

In the sections below I describe how I have extracted the surface area and rate of change volume from my fluorescence dilution assay to calculate an apparent water permeability that I use in Chapter 6 to compare the relative water permeability of epithelial cells and fibre cell membrane vesicles in rat and mouse lenses in the absence and presence of mercury.
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5.4.1. Data collection protocol

The optimised protocol for performing osmotic cell shrinkage includes adhesion of the calcein loaded epithelial cells into a perfusion chamber pre-coated with Cell-Tak™ for 30min before washing for 3-5min with isotonic 300mOsmol/L saline containing nominally zero Ca\(^{2+}\) ions at a 5ml.min\(^{-1}\) flow rate to wash off any loosely attached cells. To estimate the initial cell surface area (\(S_m\), \(\mu\text{m}^2\)), a DIC image was taken prior to commencing the fluorescence recording. Upon starting fluorescence recording a 1-3min long recording was performed by bathing the cells in isotonic saline to establish the underlying bleach curve due to calcein photobleaching, prior to challenge with 390mOsmol/L hypertonic saline to determine the water permeability by monitoring the time course of the change in fluorescence intensity (Figure 5-9A). This is achieved by drawing a circular selection within the cell on the digitised image around its axial centre to record the average intensity of each pixel within this region of interest. The average change in fluorescence intensity in the selected analysis region was recorded using Imaging Workbench 5.2 software.

5.4.2. Calculation of surface area

The initial membrane surface area \(S_m\) (\(\mu\text{m}^2\)) was determined from the initial observation of the cross sectional area (A) and radius (r, \(\mu\text{m}\)) (Figure 1-9A) given by:

\[
S_m = 4\pi r^2
\]  

(2)

The radius (r, \(\mu\text{m}\)) of the cell was calculated by tracing the pixels within a designated region of interest using ImageJ software from the following equation:

\[
r = \frac{\sqrt{A}}{\sqrt{\pi}}
\]  

(3)

where A is the observed cross sectional area.
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Figure 5-9: Correction of photobleaching in a rat lens epithelial cell loaded with calcein. (A) DIC and fluorescence images of a representative rat lens epithelial cell before and after exposure to 390mOsmol/L saline. The DIC image was used to calculate the area of the cell, while the average change in fluorescence intensity was collected from a region of interest (black circle) in the fluorescent images. Scale bar 10µm. (B) Plots of the change in fluorescence intensity in response to a change in extracellular osmolarity are shown for the raw (grey) and bleach corrected (black) signals. The fit parameters obtained from fitting an exponential curve (orange) to the raw fluorescence signal to provide the bleach correction are shown.
5.4.3. Calculating the rate of change in volume and absolute water permeability of epithelial cells

An initial decrease of the fluorescence signal due to bleaching of the calcein dye by exposure to excitation light is shown in the uncorrected trace in Figure 5-9B. To characterise and subtract this exponential bleaching process from the induced signal change caused by the osmotic challenge, a short 1-3min recording was performed under control conditions (isotonic 300mOsmol/L saline) and an exponential curve fitted through this baseline region of the data to obtain an equation that described the bleaching process. The extracted bleach curve was then subtracted point by point from the raw data to correct for the bleaching process across the entire trace. The corrected data set, that contained the response of the cell to the change in extracellular osmolarity, could then be further analysed to calculate cell water permeability by analysis of the individual responses to the hypertonic challenge.

In order to calculate water permeability, it is necessary to measure the initial rate of shrinking of a cell in response to a change in extracellular osmolarity (Varadaraj et al., 1999). One approach commonly used in the literature (Gao et al., 2006) is to measure the maximal slope of the change in fluorescence in response to the osmotic challenge. However, in my system I found that due to inherent noise in the data there was a lot of scatter in the first derivative extracted from the data trace. This made it an unreliable parameter to utilise in the subsequent calculation of water permeability. Instead, I fitted an exponential curve through all of the values recorded since the time constant is a parameter which describes the response of the cell to a complete volume decrease and averages any inherent noise associated with the measurement of the change in volume (Figure 5-10).

To calculate the initial volume (V1) and final volume (V2) I used the linear equation Y=0.2447X+0.76 obtained from the best fitted line of the relationship between fluorescence (F0/Ft) and osmolarity in Figure 1-7C. Since X=Osm0/Osm=0.76, substituting X in the linear equation Y=0.2449*0.76+0.7361=0.92 and V1/V2=0.92 therefore V2=V1/0.92=1.08V1.

Using the best fit values for the time constant (τ) and the initial volume (V1) and final volume (V2), the rate of change in volume can be calculated as:

\[
\frac{dV(t)}{dt} = \frac{V2-V1}{\tau}
\]
By substituting equation 4 into equation 1 and using the parameters \( C_{\text{H}_2\text{O}} \approx 55\text{M} \) as the concentration of water, \( C_i(0) = 300\text{mOsmol/L} \) as the initial bathing solution equal to the initial internal osmolarity of the cell and \( C_0 = 390\text{mOsmol/L} \) it is possible to calculate the absolute water permeability in \( \mu\text{m/s} \). For the epithelial cell shown in Figure 5-9 the absolute \( P_{\text{H}_2\text{O}} = 17.9\mu\text{m/s} \), while the average for 9 epithelial cells was \( 18.4 \pm 1.1\mu\text{m/s} \).

Figure 5-10: Extraction of the time constant that describes the rate of change in fluorescence in a rat lens epithelial cell. A plot of bleach corrected normalised fluorescence intensity versus time recorded from a single rat lens epithelial exposed to hypertonic challenge (black). The change in signal intensity in response to the addition of hypertonic solution has been fitted with an exponential curve (orange) to extract the time constant, \( \tau \).
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This approach to calculating the absolute water permeability depends on the ability to convert the rate of change in fluorescence intensity into a change in cell volume. Unfortunately, I was unable to obtain a full calibration of this relationship in fibre cell membrane vesicles due to the bursting of the vesicles upon exposure to hyposmotic challenge. Therefore rather than calculating the absolute water permeability of fibre cell membrane vesicles I had to calculate an apparent water permeability as outlined below.

5.4.4. Calculation of apparent water permeability

To estimate the apparent water permeability I used the difference in the initial and final fluorescence from the rate of change in fluorescence fitted to the following equation:

$$\frac{dF_i(0)}{dt} = \frac{F_2 - F_1}{\tau}$$  \hspace{1cm} (5)

where $F_1$ and $F_2$ are the best fit values for the initial and final fluorescence, respectively; and $\tau$ is the time constant extracted from the rate of change of fluorescence induced by hyperosmotic challenge. By implementing the rate of change in fluorescence into Eq. 6 an apparent water permeability can be calculated which for the epithelial cell illustrated in Figure 1-9 is $P_{H2O} = 14.9 \times 10^{-3}$ which is expressed in arbitrary units (AU).

$$P_{H2O} = \frac{1}{S_m} \frac{C_{H2O}}{C_i(0) - C_o} \frac{dF_i(0)}{dt}$$  \hspace{1cm} (6)

In the absence of a calibration to convert the rate of change in fluorescence into a change in volume the water permeability calculated for fibre cell membrane vesicles can only be expressed as apparent water permeability. While not an absolute water permeability this approach still allows me to compare the relative permeability between epithelial cells and fibre cell membrane vesicles and whether the permeability is sensitive to mercury inhibition.
5.5. Summary

In this chapter I have described the development and optimisation of a fluorescence dye dilution method to measure the water permeability of rat lens epithelial cells and fibre cell membrane vesicles. This process involved:

- The design and construction of an optimal recording chamber and perfusion system that provided fast solution exchange with laminar flow
- The use of Cell-Tak™ to adhere cells and vesicles to the bottom of the recording chamber, thereby increasing cellular retention and reducing movement artefacts
- Evaluating potential effects of the known limitations of the fluorescence dilution method (dye leakage, location of the region of interest within a cell and dye compartmentalisation) on the accuracy of measurements of cell volume in lens epithelial cells and fibre cell membrane vesicles
- Establishing a linear relationship between the change in fluorescence signal and a change in extracellular osmolarity for epithelial cells, but not for fibre cell membrane vesicles
- Using the time course of change in fluorescence in response to a hypertonic challenge in a single rat lens epithelial cell to demonstrate how the absolute and apparent values for the water permeability of a cell membrane can be calculated

Using my fluorescence dilution assay I have calculated an average absolute water permeability of 18.4 ± 1.1µm/s for rat lens epithelial cells. In contrast, Varadaraj et al., using a morphometric approach have calculated for rabbit epithelial cells ($P_{H2O}=136±17\mu m/s$) that is 7.5 fold higher (Varadaraj et al., 1999). This difference is most likely due to the inherent assumptions associated with using the two different experimental approaches (Table 1). The morphometric method uses 2D images of cell area to extract changes in cellular radius that allow the rate of change of cell volume to be calculated by assuming an isotropic change in volume, a condition which is generally not applicable to adherent cells. While the use of the fluorescence dilution approach to calculate absolute water permeability has its own technical limitations, the ultimate calculate of an absolute value for water permeability using this approach is reliant on the accuracy of conversion of the change in fluorescence intensity into a change in cell volume. While I have been able to obtain a calibration for rat epithelial cells
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to make this calibration, I was unable to quantify the relationship between the change in fluorescence and volume in fibre cell membrane vesicles due to the fragility of vesicles following exposure to hyposmotic challenge. Hence rather than calculating an absolute water permeability that will be potentially imprecise, I have instead chosen to use the rate of change in fluorescence to calculate an apparent water permeability.

The apparent water permeability is expressed in arbitrary units and is effectively a parameter that allows the rate at which cells/vesicles change their volume in response to an applied osmotic gradient to be calculated thereby facilitating comparison between cell types and/or the effects of pharmacological reagents that alter membrane water permeability. Having optimised and characterised the fluorescence dilution assay I will use it in the next Chapter to calculate the apparent water permeability of epithelial cells and fibre cell membrane vesicles isolated from rat and mouse lenses that exhibit different subcellular distributions of AQP5. Furthermore, by incubating lens cells from the two species in the absence and presence of mercury, the apparent water permeability can be used to confirm if the Hg^{2+}-sensitive water channel, AQP5, contributes to the water permeability of lens fibre cells.
Chapter 6. Water permeability of lens cells

In pervious chapters I have used immunolocalisation data to advance various hypotheses about the relative contributions made by AQP0 and AQP5 to fibre cell function in the different regions of the lens. While in the last chapter I developed, optimised and tested a fluorescence dye dilution assay that allows the relative water permeability of isolated epithelial cells and fibre cell membrane vesicles to be compared, in this chapter I have applied this assay to lens cells isolated from the outer cortex of the rat and mouse lenses in order to determine the relative contributions of AQP5 and AQP0 to the water permeability in this specific region of the lens. To functionally distinguish between the two AQPs in the outer cortex, I adopted two approaches. The first involved a pharmacological approach that utilised the differential sensitivity of the two water channels to mercury compounds and allowed functional AQP5 channels to be detected as a Hg$^{2+}$-sensitive water permeability. The second approach utilised my finding that the membrane localisation of AQP5 in the outer cortex of the rat and mouse lens is different (Figure 3-3 & Figure 3-7). Using these two approaches, I show that epithelial cells isolated from both the rat and mouse lens have high water permeability that is blocked by mercury, as would be expected by the expression of the Hg$^{2+}$-sensitive water channels AQP1 and AQP5 in these cells. In contrast, fibre cell membrane vesicles isolated from the outer cortex of the rat lens had low water permeability relative to the two epithelial cells and were not sensitive to mercury. Mouse fibre cell membrane vesicles exhibited a higher water permeability that was significantly inhibited by mercury. In this section, I first present results that show lens epithelial cells contain the Hg$^{2+}$-sensitive water channels AQP1 and AQP5 in order to establish appropriate parameters for my pharmacological approach, before concentrating on comparing the water permeability of fibre cell membrane vesicles prepared from the outer cortex of both species of lens incubated in the absence and presence of mercury.
6.1. The water permeability of lens epithelial cells

Lens epithelial cells are known to express both AQP1 (Varadaraj et al., 2007) and AQP5 (Figure 3-6). Both AQP1 and AQP5, due to the presence of a common cysteine located close to the NPA motif in the water pore (Figure 6-1), are sensitive to inhibition by mercury compounds (Chandy et al., 1997, Krane et al., 2001). In contrast, AQP0 does not contain this critical cysteine residue (Figure 6-1), and therefore AQP0 water channels either expressed in oocytes (Mulders et al., 1995,) or fibre cell vesicles (Mulders et al., 1995, Varadaraj et al., 1999) are insensitive to mercury compounds.

![Figure 6-1: Mercury sensitivity in AQPs. Alignment of the amino acid sequence of the human AQP0, AQP1 and AQP5 around the conserved NPA motif (orange box) showing the location of the cysteine residue 181 (red box) in AQP1 and AQP5 that confers sensitivity to mercury compounds, but is absent in AQP0 which has an alanine at this position (blue box). The stars represent conserved amino acid residues. The alignment was performed on ClustalW at http://www.genome.jp/tools/clustalw/.

While this differential sensitivity to mercury compounds could be potentially used to determine the relative contributions of AQP0 and AQP5 to overall water permeability in fibre cell membrane vesicles, I first wanted to establish the optimal conditions for utilising mercury to inhibit water permeability using my fluorescence dye dilution technique. To establish these parameters, I initiated a study into the effects of different mercury concentrations on the water permeability of lens epithelial cells isolated from the rat lens.
6.1.1. Isolation and characterisation of rat lens epithelial cell morphology

Lens epithelial cells were obtained by dissociating the cells that adhered to the lens capsule following its removal from the underlying fibre cell mass with a pair of sharpened forceps (Chapter 2, 2.6.2). The resultant population of isolated cells had a predominantly circular shape (Figure 6-2A), although a few ovoid cells were detected (Figure 6-2, insert a) that were representative of equatorial epithelial cells that had started to differentiate and elongate into fibre cells. Isolated epithelial cells loaded with calcein-AM produced bright fluorescent labelling, indicating a strong intracellular esterase activity which was indicative of the viability of the isolated cells (Figure 6-2B). In this context, dying or dead cells are recognised in the fluorescent images as an absence of calcein loading (Figure 6-2B, arrow). A defining feature of epithelial cells that distinguishes them from fibre cell membrane vesicles is the presence of cell nuclei. Using DIC optics, the differences in refractive properties of the nuclei and cellular cytoplasm are usually apparent and therefore serve as a robust method to distinguish between epithelial cells and fibre cell membrane vesicles. This is further illustrated in a representative cluster of 4 cells that have been stained with the nuclear stain Hoechst 33258 (Figure 6-2, insert c). Finally, to facilitate future comparisons between epithelial cells and fibre cell membrane vesicles, I conducted an analysis of the size distribution of rat epithelial cells (Figure 6-3), which revealed a somewhat skewed distribution. On average, rat epithelial cells had a radius of 7.3 ± 0.1µm, but a large subgroup of epithelial cells, that most likely represented those equatorial cells which are starting to elongate into fibre cells, were also present.
Figure 6-2: Characterization of isolated rat lens epithelial cells. (A) An overview DIC image shows that rat epithelial cells have a predominantly circular morphology, although ovoid-shaped cells that are elongating into fibre cells are also present (a-c). (B) Fluorescent image of the cells shown in A that shows strong calcein loading into viable epithelial cells, while dead or dying cells lack calcein fluorescence (arrow). Note the variation in the intensity of the dye labelling due to the difference in degree of de-esterification of the dye. The lower left inset shows a representative image of four cells in DIC (a), Calcein-AM (b) and nuclei marker Hoechst 33258 (c) to confirm presence of cell nuclei.
Figure 6-3: Morphological analysis of the size distribution of epithelial cells isolated from the rat lens. Frequency distribution histogram showing that, on average, epithelial cells are $7.3 \pm 0.1\mu\text{m}$ in radius although some larger cells are present that presumably represent equatorial cells that are starting to elongate into fibre cells.

6.1.2. Measurement of the water permeability in rat epithelial cells

The response of a rat epithelial cell, incubated in the absence of mercury, to hyperosmotic challenge is shown in Figure 6-4 to illustrate my typical experimental protocol. Initially, cells are bathed in isotonic saline to allow baseline DIC and fluorescence images (Figure 6-4A) to be collected to enable the initial surface area ($S_m$) of the cell, and bleach curve, respectively, to be calculated prior to the onset of the hyperosmotic challenge. Upon changing the osmolarity of the bath to 390mOsmol/L, the cells rapidly shrunk to reach a new steady state volume within 30s, which was evident as an apparent isotropic reduction in cell size (Figure 6-4A) and an increase in normalised fluorescence ($F_t/F_0$) intensity (Figure 6-4B). These changes were reversed upon changing the bath osmolarity back to 300mOsmol/L. The rate of change in fluorescence was then fitted with an exponential curve to extract the time constant ($\tau$) of the change in fluorescence intensity, which reflects the change in cell volume (Figure 6-4C). Finally, water permeability was determined from the estimated initial surface area ($S_m$), the rate of change in cell volume ($\tau$), and the osmotic gradient ($\Delta\pi$) to yield an apparent $P_{\text{H2O}} = 14.2 \times 10^{-3}$ (AU). This experimental protocol was performed on a further 31 epithelial cells derived from 3 different lenses, and mean results are summarised in Figure 6-8.
Figure 6-4: Response of a rat epithelial cell to hyperosmotic challenge in the absence of mercury. (A) DIC and fluorescence images of rat epithelial cells taken prior (t=25 s) during (t=75 s) and after (t=145 s) exposure to 390mOsmol/L saline. Scale bar, 5 µm. (B) Time course of the change in normalized fluorescence (Ft/F0) intensity in response to hyperosmotic challenge. (C) Analysis of the initial change in measured fluorescence by fitting an exponential curve to the data points to extract the time constant, (𝜏), used to calculate water permeability.
Next, the ability of mercury to inhibit AQP-mediated water permeability in rat lens epithelial cells was investigated. Initial attempts to study the effects of 1mM HgCl₂ on water permeability in the same experiment, using the same group of cells, were not successful since the cells did not tolerate multiple exposures to hypertonic challenge in the absence and then presence of mercury. Unfortunately, mercury is highly toxic (Aduayom et al., 2005), and in my experiments this toxicity often manifested itself as membrane blebbing and/or membrane rupture and loss of dye (Figure 6-5). To alleviate the toxic effects of mercury, I chose to perform measurements on separate pools of control and mercury-treated epithelial cells. My protocol for the addition of mercury to lens cells is illustrated in Figure 6-6. It consisted of pre-incubating cells in the presence of two different concentrations (0.3 and 1mM) of HgCl₂ for 5min prior to the introduction of hyperosmotic challenge. Furthermore, only those cells that did not exhibit membrane blebbing or loss of calcein dye were chosen for analysis.
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Figure 6-6: Protocol for monitoring the fluorescence response of mercury-treated lens cells. The protocol involves pre-incubation in mercury for 5min and then the introduction of a hypertonic challenge (390mOsmol/L) in the continued presence of mercury for 1 to 2min before washing off the mercury by returning the cells to an isotonic solution (300mOsmol/L). Fluorescence recording starts after 3min of mercury pre-incubation and stops 1 min after the return to isotonic saline.

Representative results from three cells exposed to hypertonic challenge in the absence (control) or presence of either 0.3 or 1mM HgCl$_2$ are shown in Figure 6-7. While these cells had slightly different volumes and fluorescence intensities, the subsequent analysis of the change in normalised fluorescence (F$_t$/F$_0$) intensity induced by hyperosmotic challenge revealed that although the cells all exhibited the same steady state change in volume, the rate of change ($\tau$) between control and Hg$^{2+}$-treated cells was different. In these three cells the fitted time constants ($\tau$) were 6.0, 9.2 and 14.4s respectively for cells incubated in the absence or presence of either 0.3 or 1 mM HgCl$_2$.

A summary of the changes in steady state F$_t$/F$_0$, $\tau$ and apparent P$_{H2O}$ in response to hypertonic challenge in the absence and presence of mercury are shown in Figure 6-8. There was no statistically significant difference in the magnitude of the response between control and Hg$^{2+}$-treated cells, supporting the idea that blocking water channels does not affect the magnitude of the change in cell volume in response to the applied change in transmembrane osmotic gradient (Figure 6-8A), but that the time taken to reach the new steady state volume ($\tau$) is statistically slower in the presence of mercury (Figure 6-8B). The increase in average $\tau$ observed for Hg$^{2+}$-treated epithelial cells was inversely related to the observed decrease of P$_{H2O}$ (Figure 6-8C) and indicates that the addition of mercury is reducing water permeability by blocking water channels and therefore my assay system is able to detect this change. Also, since there was no statistically significant difference between P$_{H2O}$ values measured in the presence of 0.3mM and 1mM HgCl$_2$, this indicates that at these concentrations, mercury had potentially blocked all AQP1 and AQP5 Hg$_2^+$-sensitive water channels that contribute to
water permeability in rat lens epithelial cells. This suggests that the Hg$^{2+}$-insensitive component of the $P_{H_2O}$ measured in epithelial cells is due to the inherent permeability of the lipid bilayer to water. Furthermore, since 0.3mM HgCl$_2$ was as effective as 1mM HgCl$_2$ and has a lower toxicity on cells, this lower concentration was adopted for all subsequent experiments that utilised fibre cell membrane vesicles derived from both rat and mouse lenses.
Figure 6-7: Changes to cell volume in rat lens epithelial cells in the absence and presence of mercury. (A) DIC images of three epithelial cells incubated in 300mOsmol/L isotonic saline in the absence or presence of either 1mM (b) or 0.3mM (c) HgCl₂. Representative fluorescence images of the same cells taken before (t=5s) and after (t=55s) the addition of 390mOsmol/L hypertonic saline to the recording chamber. Scale bar 5µm. (B) The timecourse of the change in normalised fluorescence ($F_t/F_0$) of cells exposed to hypertonic saline in the absence (control) and presence of 0.3 and 1 mM HgCl₂. Iso-Isotonic saline, Hypertonic-Hypertonic saline
Figure 6-8: Summary of the effects of mercury on the water permeability of rat lens epithelial cells. (A) Histogram showing no statistical significance between the steady state change in the magnitude of $F_t/F_0$ in response to hypertonic challenge in the absence and presence of either 0.3 or 1mM HgCl$_2$. (B) Histogram of the time constant ($\tau$) describing the rate of cell shrinkage in response to hypertonic challenge in the absence and presence of either 0.3 or 1mM HgCl$_2$. (C) Histogram comparing the apparent $P_{H2O}$ calculated for rat lens epithelial cells in the absence and presence of either 0.3 or 1mM HgCl$_2$. Results are shown as Mean ± SEM where *** represents a statistically significant difference ($P < 0.0001$) and NS represents not significantly different from the respective control groups. The number of cells analysed was: control (n=31), 0.3 mM HgCl$_2$ (n=19) and 1 mM HgCl$_2$ (n=25).
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6.2. The water permeability of lens fibre cell membrane vesicles

Having established that I can detect a Hg^{2+}-sensitive water permeability in lens epithelial cells, my next goal was to determine whether I could detect a similar sensitivity to mercury in fibre cell membrane vesicles to confirm that, in addition to AQP0, AQP5 contributes to the water permeability of lens fibre cells. This strategy was applied to fibre cell membrane vesicles derived from both rat and mouse lenses since the two species exhibit differences in the degree to which AQP5 is located in the plasma membrane of cortical fibre cells. Based on this difference, I would expect to see marked differences in the contribution made by Hg^{2+}-sensitive AQP5 water channels to water permeability in the two species. I began this investigation by first characterising the morphology, size distribution, and degree of calcein loading in the membrane vesicles isolated from the outer cortex of both species of lenses.

6.2.1. Isolation and characterisation of fibre cell membrane vesicles

The elongated shape of lens fibre cells (Figure 6-9A) renders them unsuitable for use in the fluorescence dilution assay. However, as outlined previously, if fibre cells are isolated from the overlying epithelium that maintains the lens potential they are unable to maintain a negative membrane potential and initiate a cascade of events (Bhatnagar et al., 1995, Varadaraj et al., 1999) that results in depolarisation, cell swelling, Ca^{2+}-influx and activation of Ca^{2+}-dependent proteases that causes elongated fibre cells to spontaneously form membrane vesicles (Figure 6-9B). These vesicles unfortunately only tend to form from the outer cortex, thereby effectively limiting my studies of water permeability to this peripheral region of the lens. These vesicles take up calcein-AM and cleave it to render it fluorescent, proving that these membrane vesicles are correctly orientated and contain a cytoplasm that retains intracellular esterase activity (Figure 6-9C). Like epithelial cells, fibre cell membrane vesicles loaded with calcein differ in their brightness, which reflects the extent of dye uptake in individual vesicles. However, in general, the intensity of calcein loading in fibre cell membrane vesicles was lower than that observed in epithelial cells, presumably due to a lower esterase activity of fibre cells (Rhodes and Sanderson, 2009). To compensate for this, I loaded fibre cell vesicles in the presence of twice the concentration of calcein-AM that was used to load epithelial cells to ensure equivalent dye loading between the two cellular preparations. This differential esterase activity also provided a convenient means to discriminate between epithelial cells and membrane vesicles, since any epithelial cells that happened to be present.
in a vesicle preparation appeared extremely bright relative to fibre cell membrane vesicles (Figure 6-10).

Figure 6-9: Morphology of rat fibre cells and fibre cell vesicles. (A) DIC image of an isolated cluster of rat fibre cells (arrow) that maintained their elongated cellular morphology in the absence of extracellular Ca$^{2+}$. (B) In the presence of 5mM CaCl$_2$, isolated fibre cells spontaneously disintegrate forming an inhomogeneous population of membrane vesicles. A “ghost” vesicle (arrowhead) displays a visible membrane boundary without any apparent cytoplasmic content. (C) Fluorescence image of the cells shown in B to visualize the extent of calcein loading in membrane vesicles. Note that the “ghost” vesicle (arrowhead), does not load with calcein dye.
Figure 6-10 Differential dye loading discriminates between epithelial cells and fibre cell membrane vesicles. (A) DIC image of a vesicle preparation showing an epithelial cell (arrow) and two fibre cell membrane vesicles (arrowheads). (B). A fluorescence image optimised to detect the two fibre cell membrane vesicles (arrowheads) loaded with calcein results in the epithelial cell (arrow) being greatly saturated due to a greater uptake of calcein. Scale bar, 5µm.
Like the rat, the isolated fibre cells from the mouse lens readily formed membrane vesicles when exposed to Ca$^{2+}$. The size distributions of both rat and mouse fibre cell membrane vesicles are shown in Figure 6-11. The average radius of membrane vesicles from the rat was $9.7 \pm 0.2\mu$m, while membrane vesicles from the mouse were $9.0 \pm 0.2\mu$m.

\textbf{Figure 6-11:} Size distribution of fibre cell membrane vesicles isolated from rat and mouse lenses. Frequency distribution histogram of rat (A) and mouse (B) fibre cell membrane vesicles used to measure water permeability showing that, on average, vesicles from the rat ($9.7 \pm 0.2\mu$m, $n=75$) and mouse ($9.0\mu$m $\pm 0.2\mu$m, $n=60$) lens had similar radii. Data was collect from at least 3 different experiments.
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Having examined the dye loading and size distribution of the rat and mouse fibre cell membrane vesicles, I then wanted to determine whether the subcellular localisation patterns I had observed in lens sections were preserved in membrane vesicles isolated from the outer cortex of the two lens species. To achieve this, I performed immunolabelling for AQP5 and AQP0 on fibre cell membrane vesicles derived from the outer cortex of both species, and representative images of the distribution patterns observed are shown in Figure 6-12. In membrane vesicles isolated from the mouse lens, the majority of vesicles showed AQP5 labelling of the membrane, but in a small number of vesicles AQP5 labelling was more strongly associated with the cytoplasm (Figure 6-12A). In contrast, the majority of rat membrane vesicles exhibited cytoplasmic AQP5 labelling and only rarely did it co-localise with the membrane (Figure 6-12B). In contrast to AQP5, AQP0 labelling in both mouse and rat membrane vesicles was always detected in the membrane. This pattern of AQP5 localisation, predominantly in the membranes of mouse vesicles but in the cytoplasm of rat vesicles, is similar to the distribution pattern observed for AQP5 in sections taken through the outer cortex of the mouse and rat lenses, and confirms that the membrane vesicles derived from the outer cortex are retaining the in vivo differences in the subcellular location of AQP5 in the two species. Having confirmed this retention of the differential localisation of AQP5, I was then able to determine whether the subcellular location of AQP5 affects its contribution to the water permeability of fibre cell membrane vesicles in the two species of lens.
Figure 6-12: Differential subcellular distribution of AQP5 and AQP0 in fibre cell membrane vesicles in the mouse and rat lens. Representative images of fibre cell membrane vesicles derived from the mouse (A) or rat lens (B) labelled with AQP5 (green) and AQP0 (red) (A) Immunolabeling of mouse membrane vesicles showed a predominantly membrane localisation of AQP5 (left panel) that colocalised with the membrane labelling of AQP0 (middle panel) although some vesicles with a cytoplasmic (right panel) labelling pattern were also present. (B) Immunolabelling of rat membrane vesicles showed AQP5 labelling was predominantly found in the cytoplasm (left and middle panels) and rarely in the membrane (right panel); while AQP0 was always associated with the membrane (middle panel). Scale bar 5µm.
6.2.2. Measurement of the water permeability in rat fibre cell membrane vesicles

Based on my immunolocalisation data from both cryosections and membrane vesicles, AQP5 in the outer cortex is predominantly cytoplasmic, while AQP0 is membranous. Hence I would expect that fibre cell vesicles derived from the outer cortex of the rat lens to have a relatively low water permeability that is not sensitive to mercury. To test this hypothesis, I exposed fibre cell membrane vesicles derived from the outer cortex of the rat lens to hyperosmotic challenge in the absence or presence of 0.3mM HgCl₂. As observed for rat epithelial cells, the addition of mercury to the vesicles was somewhat problematic, with the pre-incubation in mercury followed by hypertonic challenge inducing a transient membrane infolding or collapse in 37% of vesicles after subsequent introduction of hypertonic challenge (Figure 6-13). These “collapsed” vesicles were excluded from any further analysis.

Figure 6-13: Effects of mercury on the morphology of rat fibre cell membrane vesicles. A representative response of a rat fiber cell membrane vesicle that is exposed to 0.3mM HgCl₂ contained within the isotonic bath and subsequent hypertonic manipulation shows that the vesicle was initially round (t=32s) and following the 390mOsmol/L hypertonic challenge (t=43 to 131s) the bottom half folded in, causing an artefactual displacement of the calcein dye into the top half of the vesicle. The vesicle resumed an oval shape post challenge without an indication of calcein dye loss. Scale bar, 5µm. Iso-isotonic saline, Hyper-hypertonic saline.
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The responses of two rat fibre cell membrane vesicles that responded to hypertonic challenge in the absence and presence of 0.3mM HgCl₂ with the expected isotropic shrinkage are shown in Figure 6-14. For these two vesicles, the time constants (τ) that described the rate of change in volume in response to hyperosmotic challenge were 9.2s and 11.3s in the absence and presence of 0.3mM HgCl₂, respectively (Figure 6-14B). By repeating these measurements on multiple vesicles, I was able to extract average values for the water permeability in the presence and absence of 0.3mM HgCl₂, and determine whether a statistically significant Hg²⁺-sensitive water permeability is present in fibre cell vesicles derived from the outer cortex of the rat lens (Figure 6-15). This analysis showed that there was a significant difference between the time constant (τ) of control (16.5 ± 0.9s, n=30) and Hg²⁺-treated vesicles (11.9 ± 1.2s, n=15), while the magnitude of the response (control=0.1 ± 0.07 and Hg⁺-treated=0.1 ± 0.01) and apparent water permeability (control=4.8 ± 0.6x10⁻³ (AU) and Hg⁺-treated=3.9 ± 0.4x10⁻³ (AU)) had no significant difference (Figure 6-15). On a closer inspection of the distribution of the magnitude, it appeared that although there was no significant difference between the two groups of vesicles, the magnitude of the Hg²⁺-treated vesicles had a higher standard deviation and consisted of vesicles with predominantly low magnitude, which may explain why they had faster time constants than the control group of vesicles.

The water permeability and its sensitivity to mercury in rat lens fibre cell membrane vesicles is very different to that found in the epithelial cells (Figure 6-16). A comparison of the mean values for water permeability for each cell type showed that fibre cell membrane vesicles displayed a 3 fold lower water permeability than that measured in epithelial cells (Figure 6-16A). To investigate the variation in the different data sets, I have re-plotted the water permeability for each treatment group as a scatter plot (Figure 6-16B). This analysis revealed five membrane vesicles as apparent outliers which exhibited high values for P_H₂O (about 12 x10⁻³ (AU)) that were more like those obtained for epithelial cells (Figure 6-16B). While this subset of membrane vesicles could be due to the presence of epithelial cells in the vesicle preparation, this is highly unlikely since the protocol to produce vesicles involves discarding the capsule and its adherent epithelium. In addition, contaminating epithelial cells could be distinguished from vesicles by the presence of cell nuclei and/or higher levels of dye fluorescence (Figure 6-10). Thus it is more likely that these five outliers represent a subset of vesicles that have higher water permeability due to the presence of AQP5 in their membranes (Figure 6-12B). To confirm this conclusion, the sequential application of mercury to the same
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vesicle population is required, a protocol which proved impossible to perform (see section 6.1.2). It is however interesting to note the absence of the subpopulation of outliers in the mercury-treated fibre cell membrane vesicles as indicated by a reduction in the degree of scatter in this treatment group.

Figure 6-14: Effects of mercury on the response of rat fibre cell membrane vesicles to hyperosmotic challenge. (A) Examination of the morphological changes of vesicles undergoing hypertonic challenge in absence (a) and presence (b) of 0.3mM HgCl₂ shows an isotropic shrinkage. (B). Time course of the change in normalised fluorescence ($F_t/F_0$) of control and mercury-treated cells in response to hypertonic challenge.
Figure 6-15: Summary of the effects of mercury on the water permeability rat lens fibre cell vesicles. (A) Histogram showing no statistical significance between the steady state change in the magnitude of $F_t/F_0$ in response to hypertonic challenge in the absence and presence of either 0.3mM HgCl$_2$. (B) Histogram of the time constant ($\tau$) describing the rate of cell shrinkage in response to hypertonic challenge in the absence and presence of either 0.3mM HgCl$_2$. (C) Histogram comparing the $P_{H2O}$ calculated for rat lens fibre cell membrane vesicles in the absence and presence of 0.3mM HgCl$_2$. **significantly ($P < 0.001$) different from the respective control group. NS, not significantly different from the respective control group. Results shown as Mean $\pm$ SEM. The number of cells analysed was: control (n=30), 0.3 mM HgCl$_2$ (n=15).
Figure 6-16: Comparison of the effect of mercury on the water permeability of rat lens epithelial cells and fibre cell membrane vesicles. (A) Histogram showing the mean water permeability ($P_{H2O}$) for each treatment group. (B) Scatter plot showing the range of $P_{H2O}$ values obtained for each treatment group. This analysis shows that rat fibre cell membrane vesicles have a significantly lower $P_{H2O}$ than epithelial cells and, unlike epithelial cells, this $P_{H2O}$ is not blocked by the addition of mercury. ***significantly different ($P < 0.0001$) from the respective control epithelial cells. NS, not significantly different from the respective control vesicle group. The number of samples analysed was: control rat epithelial cells ($n=31$), epithelial cells + 0.3mM HgCl$_2$ ($n=19$), control rat vesicles ($n=30$), rat vesicles + 0.3mM HgCl$_2$ ($n=15$). Bars show Mean ± SEM.
In summary, my functional analysis of the water permeability of cells from the rat lens has corroborated my immunomapping of the subcellular expression of AQP0 and AQP5. Rat epithelial cells express AQP1 and AQP5 water channels that exhibit a high water permeability that is blocked by mercury. In contrast, fibre cells express both AQP0 and AQP5, but exhibit a mean water permeability that is 3-4 fold lower than that observed for epithelial cells, and which is insensitive to mercury. Taken at face value this result shows that AQP5 water channels do not contribute to the water permeability of fibre cell membrane vesicles derived from the outer cortex of the rat lens. This result is consistent with my immunolocalisation data that shows AQP5 in this region of the rat lens is predominantly confined to a cytoplasmic pool of water channels that do not significantly contribute to the water permeability of the plasma membrane of differentiating fibre cells.

Therefore, to determine whether AQP5 forms functional water channels in the rat lens requires the use of membrane vesicles from the inner cortex or core of the rat lens where AQP5 is predominantly found associated with the membranes of mature fibre cells. Unfortunately, obtaining vesicles from the core of the lens was not possible (Varadaraj et al., 1999). Therefore, an alternative approach was trialled which involved pre-incubating lenses in a hyperosmotic solution to induce membrane insertion of AQP5.

6.2.2.1. Effect of pre-incubation of the rat lens in hypertonic solution on the water permeability of fibre cell membrane vesicles

To potentially increase the water permeability of fibre cell membrane vesicles derived from the outer cortex of the rat lens, AQP5 insertion into the plasma membrane was induced by organ culturing rat lenses in hypertonic solution prior to making membrane vesicles. As shown in Chapter 3, this procedure increased the insertion of AQP5 into the membranes of elongating fibre cells in the mouse lens (Figure 3-10) and a similar phenomenon was reported by others in the laboratory for the rat lens (Kerry Walker, personal communication). If the pre-incubation step resulted in an insertion of functional AQP5 water channels into the membranes of fibre cell vesicles, I should be able to detect this as an increase in the $P_{H2O}$ of rat fibre cell vesicles, and this increase should be sensitive to mercury. Unfortunately, although the pre-incubation of rat lenses in hypertonic solution did produce an increase in the average $P_{H2O}$, this increase was neither significant, nor blocked by the addition of mercury.
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(Figure 6-17). In fact, mercury appeared to paradoxically increase the $P_{H2O}$ of rat fibre cell membrane vesicles pre-incubated in hypertonic solution.

Figure 6-17: Effect of pre-incubation of the rat lens in hypertonic solution on the water permeability of fibre cell membrane vesicles. Histogram comparing the mean water permeability ($P_{H2O}$) calculated for fibre cell membrane vesicles obtained from rat lenses with no prior pre-incubation (control) or following pre-incubation in a hypertonic saline for 2 hours in the presence and absence of 0.3mM HgCl$_2$. **significantly different ($P < 0.001$) from respective control groups, NS-not statistically different from respective control group. Number of vesicles analysed: control rat vesicles (n=30), rat vesicles treated with mercury (n=15) pre-incubated vesicles (n=19) and mercury treated pre-incubated vesicles (n=15).
This unexpected negative result could be due to two reasons. Firstly, I have relied on the assumption that my observation of AQP5 membrane insertion in the organ cultured mouse lens can be extrapolated to the rat lens. While others in the lab (Kerry Walker, personal communication) have observed membrane insertion in cultured rat lenses, this assumption requires more rigorous testing. Secondly, the addition of 0.3mM HgCl₂ to membrane vesicles derived from rat lenses organ-cultured in hypertonic solution actually caused a significant increase in \( P_{H2O} \). This suggests that the pre-incubation of lenses in hypertonic solution may be sensitising the membrane vesicles to the toxic effects of HgCl₂ (Aduayom et al., 2005) inducing a non-specific increase in the permeability of the membrane to water. While the regulation of AQP5 membrane insertion is an area I wish to pursue further, I currently do not have enough background information on the dynamic insertion process in the rat lens to optimise my functional assay. Instead, I chose to utilise the membrane vesicles isolated from the mouse lens which I have shown exhibit a higher degree of AQP5 in their plasma membrane than the rat lens.

### 6.2.3. Measurement of the water permeability in mouse fibre cell membrane vesicles

Relative to the rat lens, the mouse lens has higher expression levels of AQP5 and, apart from the most peripheral elongating fibre cells, the majority of AQP5 labelling is associated with the membranes of fibre cells in the outer cortex from which membrane vesicles are derived. Thus I would predict that fibre cell membrane vesicles derived from the mouse lens would have, on average, higher water permeability than observed for the rat lens, and furthermore the water permeability of mouse vesicles would exhibit an Hg²⁺-sensitive component that reflects the joint contribution of both AQP0 and AQP5 to overall water permeability. With regard to the first prediction, a comparison of mouse and rat vesicles shows that the mean water permeability in mouse vesicles is significantly higher \((7.4 \pm 0.7 \times 10^{-3} \text{ (AU)})\) than in rat vesicles \((4.8 \pm 0.6 \times 10^{-3} \text{ (AU)})\), having a 1.5 fold difference, and the range of \( P_{H2O} \) values in mouse is considerably higher than in rat vesicles (Figure 6-18).
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Figure 6-18: Comparison of the water permeability of fibre cell membrane vesicles derived from the outer cortex of the rat and mouse lenses. Mouse vesicles exhibited a wider range of water permeability ($P_{\text{H}_2\text{O}}$) and a significantly higher mean $P_{\text{H}_2\text{O}}$ than rat vesicles. *significantly ($P < 0.05$) different. Number of rat vesicles ($n=30$) and mouse vesicles ($n=39$). Bars show Mean ± SEM.
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This wider range of water permeability values calculated for mouse membrane vesicles was also evident when compared to mouse epithelial cells (Figure 6-19). In this scatter plot it is apparent that approximately one third of mouse membrane vesicles had water permeability values that were equivalent of that recorded for epithelial cells. As stated before, the cross-contamination of vesicles with epithelial cells is highly unlikely due to the difference in the appearance of the epithelial cells and vesicles when imaged with DIC microscopy and due to the difference in the degree of calcein loading (Figure 6-10). The observation of the increased $P_{H2O}$ rates in mouse vesicles, and a significant shift towards lower $P_{H2O}$ values after inhibition with mercury (Figure 6-19), suggest the presence of a Hg$^{2+}$-sensitive water flux in this sub-group of mouse membrane vesicles, which is mediated by AQP5.
Figure 6-19: Comparison of the water permeability of mouse epithelial cells and mouse fibre cell membrane vesicles. The scatter plot shows that the water permeability ($P_{H2O}$) of mouse epithelial cells is significantly different from the $P_{H2O}$ of mouse fibre cells vesicles, and further that the $P_{H2O}$ in mouse membrane vesicles is significantly reduced by mercury. **significantly ($P < 0.001$) different from control epithelial cells. *significantly ($P < 0.05$) different from control vesicle group. The number of samples analysed was: control mouse epithelial cells (n=13), control mouse vesicles (n=39) and 0.3mM HgCl$_2$ treated vesicles (n=20). Bars show Mean $\pm$ SEM.
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6.3. Conclusion

In this chapter I have measured the water permeability of epithelial cells and fibre cell membrane vesicles in both the rat and mouse lens, two species that my earlier results showed have distinctly different subcellular localisations of AQP5 in the membranes of differentiating fibre cells in the outer cortex.

In summary I found that:

Lens epithelial cells isolated from both the rat and mouse lens exhibit high $P_{H2O}$ that was blocked by the addition of the water channel inhibitor mercury, results that are consistent with the expression of the Hg$^{2+}$-sensitive water channels, AQP1 and AQP5, in this cell type.

Rat epithelial cells exhibited higher water permeability (14.4 ± 0.9x10$^{-3}$ AU) that was significantly reduced with 2.5 fold by using 1mM HgCl$_2$ block (5.7 ± 0.5x10$^{-3}$ AU) and with 2.1 fold by applying 0.3mM HgCl$_2$ block (6.8 ± 0.9x10$^{-3}$ AU) a result consistent with blocking epithelial cells water permeability with mercury by previous studies (Varadaraj et al., 1999). There was no statistical difference between the 1mM and 0.3mM Hg$^{2+}$ block suggesting that at this concentrations mercury had a saturation effect on the $P_{H2O}$ of rat epithelial cells. Further examination of the morphology and behaviour of rat epithelial cells showed that although successful in blocking their water permeability the use of 1mM Hg$^{2+}$ caused membrane blebbing and in some instances membrane damage and release of the intracellular calcein dye affecting 40% of the cells. 0.3mM Hg$^{2+}$ block had a milder effect on the morphology of the cells therefore I selected 0.3mM concentration as preferred pharmacological blocker. These observed morphological features may be attributed to the toxic effect of Hg$^{2+}$ that is known to cause changes in the properties/permeability of cellular membranes (Aduayom et al., 2005). Next I measured the water permeability of rat fiber cell vesicles and have found that:

- Rat fibre cell membrane vesicles exhibited a lower $P_{H2O}$ than rat epithelial cells that was not blocked by mercury, a result consistent with the cytoplasmic location of the AQP5 in this region of the rat lens.

Similar to previous publications (Varadaraj et al., 1999, Varadaraj et al., 2007) rat epithelial cells displayed 4 fold higher water permeability than rat membrane fibre cell vesicles (4.8 ±0.6x10$^{-3}$ AU). Blocking rat fibre cell membrane vesicles with 0.3mM Hg$^{2+}$ had no effect on their $P_{H2O}$ level also in line with these studies (Figure 6-16). On a closer inspection of the
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range/variety of $P_{H2O}$ I found that although the majority of the vesicles exhibited low $P_{H2O}$ levels there were 5 outliers with high $P_{H2O}$ similar to the $P_{H2O}$ of rat epithelial cells. This result may be expected since my immunomapping data showed that AQP5 was predominantly cytoplasmic in this peripheral region of the lens therefore AQP5 is not contributing to the water permeability of these vesicles and they display low $P_{H2O}$ levels. The observation of 5 outliers may indicate that these vesicles were derived from deeper cortical region of the lens where AQP5 may be inserted in the membranes. To investigate this hypothesis further, vesicle from the inner cortex may be made and their $P_{H2O}$ level tested. Such comparative measurement of the water permeability of fibre cell vesicles from the outer cortex and inner cortex have been done with rabbit lenses (Varadaraj et al., 1999). It was found that the inner cortical fibre cell vesicles had a 3 fold reduced $P_{H2O}$ in comparison to the outer cortical vesicles. In this regard, finding a difference between the $P_{H2O}$ levels from these specific lenticular regions, it would be interesting to investigate the subcellular localisation of AQP5, which in addition to the lower abundance of AQP0 expression in inner cortical fibre cells may explain the reduced level of $P_{H2O}$ in the inner cortex of rabbit lenses. An attempt to upregulate the insertion of AQP5 into the membranes of rat fibre cells using organ cultured rat lenses in hypertonic incubation did not show a significant difference with the water permeability of rat vesicles derived from non-incubated lenses and that their water permeability was not blocked by mercury. Although negative in nature this observation may be related to a predicted behaviour of AQP5 expression from extrapolated results obtained for mouse organ cultured lenses therefore a future rigorous test are needed to investigate this phenomenon.

To further establish the contribution of AQP5 in the outer cortex of mouse lenses I measured the water permeability of mouse fibre cell vesicles derived from the outer cortex and found:

- Mouse fibre cell vesicles had a significantly lower $P_{H2O}$ than mouse epithelial cells, although they revealed a higher variation in their $P_{H2O}$ level which was overlapping with the $P_{H2O}$ levels of mouse epithelial cells
- Mouse membrane vesicles showed a higher $P_{H2O}$ than rat vesicles that was successfully blocked by mercury, which indicated that in the mouse a sub-group of vesicles may express membrane AQP5 which confers the Hg$^{2+}$-sensitive water component
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Like rat, mouse epithelial cells had significantly higher water permeability (11±0.8) than mouse fibre cell vesicles (7.4±0.8). It appeared that the $P_{H2O}$ of the mouse vesicles had a higher spread/variety and that a third of the vesicles displayed $P_{H2O}$ level within the range of the epithelial cells. Furthermore applying 0.3mM HgCl$_2$ block significantly reduced the water permeability of mouse fibre cell vesicles possibly because of insertion of AQP5 to the membranes of differentiating fibre cells in the outer cortex. This result differed from a recent study investigating the functional contribution of AQP0 in the mouse lens by Varadaraj et al., (Varadaraj et al., 2007) where it was shown that the $P_{H2O}$ of mouse fibre cell vesicles was not blocked by mercury. This difference may stem from the high number of vesicles used in my study which allowed me to find/pick on a sub-population of vesicles that displayed a high water permeability which was Hg$^{2+}$-sensitive suggesting that this vesicles may express membrane AQP5 that was contributing to the increased level of water permeability.

Having concluded the main findings of this chapter, I will next discuss the contribution of AQP5 and AQP0 to the normal physiology of the lens in chapter 7 of my thesis.
Chapter 7. Concluding Discussion

In this final chapter I will first summarise the key conclusions of the previous chapters before discussing the relevance of my findings to a broader understanding of how AQP0 and AQP5 contribute to overall lens structure and function.

7.1. Summary of key findings

Differentiation dependent changes in the distribution of AQP5 in the mammalian lens:

In all species of lens studied:

- AQP5 was expressed throughout all regions of the mammalian lens
- The C-terminal tail of AQP5 does not undergo extensive C-terminal truncation in the core of the lens
- In equatorial sections AQP5 was initially found in a cytoplasmic pool in peripheral differentiating fibre cells of all lens species, but with distance into the lens AQP5 undergoes a differentiation dependent translocation into the plasma membrane, the location of which was species dependent.

While specifically in the mouse lens I have shown that:

- In axial sections translocation of AQP5 to the plasma membranes of differentiating elongating fibre cells increases towards both anterior and posterior poles.
- The cytoplasmic pool of AQP5 in peripheral fibre cell can be induced to insert into the plasma membrane by organ culturing lenses in solutions of different extracellular osmolarity.

Developmental cues determine the change in subcellular distribution and post-translational modification of AQP5 and AQP0 during embryonic and subsequent postnatal growth of the mouse lens:

- AQP0 expression was first detected at E11 in the membranes of primary fibre cells that had started to elongate to fill the lens vesicle
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- AQP5 expression preceded the expression of AQP0 expression being detected at E10, but was only associated with the cell cytoplasm.

- The pattern of membranous AQP0, and cytoplasmic AQP5 labelling was maintained throughout embryonic development.

During the period of postnatal development in the mouse lens I found that:

- The initial embryonic pattern of membranous AQP0, and cytoplasmic AQP5 labelling was maintained into the early postnatal period (P1-P6).

- From P6 to P15 loss of AQP0 labelling by the C-terminal antibody in the nucleus occurred, which was mirrored by an increase in the association of AQP5 with the membranes of primary fibre cells and mature fibre cells in the nucleus.

Following eye opening in the adult mouse lens I showed that:

- AQP5 continues to translocate to the membranes of fibre cells throughout lens aging in the absence of further AQP0 truncation in the lens cortex.

- The loss of AQP0 signal in the lens nucleus was due to truncation of the C-terminus, since a polyclonal antibody raised against the whole protein labelled AQP0 in the nucleus of the mouse lens.

Differential contribution of AQP5 and AQP0 to the water permeability of fibre cells in the different regions of the lens:

- Rat epithelial cells have an apparent $P_{H2O}$ that is both higher than the $P_{H2O}$ in rat fibre cell membrane vesicles and which is sensitive to mercury inhibition.

- Rat fibre cell vesicles apparent $P_{H2O}$ was not blocked by mercury.

- Mouse epithelial cells have a significantly higher apparent $P_{H2O}$ than mouse fibre cells vesicles although a third of the vesicles displayed $P_{H2O}$ levels within the range of $P_{H2O}$ of the epithelial cells.

- Mouse fibre cell vesicles had a significantly higher apparent $P_{H2O}$ than rat fibre cell vesicles.
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- Mouse fibre cell vesicles apparent $P_{H2O}$ was significantly reduced after inhibition with mercury

Taken together my results show that AQP5 and AQP0 exhibit regional differences in their subcellular location (cytoplasmic versus membrane) and their extent of post-translational modification, which change during the course of lens development and postnatal growth. Functionally, it appears that this localisation of AQP5 with the membrane coincides with the addition of a Hg$^{2+}$-sensitive contribution to the total water permeability of fibre cell membranes vesicles. Furthermore, I have shown that at least in the outer cortex of the mouse lens, changes in to the membrane location of AQP5 can be dynamically increased by organ culturing lenses in the presence of an osmotic challenge. In the next sections I will discuss my findings in the context of the relevant literature in order to develop a series of hypotheses that integrate my results into our current understanding of lens structure and function, and where relevant I will highlight areas that will require further work to test these new insights into the maintenance of lens transparency.

7.2. Hypothesis one: Membrane insertion of AQP5 from a cytoplasmic pool of inactive water channels increases the water permeability of fibre cell membranes

A common observation to emerge from all experiments that mapped the distribution of AQP5 in the different species of adult lens was the existence in differentiating fibre cells of a cytoplasm pool of AQP5, and the relative absence of AQP5 labelling in the plasma membrane of these cells. However, as these cells underwent further differentiation there was a shift from a cytoplasmic labelling pattern to a membranous labelling pattern. This phenomenon of differentiation dependent membrane insertion has also been shown for a variety of other membrane proteins expressed in the lens. (Grey et al., 2003, Lim et al., 2006, Lim et al., 2007). Such an observation may be explained by the existence of a cytoplasmic pool of inactive membrane proteins that are inserted into the plasma membrane at specific stages of fibre cell differentiation, thereby delivering a functionally active membrane protein that can alter the function of differentiating fibre cells. Often this membrane insertion event occurred at the transition between differentiating and mature fibre cells.
Since the mature fibre cells lack the necessary cellular organelles required to perform *de novo* protein synthesis, this membrane insertion potentially represents a mechanism by which the functional properties of mature fibre cells can be altered to adjust to changes in their environment as they are internalised into the lens inner cortex (Donaldson et al., 2004). Progressive membrane insertion of AQP5 occurred not only along the equatorial axis but was also observed in the anterior-posterior direction where young fibre cells undergo extensive elongation of their lateral surfaces by extending their tips towards the poles of the lens. This raises the question of the role of this axial AQP5 membrane insertion which requires further investigation since it may have implications in directing fibre cell migration during growth of the adult lens.

The development of a functional assay to measure water permeability allowed me to compare the Hg$^{2+}$-sensitivity of fibre cell membrane vesicles derived from the outer cortex of either the rat or mouse lens that exhibit differences in the subcellular location of AQP5. Despite the presence of cytoplasmic labelling in the very peripheral fibre cells, in the outer cortex of the mouse lens AQP5 was predominantly membranous, whereas in the rat AQP5 was primarily associated with the cytoplasmic pool. I therefore hypothesised that AQP5 would significantly contribute to the water permeability of outer cortical mouse fibre cells, but not to the water permeability of the fibre cells from the same region of rat lens. Subsequent experiments confirmed this prediction and revealed a significant Hg$^{2+}$-sensitive contribution to the average water permeability of fibre cell membrane vesicles derived from the mouse, but not the rat lens, a result consistent with the subcellular localisation of AQP5 in the two species of lens.

Within the data sets collected from both species there was a sub-set of fibre cell membrane vesicles (5 of 30 in rat and 13 of 39 in mouse) that displayed high *apparent* $P_{\text{H}_2\text{O}}$ values. Since this sub-set of high values were not present following the addition of mercury it is reasonable to assume that these vesicles contained the Hg$^{2+}$-sensitive AQP5 water channel that has a water permeability some 20 times higher than AQP0 (Yang and Verkman, 1997). AQP0 protein is approximately 100 times more abundant than AQP5 (Bassnett et al., 2009) so it is not surprising that there was a cluster of vesicles with a very low *apparent* $P_{\text{H}_2\text{O}}$ values that were not Hg$^{2+}$-sensitive, presumably due to the presence of AQP0 in the plasma membrane and AQP5 in the cytoplasmic inactive pool. Having shown that AQP5 contributes to the water permeability of fibre cells it is reasonable to assume that the insertion of AQP5 from the inactive cytoplasmic pool will serve to increase the water permeability of fibre cell
membranes above a basal level provided by AQP0 water channels (Figure 7-1A). It follows therefore, that the observed developmental and differentiation dependent insertion of AQP5 would increase (or maintain) water permeability in different regions of the lens, while the dynamic insertion (and potential removal) of AQP5 would serve to modulate water permeability in peripheral lens fibre cells. Both phenomenon raise questions about the pathways that regulate AQP5 membrane insertion in the lens.

![Figure 7-1](image_url)

Figure 7-1: Regulation of AQP5 membrane translocation. (A) In discrete zones in the outer cortex of the mouse and rat lenses AQP5 is predominately localised to a cytoplasmic pool of inactive water channels. In these cells basal water permeability is mediated by AQP0 water channels and the inherent low permeability of the lipid bilayer to water. Total water permeability can be increased by the insertion of AQP5 channels into the plasma membrane in response to osmotic perturbations and/or signalling pathways associated with fibre cell differentiation. (B) In other cell types, translocation of AQP5 to the membranes has been shown to be regulated by phosphorylation of AQP5 via multiple pathways (see text for details). Legend: AQP-aquaporin, H2O-water, Osm-osmolarity, ACh-acetylcholine, M3-M3 muscarinic receptor, PKA-phosphokinase A, ERK- extra cellular signal-regulated kinase, TRPV4-transient receptor potential vanilloid 4, P-phosphorilation

While the regulation of AQP5 translocation to the cellular membranes in the lens has yet to be identified, in other tissues there is accumulating evidence that a variety of signalling pathways can modulate water permeability by increasing the expression and insertion of AQP5 to the plasma membranes (Figure 7-1B). Similar to the shuttling mechanism described for AQP2 in the renal collecting duct (Noda and Sasaki, 2005), AQP5 has been found to insert into the apical plasma membranes of murine lung epithelial cells (MLE-12) following phosphorylation through the cAMP-dependent PKA pathway (Yang et al., 2003). In addition to the cAMP-dependent PKA pathway a number of other stimuli have been shown to increase...
Chapter 7 – Concluding Discussion

water permeability by increasing AQP5 expression at the mRNA and protein levels and via increased membrane insertion. Exposure to hypertonic challenge was shown to up-regulate AQP5 protein expression through an Extracellular signal-Regulated Kinase (ERK) dependent pathway in mouse lung epithelial (MLE-15) cells (Hoffert et al., 2000). While, a cholinergic stimulation of mouse parotid (salivary) glands, has been found to increase salivary secretion and translocation of AQP5 to the apical membrane after binding of acetylcholine (ACh) to M3 muscarinic receptors, via a process that require the elevation of cytosolic Ca\(^{2+}\) levels, but did not involve PKC activation. Interestingly this translocation of AQP5 to the apical membranes was transient and the amount of AQP5 bound to the apical membranes decreased following longer ACh exposures. In this regard the appearance of a transient level of insertion of AQP5 into the plasma membranes is similar to the shuttling/trafficking of AQP2 from storage cytoplasmic vesicles to the membranes via insertion and then subsequent retrieval back to the cytosol via endocytosis. Although potentially similar in nature to AQP2, the exact molecular mechanisms that regulate the dynamic trafficking of AQP5 to the membrane in response to ACh stimulation needs to be further elucidated.

The above studies demonstrate that the phosphorylation of AQP5 in the lens might be an important molecular mechanism that regulates AQP5 insertion in the plasma membrane to increase water permeability following osmotic perturbations. Although the phosphorylated form of AQP5 has been reported in the mouse lens (Kumari et al., 2012), the peptides that encompass the two predicted phosphorylation sites of AQP5 (S156, T259) were not present in the mouse, bovine or human lenses membrane proteome by mass spectrometry (Wang et al., 2008, Bassnett et al., 2009, Grey et al., 2013). Further investigation is required to establish the phosphorylation status of cytoplasmic and membraneous pools of AQP5, in order to understand the mechanisms that control both the dynamic insertion of AQP5 observed in response to osmotic/mechanical challenge and the membrane insertion that occurs in specific regions of the lens during postnatal development and growth of the lens.

Intriguingly recent reports have revealed a synergistic association between the mechano- and osmo-sensitive transient receptor potential vanilloid (TRPV4) channel. TRPV4 is a non-selective Ca\(^{2+}\) entry channel (Nilius et al., 2004) and co-expression of either AQP5 in acinar salivary cells (Liu et al., 2006), AQP4 in Müller cells of the retina (Jo et al., 2015), or AQP2 in renal cells (Galizia et al., 2012) have been shown to be important in activation of volume regulatory process in these different cell types. The functional pairing of TRPV4 and AQP channels appear to be important for the eliciting a cellular response to hyposmotic swelling.
that was dependent on a Ca\(^{2+}\) influx, since deletion of either TRPV4 or AQP5 resulted in reduced Ca\(^{2+}\) entry and loss of regulatory volume decrease. Thus it appears that the initiation of a volume regulatory responses require a physical association (Liu et al., 2006) between the osmosensing TRPV4 and AQP water channels to trigger appropriate activation of ion transport and water fluxes to restore cell volume. The potential relevance of this association between TRP channels and AQP5 will be discussed in section 7.4.

In conclusion the observed changes in the water permeability of mouse and rat fibre cell vesicles suggest that the insertion of AQP5 in mouse fibre cells in the outer cortex delivers functional AQP5 water channel that increase the water permeability of fibre cells in this region of the mouse lens, while AQP5 in rat vesicles due to its cytoplasmic localisation is not functional resulting in vesicles with an inherently lower water permeability. These species differences in AQP5 distribution can now be exploited to study the signalling pathways that regulate AQP5 insertion in response to external stimuli to determine how the dynamic regulation of fibre cell water permeability affects the overall function of the lens.

7.3. Hypothesis two: Changes in the relative contributions of AQP0 and AQP5 to the water permeability of fibre cell membranes in different regions of the lens occur throughout lens development and postnatal growth

The water fluxes predicted by the model of the circulation system to exist in the lens (Mathias et al., 1997, Mathias et al., 2007, Donaldson et al., 2010) have recently been experimentally confirmed (Gao et al., 2011, Candia et al., 2012, Vaghefi et al., 2012). In this system water follows ion fluxes and crosses cell membranes via water channels. It is important therefore to examine how changes in the subcellular localisation and functionality of AQPs in different regions of the lens may contribute to the directionality of the water fluxes generated by the fluid circulation system. In this section I will discuss how the observed changes to the spatial and temporal localisation patterns of AQP5 and AQP0 and the post-translational modification to AQP0 that occur during lens development and postnatal growth fit into the overall function of the lens.

From the present and previous studies, AQP0 expression was first detected from E11, while AQP5 was already present at E10, but only in the cell cytoplasm. This subcellular distribution
of AQP5 suggests that it does not contribute to cell membrane water permeability at this early developmental stage. Given that the size of the E10/E11 lens is small, and that during embryonic and early postnatal development the lens is nourished by the hyaloid vascular system (HVS), this suggests that nutrient delivery to the developing lens can be achieved in the absence of a microcirculation system. This is consistent with a previous study that proposed the lens microcirculation was not initiated until later in embryonic development (E17.5), when AQP1 protein is first expressed in the lens epithelium (Varadaraj et al. 2007). The same study suggested that the primary function of AQP0 in lens development is structural, providing cell-to-cell adhesion to facilitate development of the ordered cellular architecture of the adult lens, while AQP0 takes on a water transport role later in postnatal development to maintain homeostasis in the growing lens fibre cell mass. This hypothesis is consistent with studies that show AQP0 is essential for the ordered development of the lens since mutations in, or removal of, the AQP0 gene lead to significant lens structural defects (Shiels et al., 1991, Al-Ghoul et al., 2003). From P6 to P15 changes in the observed distribution are obvious since AQP0 undergoes a developmentally programmed C-terminal truncation in the core of the lens which is mirrored by an increase in the association of AQP5 with the membranes of primary and mature fibre cells initially in the core, and then progressively in the outer cortex. In this regard it appears that the most significant spatial distribution and modification changes to both AQPs take place during postnatal development of the lens to accommodate the growing fibre cell mass and to prepare for eye opening. While changes in the localisations of the AQPs that mediate water/circulating fluxes in the adult lens are being established during the period in which the HVS begins to regress (Mitchell et al., 1998). The loss of the HVS results in a switch from a vascular tissue to an avascular tissue that is solely dependent on the fluid microcirculation system to maintain transparency of the lens post eye opening. The changes between the observed expression of AQP5 and AQP0 in the different regions of the lens and the regression of the HVS are shown in Table 6.

While the translocation of AQP5 from the cytoplasm to the membranes of fibre cells first occurs at P6 when AQP0 is first truncated in the lens nucleus, with advancing age AQP5 also translocates to the cell membranes of cortical and peripheral differentiating fibre cells in regions where AQP0 C-terminal truncation does not occur. Thus there appear to be two temporally and spatial resolved zones of AQP5 translocation. The first involves an initial translocation event that starts around P6 and results in the original cytoplasmic pool of AQP5 to be inserted into the membranes of mature fibre cells in the nucleus, and then later into the
membranes of differentiating fibre cells in the cortex. The second zone occurs as a result of the differentiation of epithelial cells into fibre cells a process that continues throughout life and adds additional layers of fibres cells to the lens. Post eye opening these newly derived fibre cells contain a substantial pool of cytoplasmic AQP5 labelling, which is maintained as the differentiating fibre cells elongate towards both poles of the lens and increasingly become internalised by the formation of additional superficial layers of younger differentiating fibre cells. However, at a discrete stage in this continuous process of fibre cell differentiation there is a translocation of AQP5 to the cell membrane.

In the adult mouse lens the transient decrease of AQP5 labelling in the inner cortex, detected with two different C-terminal antibodies, seems to coincide with the re-arrangement of AQP0 expression in alternating junctional plaques/aggregates a result which in the rat lenses have been shown to coincide with a restriction of the extracellular space that results in the formation of extracellular diffusion barrier to larger molecules (Grey et al., 2009). This attenuated AQP5 labelling might be due to a decreased expression of AQP5 in this region of the lens, however no such decrease in labelling intensity was observed in the relevant region of the postnatal lens. It is therefore more like that the decrease in labelling reflects a masking of the antibody epitope presumably due to the binding of an accessory potentially regulatory protein in the this region in the adult lens. Regardless of the exact mechanism the decrease in the intensity of AQP5 labelling in the inner cortex would be expected to decrease the water permeability of fibre cell membranes in the inner cortex a region where water is being removed from the lens core via an intracellular pathway mediated by gap junction channels. Currently this attenuation of AQP5 labelling in the inner cortex is only a passing observation, but it deserves further investigation to confirm its origin and to determine its significance to the overall function of the lens.
Table 6: Correlation between the observed subcellular distribution of AQP0 and AQP5 during lens development and postnatal growth and the regression of the hyaloid vascular system in the mouse lens.

<table>
<thead>
<tr>
<th>Age</th>
<th>Milestone</th>
<th>HVS status</th>
<th>Protein Expression Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AQP0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cortex</td>
</tr>
<tr>
<td>E11.5</td>
<td>Lens Vesicle Formation</td>
<td>Forming</td>
<td>M</td>
</tr>
<tr>
<td>E13.5</td>
<td>Vesicle Lumen Disappears</td>
<td>Present</td>
<td>M</td>
</tr>
<tr>
<td>E17.5</td>
<td>AQP1 protein expression a</td>
<td>Present</td>
<td>M</td>
</tr>
<tr>
<td>P0</td>
<td>Birth</td>
<td>Regressing</td>
<td>M</td>
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<tr>
<td>P14</td>
<td>Eye Opening</td>
<td>Regressing</td>
<td>M</td>
</tr>
<tr>
<td>P21</td>
<td>Weaning</td>
<td>Absent</td>
<td>M</td>
</tr>
<tr>
<td>P30</td>
<td>Maximal AQP1 expression</td>
<td>Absent</td>
<td>M</td>
</tr>
<tr>
<td>P42</td>
<td>Animal reaches adulthood</td>
<td>Absent</td>
<td>M</td>
</tr>
</tbody>
</table>

* data from (Varadaraj et al., 2007); C - cytoplasmic, M - membranous, T – truncated, HVS-hyaloid vascular system

From these observations it is evident that both AQPs are subjected to distinctly different post-translational modifications that are abruptly initiated during the period of postnatal development that precedes eye opening. It has been extensively reported in a variety of species that AQP0 in mature fibre cells undergoes a series of cleavage events that removes C-terminal tail residues 232-263 (Schey et al., 1999, Grey et al., 2009) and 247-263 (Schey et al., 2000) from the rat and human lens, respectively. Such a large truncation would be expected to change either the properties of AQP0, or its interaction with other proteins. Indeed it has been proposed that AQP0 C-terminal truncation occludes the water pore, (Gonen et al., 2004, Harries et al., 2004, Palanivelu et al., 2006), however, water permeability was shown to be maintained in C-terminally truncated AQP0 up to residue 243 (Ball et al., 2003, Kumari and Varadaraj, 2014). The major truncation products observed in the rodent lens, AQP0 1-234 and AQP0 1-238 (Grey et al., 2009), did not traffic to the membrane and therefore their ability to transport water could not be effectively tested. However, irrespective of the direct consequence of AQP0 C-terminal truncation on its inherent water permeability, the interaction of AQP0 with cytoskeletal elements, and/or regulatory proteins such as AKAP2 (Gold et al., 2012) and calmodulin (Németh-Cahalan and Hall, 2000) that bind to the C-terminus of AQP0, would be lost in mature fibre cells in the lens nucleus and suggests that truncation of AQP0 changes its functionality relative to the cortex. In this project I have shown for the first time that AQP0 truncation begins at P6 suggesting that this is a
developmentally programmed truncation and not the result of age-related, non-enzymatic peptide backbone cleavage, as has been reported for AQP0 in other species (Schey et al., 2000). The observed AQP0 C-terminal truncation is mirrored by a translocation of AQP5 to the membranes of mature fibre cells. This sequence of events suggests that the membrane insertion of AQP5 into the membrane may be compensating for a loss in functionality provided by full length AQP0 in the nucleus of the lens.

Based on the observed changes in the subcellular distribution patterns AQP5 and AQP0 and post-translational modifications to AQP0 in the different regions of the adult lens a proposed model of how potential changes in water permeability may contribute to circulating water fluxes in the lens is presented in Figure 7-2. In this model, the water efflux in the outer cortex of the lens is mediated by constitutively expressed AQP0 in fibre cell membranes that provides basal water permeability along with a slow diffusion of water via the lipid bilayer, while AQP5 insertion into the membranes may be regulated, possibly by a PKA dependent pathway (Kumari et al., 2012), to increase the water permeability of peripheral fibre cells in response to osmotic perturbations (Figure 7-2A). In the inner cortex (Figure 7-2B) of the lens redistribution of AQP0 protein in the plasma membrane results in the formation of junctional structures that are suggested to be involved in restriction of the extracellular space to create an extracellular diffusion barrier (Grey et al., 2009). In this region AQP5 immunolabelling signal intensity is reduced, possibly due to association of AQP5 with another protein, that may represent a mechanism to reduce the water permeability of non-junctional membranes in a region where the preferentially movement of water is via an intracellular pathway mediated by gap junctions. In the core of the lens (Figure 7-2C) AQP0 undergoes an extensive cleavage to its C-terminus that we expect would change its water permeability and/or junctional properties, while AQP5 remains uncleaved. Although AQP5 is less abundant than AQP0 (Grey et al., 2013), because it is 20x more permeable to water than AQP0 (Yang and Verkman, 1997), it is interesting to speculate that AQP5 insertion maintains or even increases water permeability in the lens core.
Figure 7-2: Schematic diagram detailing the relative contributions of AQP0 and AQP5 to fluid circulation in the lens. (A) In the outer cortex water efflux is mediated by the basal water permeability ($P_{H2O}$) of water channels formed from AQP0 (green, open structure). In this region of the lens AQP5 (purple) water channels are predominantly present as an inactive cytoplasmic pool of water channels that can be shuttled to the membrane to form active water channels following phosphorylation by signalling pathways to increase water efflux from the lens. (B) In the inner cortex water fluxes are preferentially carried away from the lens core via an intracellular pathway mediated by gap junction channels. In this region AQP0 functionality shifts from being a water channel to a junctional protein (green, closed structure) that reduces $P_{H2O}$ and narrows the extracellular space to form a barrier to extracellular diffusion. In this region the intensity of AQP5 labelling in the membrane is reduced which may represent a reduction in the contribution of AQP5 to $P_{H2O}$ of fibre cells in this region of the lens. (C) In the core of the lens mature fibre cells accumulate fluid delivered to them via the sutures. In this region the C-terminus of AQP0 is cleaved (dark green, closed structure), while AQP5 remains uncleaved and membranous. In this region the post translational changes to AQP0 are expected to alter the regulation of AQP0, and therefore AQP5 may compensate for any loss in the contribution of AQP0 to the $P_{H2O}$ of mature fibre cells.
In conclusion, this comparative mapping of the spatial distributions of AQP5 and AQP0 at subcellular resolution in embryonic and postnatal lenses has revealed that the distinct spatial distributions of the two AQPs observed in the adult lens are abruptly established during a narrow window of postnatal development and coincide with the regression of the HVS prior to eye opening. I propose that these changes in the distribution of AQPs are critical for the development of the lens microcirculation system that enables the avascular adult lens to maintain its transparency. Taken together my results suggest that the truncation of AQP0 and the translocation of AQP5 in mature fibre cells act to maintain or even increase water permeability of mature fibre cell membranes in the core of the adult mouse lens, and that with advancing age the contribution of AQP5 to overall water permeability in the lens increases. To test these hypotheses the development of methods that allow water permeability measurements to be performed on membrane vesicles derived from the mature fibre cells in the lens core are required and will be the focus of ongoing work in our laboratory.

7.4. **Hypothesis three: Dynamic membrane trafficking of AQP5 can alter the water permeability of outer cortical fibre cells to modulate circulating water fluxes in the lens and thereby maintain the optical properties of the lens**

The initial identification of AQP5 expression in the lens has raised questions about why lens fibre cells require a second water channel in addition to the highly abundant AQP0. Indeed while the deletion or mutation of AQP0 (Shiels et al., 1991, Berry et al., 2000) causes lens cataract no structural or morphological differences were observed \textit{in-vivo} and \textit{ex-vivo} between AQP5 KO and wild type lenses (Kumari and Varadaraj, 2013). Furthermore, the knock-in of AQP1 into the AQP0 knockout mice did not rescue the structural damage in the core of AQP0 knockout lenses suggesting that the functional role played by AQP0 in this region of the lens is a structural one (Varadaraj et al., 2010). Taken together these studies point to AQP0 acting as a multifunctional protein with both water channel and structural properties that are essential for basic lens transparency, with AQP5 playing an ancillary role to fine tune water permeability in different regions of the lens (Figure 7-2). In this section, I discuss the possible involvement of AQP5 water channels located in the lens outer cortex in a feedback system that dynamically regulates lens hydrostatic pressure, a key parameter that
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drives the removal of water from the lens core, and therefore helps to maintain the gradient in refractive index which is so critical to the refractive properties of the lens.

A hydrostatic pressure gradient has been measured in the lens and has been shown to be generated by the flow of water through gap junction channels (Gao et al., 2011). This hydrostatic pressure gradient of 0mmHg in the periphery to 335mmHg in the lens centre is thought to drive intracellular flow of fluid from the lens core to the periphery, and is remarkably preserved in several different species (Gao et al., 2013). This conservation of the pressure gradient among species has led to the suggestion that the gradient is actively modulated and controls the water content in the lens core and therefore sets the water/protein ratio that determines the gradient of refractive index that in turn contributes to the optical properties of the lens (Gao et al., 2013).

Membrane transport of Na⁺ drives the circulating ionic currents that drive the fluid fluxes that generate intracellular hydrostatic pressure gradient. Hence, changes in hydrostatic pressure can be used as an indirect indicator of changes in water flow. The magnitude of the circulating Na⁺ currents are the net result from the balanced activities of the Na⁺/K⁺-ATPase, the fibre cell Na⁺ leak conductance, and gap junction channels. Maintaining constant water flow/hydrostatic pressure requires that the activities of these transport proteins to be synchronized. In this regard the regulation of the Na⁺/K⁺ATPase in the lens has been well studied (Shahidullah et al., 2012). Shahidullah et al have shown that in response to hyposmotic challenge TRPV4 channels are activated by the change in cell volume and initiate a signalling cascade that involves the release of ATP into the extracellular space via connexin hemichannels, the activation of ATP sensitive P2Y receptors that increases the activity of a Src family kinase (SFK), that ultimately increases Na⁺/K⁺ATPase activity (Shahidullah et al., 2012). Conversely, Sellitto et al. have shown that Na⁺/K⁺ATPase activity in the lens can be decreased through a mechanism dependent on PI3K (Phosphoinositide 3-kinase)/Akt (Protein kinase B) signal transduction pathway in lens epithelial cells (Sellitto et al., 2013) although the upstream activation of this pathway was not identified in this study. Interestingly, in other tissues TRPV1 has been shown to play an opposing role to TRPV4 in osmoregulation (Liedtke, 2007, Xu et al., 2007) and TRPV1 is known to be expressed in lens epithelial cells (Martinez-Garcia et al., 2013). Recent data presented by Professor Rick Mathias has demonstrated a link between activation of TRP channels and modulation of hydrostatic pressure in the lens. Mathias et al., showed that the application of specific TRPV1 and TRPV4 agonists to the mouse lens resulted in initial decreases and increases in
hydrostatic pressure, respectively. These results suggested that TRPV1 and TRPV4 acts as the initial sensors of two opposing arms of a feedback system that utilise distinct signalling pathways to keep lens hydrostatic pressure constant by modulating Na+/K+ATPase activity (Figure 7-3).

![Proposed feedback control mechanism to maintain constant hydrostatic pressure and fluid transport in the lens.](image)

While Figure 7-3 focusses on regulation of the ionic fluxes that drive water flow through the modulation of the Na+/K+ATPase activity, it is interesting to speculate that changes to the water permeability of fibre cells at the lens surface will also be required to maintain a constant internal hydrostatic pressure in response to changes in Na+ pump rate. In this scenario the observed translocation of AQP5 into the membranes of peripheral fibre cells in organ cultured in the presence of an osmotic challenge takes on a wider significance to overall lens function. As a working hypothesis it is intriguing to speculate that the dynamic regulation of AQP5 membrane trafficking alters the water permeability of outer cortical fibre
Figure 7-4: Proposed relationship between AQP5 mediated changes in water permeability and the regulation of the optical properties of the lens. To improve its optical properties the lens establishes a gradient of refractive index which it actively maintains by removing water from the lens core. Since the removal of water occurs via an intracellular route mediated by gap junction channels, a hydrostatic pressure gradient is generated. The magnitude of this pressure gradient is held relatively constant by two arms of a signalling pathway, that work reciprocally to alter Na$^+$ efflux at the lens equator by modulating the activity of the Na$^+$ pump (See Figure 7-3). In parallel to these changes in pump rate, I propose that the membrane trafficking of AQP5 to and from the membrane alters the water permeability of peripheral fibre cells to alter water efflux, thereby modulating the water gradient so as to maintain the gradient of refractive index that ultimately determines the optical properties. E-epithelial cells, DF- differentiating fibre cells, MF-mature fibre cells, GJ- gap junction, AQP5-Aquaporin 5, P-phosphorilated form, TRPV4- transient receptor potential vanaloid 4.
cells to modulate circulating water fluxes and thereby maintain the optical properties of the lens (Figure 7-4). The optical properties of the lens are critically dependent on the gradient of refractive index that is actively maintained by the circulation system (Vaghefi et al., 2011). More recently it has been shown that blocking the Na⁺ pump increases the water content in the lens core, changes the gradient of refractive index, increases the optical power of the lens and impacts on overall vision quality (Vaghefi et al., 2015). Finally, based on the emerging synergy between TRPV4 channels and AQP water channels in other cell types in response to osmotic challenge (Liu et al., 2006, Galizia et al., 2012, Jo et al., 2015), it is interesting to speculate that TRPV1/4 channel activation in response to changes in hydrostatic pressure provides a link between changes in Na⁺/K⁺ATPase activity, the modulation of water permeability in peripheral fibre cells and the dynamic maintenance of the optical properties of the lens. Thus it would appear that the observed insertion of AQP5 into the membrane in response to osmotic challenge in the in vivo lens may actually be in response to changes in the hydrostatic pressure gradient that are sensed by TRP channels which in turn activate signalling pathways that alter both ion transport and water permeability in parallel to ensure the optical properties of the lens remain constant.

In conclusion, the significance of the existence of a dynamic feedback system that regulates optical performance of the lens is both exciting and far reaching. Not only does it present opportunities to study the system in more detail at the cellular and molecular levels, but also at the tissue and organ system level through studies on the optical properties of the lens and their contribution to the overall vision quality. Imagine pharmacological therapies that modulate visual acuity through modulation of the activity of AQP5.
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