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Roles for Cation Chloride Cotransporters in Diabetic Cataract

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Abstract

Cataract is the leading cause of blindness, and diabetes mellitus (DM) is one of the main risk factors associated with cataract development. DM/hyperglycaemia induced cortical cataract has been associated with dysregulation of cation chloride cotransporters (CCCs) that regulate lens fibre cell volume. In other cell types, the activity of the CCCs such as the Na⁺K⁺Cl⁻ cotransporter 1 (NKCC1) and KCl cotransporter (KCC) are reciprocally regulated by modulation of their phosphorylation status. I have characterised a panel of antibodies and utilised Western blotting to detect the phosphorylation status of NKCC1 and KCC3, and found that the phosphorylation status of NKCC1 is increased and decreased in response to hypertonic and hypotonic stress, respectively. Having optimised the Western blotting protocols, lenses were subsequently cultured in high glucose-artificial aqueous humour (AAH) to mimic the hyperglycaemia observed in diabetes. In these lenses, fibre cells in the lens periphery demonstrate minor swelling at 2 hours, and with increased periods (at 72 hours) of exposure to high glucose, cells lose their ability to regulate their volume resulting in cell swelling and cell damage reminiscent of diabetic cataract. At 2 hours, NKCC1 phosphorylation appeared to decrease, but this was not statistically significant. However, incubation of lenses in high mannitol-AAH, a membrane impermeable non-hydrolysable sugar, for 2 hours caused a significant increase in NKCC1 phosphorylation. My results show NKCC1 phosphorylation can be manipulated in the lens and suggests a role for the dysfunction of CCC regulation in the initiation of cell volume disruption that manifests as diabetic cortical cataract.
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<th>Description</th>
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<tbody>
<tr>
<td>AAH</td>
<td>Artificial aqueous humour</td>
</tr>
<tr>
<td>AR</td>
<td>Aldose reductase</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCC</td>
<td>Cation chloride cotransporter</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>FM</td>
<td>Fibre membrane</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>Gluc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Hyper</td>
<td>Hypertonic</td>
</tr>
<tr>
<td>Hypo</td>
<td>Hypotonic</td>
</tr>
<tr>
<td>Iso</td>
<td>Isotonic</td>
</tr>
<tr>
<td>K</td>
<td>1000</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>KCC</td>
<td>Potassium (K⁺) Chloride (Cl⁻) Cotransporter</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>m</td>
<td>Milli (10⁻³)</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>Mann</td>
<td>Mannitol</td>
</tr>
<tr>
<td>mOsmol/kg</td>
<td>Milli osmoles per kilogram</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NCC</td>
<td>Sodium (Na⁺) Chloride (Cl⁻) Cotransporter</td>
</tr>
<tr>
<td>NKCC</td>
<td>Sodium (Na⁺) Potassium (K⁺) Chloride (Cl⁻) Cotransporter</td>
</tr>
<tr>
<td>NKCC1-P</td>
<td>Phosphorylated NKCC1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NKCC1-T</td>
<td>Total NKCC1</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius</td>
</tr>
<tr>
<td>OSR1</td>
<td>Oxidative Stress Response kinase 1</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PP</td>
<td>Protein phosphatase</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RVD</td>
<td>Regulatory volume decrease</td>
</tr>
<tr>
<td>RVI</td>
<td>Regulatory volume increase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SPAK</td>
<td>STE-20 like Proline Alanine rich kinase</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>WNK</td>
<td>With-No-Lysine(K)</td>
</tr>
<tr>
<td>µ</td>
<td>Micro$(10^{-6})$</td>
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Chapter 1 – Introduction

1 Introduction

Lens cataract manifests as a clouding of the normally transparent lens due to changes in the cellular and molecular structure of the lens that scatter incident light. Cataract is the leading cause of vision loss, accounting for 51% of blindness worldwide (~25 million cases), of which 90% are from developing nations (Obrosova et al., 2010a; Pascolini and Mariotti, 2012). Population and epidemiological studies have revealed that old age and diabetes are the two main risk factors associated with the development of cataract (Forrester et al., 2016; Obrosova et al., 2010a). Hence, with increased life expectancy giving rise to an increasing elderly population, coupled with escalation in the incidence of diabetes (Obrosova et al., 2010a; Pollreisz and Schmidt-Erfurth, 2010), we are faced with a looming cataract epidemic (Donaldson and Lim, 2008). Thankfully cataract induced vision loss can be treated by the surgical replacement of the opaque lens with a plastic intraocular lens to effectively restore vision. However, cataract surgery is currently the most commonly performed surgical procedure worldwide, and while a safe and successful operation, the sheer volume of procedures performed imposes a major economic burden on healthcare systems, particularly in developing regions with the highest need (Chan, 2016). Furthermore, although cataracts can be successfully treated with surgery, it is not without complications, such as posterior subcapsular opacification, and diabetic patients in particular have an increased susceptibility to post-cataract surgery complications, and are more likely to experience poorer prognosis and visual outcomes (Stanga et al., 1999).

Hence, as we are confronted with a cataract epidemic, there is an urgent need for alternative therapeutic treatments to surgery that either delay the onset, or prevent the progression of cataract. In order to achieve this goal, it is essential to understand the underlying molecular mechanisms and pathways involved in maintaining lens transparency and how their
dysfunction lead to the onset of cataract. Findings from the Molecular Vision Laboratory (MVL) at the University of Auckland have suggested that the distinctive damage phenotype of localised fibre cell swelling observed in diabetic cataract (Bond et al., 1996) is associated with a failure of the lens to regulate lens volume (Donaldson et al., 2009). In this regard, ion transporters belonging to the cation chloride cotransporter (CCC) SLC12A family have been shown to mediate fibre cell volume regulation and maintain lens transparency (Chee et al., 2006; Chee et al., 2010; Vorontsova, 2013). The focus of this thesis is to extend this body of work by investigating the relationships between hyperglycaemia, SCL12A family cotransporter activity, and the regulation of cell volume in the lens. In this section I will outline the body of work that has led to this study by beginning with a general overview of lens structure and function before focussing on the roles played by the SCL12A family of transporters in controlling lens volume.

1.1 The Lens

Like the lens of a camera, the crystalline lens of the eye plays a critical role in refracting and focusing light rays to produce a clear image. The lens works in concert with other organs of the eye to allow precise transmission of light. Light is first refracted by the cornea, a transparent avascular tissue located anterior to the lens, and passed through the pupil, an aperture whose size is dictated by the pigmented iris. Subsequently, the lens precisely refracts these incident light rays to a specific point on to the retina by undergoing changes in shape and curvature determined by the tension applied by the zonules. This tension is controlled by contracting and relaxing the ciliary muscle (Delamere, 2009) (Figure 1.1).
Chapter 1 – Introduction

1.1.1 The lens structure

The lens is the largest avascular transparent tissue in the body. It is a biconvex oblate spheroid that is comprised of two distinct cell types: the cuboidal epithelial cells that cover the anterior surface of the lens and the highly differentiated fibre cells that account for majority of the lens mass (Figure 1.2). There are five morphologically distinct regions in the lens: (1) the capsule (2) the epithelial monolayer on the anterior pole, the cortex dividing into (3) outer cortex and (4) inner cortex, and (5) the central core or nucleus (Wride, 2011).

Figure 1.1 Schematic of the eye illustrating the position of the lens.
Chapter 1 – Introduction

**Figure 1.2 The structure of the lens.** An axial section through the mammalian lens. The lens is contained within the capsule. Immediately beneath the anterior capsule is the single layered epithelium, which begins to divide and differentiate at the “germinative zone”. Young, differentiating fibres in the outer cortex (light pink region) lose organelles and become increasingly mature in the inner cortex (dark pink) and nuclear regions (purple regions) establishing an inherent age gradient since the mature fibre cells are retained.

A flexible, protective layer known as the capsule surrounds the lens. The capsule is continually secreted throughout life by a single layer of cuboidal epithelial and fibre cells that lie immediately beneath the capsule of the anterior and posterior poles, respectively. The lens is anchored in place by thin fibrils known as zonules that attach the capsule of the lens to the ciliary body, leaving the lens suspended in the anterior of the eye, dividing the eye into anterior and posterior chambers (Donaldson et al., 2001). The anterior and the posterior surfaces of the lens are bathed in aqueous and vitreous humours, respectively.

A single layer of cuboidal epithelial cells lie beneath the capsule and line the anterior surface of the lens. The central region of this epithelial layer remains relatively quiescent, while the epithelial cells in close proximity to the anterior equator, occupy a “germinative zone”, where cells undergo mitotic division. The resultant daughter cells then enter the “transition zone” at the lens equator, and in response to signals such as growth factors from the posterior of the eye, exit the cell cycle permanently and embark upon the process of differentiation into
highly specialised fibre cells (Lovicu and Robinson, 2004). During this intricate process of differentiation, epithelial cells elongate extensively, eliminate a source of light scattering by degrading their cellular organelles and nuclei, and begin to express fibre specific cytoplasmic and membrane proteins (Donaldson et al., 2001) The elongating fibre cells from opposite hemispheres meet at the poles where they interlock with each other to form the lens sutures (Kuszak et al., 2004). Since this process of epithelial to fibre cell conversion occurs throughout life, differentiating fibre cells are laid down in successive waves in a series of concentric growth shells to produce an inherent age gradient. The youngest, differentiating fibre cells are found closer to the periphery in the lens cortex, with the oldest mature anuclear fibre cells being found in the central core, or nucleus of the lens (Lang, 1997). Since the younger cells internalise the older ones, no cells are lost and the number of cells, size and weight of the lens increases with age (Augusteyn, 2007).

The differentiating fibre cells adopt a flattened hexagonal cross sectional shape that enables the cells to pack neatly in columns, thereby minimizing light scattering by restricting the dimensions of extracellular spaces to less than the wavelength of light (Donaldson and Webb, 2010). Fibre cells communicate with adjacent cells via gap junctions that facilitate the intercellular exchange of ions, water and metabolites. They have unique cellular structures: where they lose their mitochondria, smooth and rough endoplasmic reticulums, Golgi bodies and nuclei during differentiation (Michael et al., 2003). This process compromises the ability of mature fibre cells to synthesize new proteins. Fibre cells are packed with crystallin proteins that account for 95% of total lens protein content (Beebe, 2010). These tightly packed crystalline proteins give rise to a steady refractive index that is higher than the vitreous and aqueous humours that surround the lens. Since the concentration of crystallins is highest in the lens nucleus, a radial gradient in refractive index is created that compensates for inherent spherical aberrations introduced by the cornea and thereby enhances overall
visual acuity in the eye (Vaghefi et al., 2015). In summary, the lack of a blood supply, tight extracellular space, elimination of organelles and the presence of a gradient of refractive index, are the key structural modifications utilized by the lens to eliminate light scattering and establish the transparent and refractive properties of the lens. However, the lens is not a purely passive optical element and requires a unique cellular physiology to maintain its structure and therefore its transparency (Donaldson et al., 2001).

1.2 Lens physiology

The lack of a vascular supply means that the lens has to accumulate nutrients from the aqueous humour that is secreted by the ciliary body. While the anterior epithelium and differentiating fibre cells found at the periphery of the lens are directly bathed by the aqueous and have access to glucose (and other nutrients) and oxygen, deeper lying mature fibre cells do not. It has been shown that the avascular lens is too large in size to depend on passive diffusion alone for supplying nutrients and removal of metabolic waste to and from deeper lying fibre cells (Donaldson et al., 2001). Instead it has been proposed that the lens operates a unique internal microcirculation system to deliver nutrients to and remove waste from the lens nucleus faster than would be achieved by passive diffusion alone (Mathias et al., 1997).

1.2.1 The lens microcirculation system

A series of electrical impedance measurements and theoretical modelling underpinned the initial proposal put forward by Mathias et al., for the existence of an internal microcirculation system (Mathias et al., 1997). This model is based on the observation that in all vertebrate lenses studied to date a circulating ionic current is generated by the lens (Figure 1.3B). This current primarily involves the movement of Na\(^+\), which enters the lens through extracellular clefts between fibre cells predominantly at the lens poles, crosses the plasma membrane to
enter mature fibre cells, before exiting the lens at the equator via an intercellular route mediated by gap junction channels (Mathias et al., 1997).

Figure 1.3 The lens microcirculation model. (A) Overview of the mammalian lens microcirculation current flow. The arrows indicate the direction of the convection current created due to the movement of Na\(^+\) and water in the lens. This current enters the lens at the anterior and posterior poles and passes through the tissue via gap junctions and exits the lens at the equator. (B) An equatorial cross section of the lens showing a cellular view of ion and water movement in the lens. Current and solutes are proposed to flow into the lens via the extracellular space, to cross fibre cell membranes, and to flow outward via an intracellular pathway mediated by gap junction channels. Schematic taken from (Vaghefi et al., 2012), an open access article on Biomed Central, made available under the Creative Commons Attribution License allowing material to be re-used without restrictions, and subject only to proper attribution.
It is hypothesized that this circulating current is driven by an electromotive potential difference between the surface cells (anterior epithelial and differentiating fibre cells) and the internalized mature fibre cells. The anterior epithelial cells and newly differentiating fibre cells possess Na\(^+\)/K\(^+\)ATPase and K\(^+\) channels which set up a negative intracellular membrane potential. In contrast, the internalized mature fibre cells lack Na\(^+\)/K\(^+\)ATPase and K\(^+\) channels, but have a membrane permeability dominated by Na\(^+\) and Cl\(^-\) leak conductances that produce a substantial depolarisation of the fibre cell membrane potential (Mathias et al., 2007). However, due to the extensive electrical coupling between fibre cells provided by numerous gap junction channels, a negative membrane potential is imposed on deeper fibre cells by virtue of their connection to the surface cells that contain Na\(^+\)/K\(^+\)ATPase and K\(^+\) channels. This electrical connection between the two regions of different membrane electrical properties causes a standing current to flow (Donaldson et al., 2001). Furthermore, the differentiating fibre cells in the peripheral regions of the lens express gap junctions in abundance near the equator. As a result, the intracellular current is directed towards the lens equator, where Na\(^+\)/K\(^+\)ATPase have been shown to be concentrated (Gao et al., 2000). This circulating flow of Na\(^+\) ions is thought to create an extracellular flow of water, and this flow of water in turn convects nutrients into the lens core significantly faster (~11 times faster for the frog lens) than the rate of delivery possible by simple diffusion alone (Mathias et al., 2007).

1.2.2 Roles for the microcirculation system

While not initially accepted by all (Beebe and Truscott, 2010), the evidence in favour of the lens microcirculation model of lens physiology is growing (Donaldson et al., 2010). It has been shown that fibre cells differentially express a wide range of Na\(^+\)-dependent and independent transporters that are capable of accumulating the nutrients and endogenous antioxidants convected to them via the circulation system (Li et al., 2007; Lim et al., 2005;
Lim et al., 2007; Lim et al., 2006; Merriman-Smith et al., 2003; Merriman–Smith et al., 1999). Candia et al., employed modified Ussing chambers to measure regional water fluxes and showed that water influx at the poles is equal to water efflux at the equator, data which supports the existence of a circulating water fluxes through the lens (Candia et al., 2012). Experiments utilising a novel combination of MRI and confocal microscopy (Lim et al., 2007; Vaghefi et al., 2012; Vaghefi et al., 2011) have visualized for the first time, the ion and fluid fluxes within the lens that were predicted by the circulation system. Subsequently, the prediction from the model that the circulating flux of water generated by the microcirculation would generate a hydrostatic pressure gradient (Mathias, 1985; Mathias et al., 1997) has recently been confirmed experimentally (Gao et al., 2011). Hence, there is a growing realisation that the microcirculation system is central and critical to the maintenance of the transparency and refractive properties of the lens. The key contributions of the microcirculation system to lens function are briefly outlined in turn.

**Steady state volume regulation:** The microcirculation system controls the ionic homeostasis of individual fibre cells and is therefore critical for the maintenance of steady state lens volume (Donaldson and Webb, 2010). In the cytoplasm of fibre cells, as in all cell types, there is an abundance of large negatively charged macromolecules such as phosphates and proteins that are membrane impermeable. This leads to an increased osmolarity within cells, which if not countered by an extracellular osmolyte will induce cell swelling which can lead to cell rupture. Most cells counter this colloid osmotic pressure by actively removing osmolytes from the cell. The Na⁺/K⁺ATPase by exchanging Na⁺ and K⁺ maintains ion gradients that establish a negatively resting membrane potential that restricts the movement of Cl⁻ into the cell, thereby restricted cell swelling (Hoffmann et al., 2009). Since in the lens functional Na⁺/K⁺ATPase is only found in epithelial and peripheral fibre cells (Mathias et al., 2007), the deeper lying fibre cells rely on their connection to these surface cells to maintain
the intracellular negative potential, which in turn limits the movement of water into the cell and maintains steady state fibre cell volume (Donaldson and Webb, 2010).

Nutrient delivery: It has been shown that fibre cells differentially express a repertoire of Na\(^+\)-dependent and independent transporters that are capable of accumulating the nutrients and endogenous antioxidants convected to them via the circulation system (Li et al., 2007; Lim et al., 2005; Lim et al., 2007; Lim et al., 2006; Merriman-Smith et al., 2003; Merriman–Smith et al., 1999) The energy requirements for lens growth and maintenance of transparency are derived primarily from glucose which is taken up from the aqueous humour (Berman, 2013). While the epithelium and the peripheral fibre cells have easy access to glucose, the deeper lying mature fibre cells do not. However it appears that deeper lying fibre cells require a constant supply of glucose to maintain high levels of glutathione (GSH), the primary lens antioxidant. Maintenance of GSH in the lens nucleus is critical to sustain a reduced redox environment to prevent cross-linking of proteins, crystallin aggregation and light scattering (Lou, 2003). Depleted levels of GSH in the lens nucleus can be regenerated from GSSG (oxidized GSH), via the catalytic activity of glutathione reductase. Glutathione reductase activity has been shown to persist in the nucleus of human lenses albeit at lower levels than found in the outer cortex (Zhang and Augusteyn, 1994). However, glutathione reductase requires NADPH as a reducing agent, which is produced by the metabolism of glucose via the hexokinase shunt pathway (Ganea and Harding, 2006). It is currently not clear whether the reductive environment in the nucleus is maintained by the local metabolism of glucose to maintain NADPH levels. In support of a role for local metabolism, high affinity glucose transporters GLUT3 and SGLT2 have been found to be expressed in mature fibre cells which could potentially mediate the uptake of glucose convected by the microcirculation system (Merriman–Smith et al., 1999). The persistence of glutathione reductase activity in the nucleus coupled with the production of reducing agents via anaerobic metabolism of glucose
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convected to the nucleus by the microcirculation system implies the possibility of GSH regeneration locally in the lens nucleus. These observations have led to the hypothesis that a failure of the microcirculation system to deliver sufficient levels of nutrients and antioxidants to the lens nucleus is an initiating cause of age related nuclear (ARN) cataract (Donaldson and Lim, 2008).

Refractive properties: Recent results have shown that the refractive properties of the lens are maintained by the microcirculation system (Vaghefi et al., 2015). The combined results of the optical properties of the cornea and lens produce the refractive power to enable light to be focussed on the retina. While, the cornea contributes ~2/3 of the refractive power of the eye, it also gives rise to positive spherical aberration (Artal and Guirao, 1998). This positive spherical aberration is subsequently corrected by the inherent Gradient of Refractive Index (GRIN) in the lens that is determined by the lens water to protein ratio (Smith et al., 2001). This gradient is primarily generated by regional differences in over expression of subtypes of lens crystallin proteins with different refractive indices (Pierscionek et al., 1987). The extraordinary solubility of the crystallin proteins are paramount for their function, where their concentration ranges from 240mg/mL in the cortex to 400-600mg/mL in the core (Slingsby et al., 2013). From these observations it was evident that the water/protein ratio needs to be maintained at different depths of the lens. T2 MRI studies have shown that the lens exhibits a water gradient, with high water content in the outer cortex relative to the lens core. This gradient is actively maintained by microcirculation system (Vaghefi et al., 2011), and contributes to increasing concentration of crystallin proteins with depth into the lens. Inhibiting Na+/K+ATPase, which generates the microcirculation system, leads to changes in water content that in turn alter the GRIN and the refractive properties of the lens (Vaghefi et al., 2015).
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Having reviewed the significance of the microcirculation system to overall lens function, I will now focus on how the dysfunction of this integrated cellular physiology is affected in diabetic lens cataract.

1.3 Diabetic cataract –a problem with volume regulation

Morphologically diabetic cataract, in both rat (Bond et al., 1996) and human (Al-Ghoul and Costello, 1993) lenses, manifests itself as a discrete zone of tissue liquefaction that despite the presence of numerous gap junction channels does not spread to adjacent cells, which appear to have a normal morphology. This disruption to the normally ordered tissue architecture in the lens cortex induces light scattering and a cortical opacity. In the rat lens Bond et al., showed that the zone of tissue liquefaction is initiated as discrete localised fibre cell swellings (Figure 1.4). This damage is apparently caused by the inability of the lens to regulate its volume, since diabetic lenses exhibit increases in their hydration and an inability to maintain their ion gradients (Jacob, 1999). Biochemically, diabetic cataract in the rat is associated with a substantial loss of intracellular metabolites/antioxidants (GSH, ATP, Taurine, ascorbate) over the 1-2 week period post streptozotocin-injection (Mitton et al., 1997; Mitton et al., 1999). This period precedes the observed tissue liquefaction, suggesting that the loss of intracellular metabolites/antioxidants is not due to non-specific tissue damage, but the activation of a leak pathway permeable to large molecules. It is now believed that in addition to osmotic stress, the biochemical effects of hyperglycaemia in the lens also include oxidative damage to and/or non-enzymatic glycation of key lens transport proteins involved in regulating the volume of lens fibre cells (Crabbe and Goode, 1998; Kyselova et al., 2004).
Figure 1.4 Cortical tissue liquefaction in the diabetic rat lens 4 weeks after injection with a single dose of streptozotocin. This equatorial cross-section labeled with fluorescein-conjugated wheat germ agglutinin (WGA) (green) and specifically for junctional domains with anti-connexin50 antibodies detected with a rhodamine-conjugated secondary antibody (red). Note transitions between normal fiber cells, swollen cells of increasing size, and large fluid-filled spaces within the liquefaction zone and along its borders. Scale bar = 50μm. Reproduced from (Bond et al., 1996) with permission from ARVO.

However, regardless of the actual nature of the hyperglycaemic insult (osmotic, oxidative, or non-enzymatic glycation), a purely biochemical view of diabetic cataract does not offer any insight into the specific mechanisms responsible for the changes in membrane permeability that results in the loss of ion gradients and the dumping of intracellular metabolites; or why in a highly coupled tissue like the lens (Goodenough, 1992) these changes are localised to a discrete zone in the outer cortex. To address these questions, the MVL has conducted a series of experiments to determine how the lens regulates fibre cell volume with the long term view to understanding how hyperglycaemia affects the regulation of key lens ion channels and transporters to produce the characteristic localised damage phenotype observed in diabetic cataract.
1.4 Lens volume regulation

Clinically and experimentally, osmotic stress has been associated with lens opacification and cataract (Zhang and Jacob, 1996). The crystalline lens is a highly ordered physical structure (Kuszak, 1995; Kuszak et al., 2006) where the unique cellular architecture of fibre cells is conducive to its remarkable transparency. Disruption of this cellular order of the fibre cells, due to cell swelling or dilation of the normally tight intercellular spaces, results in intralenticular light scattering (Donaldson et al., 2009; Jacob, 1999) and ultimately cataract. Therefore, volume regulation at the cellular, as well as tissue level, is crucial for lens homeostasis and transparency. As outlined above, the lens is constantly required to maintain its steady state, but in addition it appears to be able to respond to the volume and shape changes that occur during the elongation of lens epithelial cells into fibre cells (Augusteyn, 2010), and in response to lens accommodation (Zamudio and Candia, 2011). In addition, the lens is able to respond to changes to the osmolarity of the solutions that it is bathed in with a regulated volume increase or decrease (Patterson, 1981, 1983; Patterson and Fournier, 1976; Tunstall et al., 1999). Furthermore, the high concentration of crystallins in the lens core generates an osmotic gradient into the lens core, which needs to be counteracted to prevent swelling of the lens (Donaldson et al., 2001).

1.4.1 Lens volume regulation in response to osmotic challenge – lessons from other cell types

When exposed to osmotic challenge in vitro, the lens demonstrates the ability to regulate its volume (Zhang and Jacob, 1996). Just as seen in other cell types (Hoffmann et al., 2009), the lens fibre cells undergo an initial cell swelling in response to hypotonic stress, and cell shrinkage in response to hypertonic stress, followed by restoration of volume towards steady state values by undergoing a regulatory volume decrease (RVD) or regulatory volume increase (RVI), respectively (Donaldson et al., 2009). This process of RVD was shown to
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involve the loss of K\(^+\) and Cl\(^-\) ions followed by an obligatory loss of water, while RVI occurred via the intracellular accumulation of Na\(^+\), K\(^+\) and Cl\(^-\) and subsequent influx of water. Subsequent experiments on bovine (Zhang and Jacob, 1996) and rat (Chee et al., 2006; Merriman-Smith et al., 2002; Webb et al., 2004) lenses exposed to osmotic challenge in the presence or absence of pharmacological antagonists of Cl\(^-\) transport showed that volume recovery was mediated by Cl\(^-\) channels and members of the cation chloride co-transporter family (Donaldson et al., 2009).

In other cell types, the activity of the SCL12A superfamily of cation-chloride cotransporter (CCC) has been shown to work with Cl\(^-\) channels to alter the balance between entry and exit of Cl\(^-\) (Rinehart et al., 2009; Yerby et al., 1997) with the concomitant flow of osmotically obliged water. This is a widespread cellular mechanism adopted by many cells to maintain steady state cell volume. CCCs comprise of three subfamilies: the sodium potassium chloride cotransporters (NKCC) that consists of two isoforms NKCC1 and 2, the potassium chloride cotransporters (KCC) that has four main isoforms KCC1 to 4; and the sodium chloride cotransporter (NCC). These CCCs are evolutionarily conserved, tightly regulated secondary active cotransporters (Hartmann et al., 2013; Kahle et al., 2015) that are electrically silent. NKCC cotransports Cl\(^-\) ions across biological membranes against its electrochemical gradient by harnessing the potential energy stored in the transmembrane gradient of Na\(^+\) at a stoichiometry of 1Na:1K:2Cl, while KCC harnesses the energy stored in the K\(^+\) gradient to cotransport Cl\(^-\) with a stoichiometry of 1K:1Cl (Gamba, 2005; Hartmann et al., 2013). NKCC1, is the primary mediator of Cl\(^-\) influx into the cell, and is activated by hypertonic stress and cell shrinkage to facilitate RVI. In contrast, KCCs which normally mediate Cl\(^-\) efflux, are activated by hypotonic stress and cell swelling to effect RVD (Rinehart et al., 2009) to restore steady-state cell volume. NKCC and KCC activity is reciprocally regulated via a two tiered system of phosphorylation at select threonine/serine sites on the co-
transporters. Phosphorylation of select threonine/serine sites activates NKCC (Lytle and Forbush, 1992; Moriguchi et al., 2005), but inactivates KCC, while dephosphorylation of these sites inactivates and activates NKCC and KCC, respectively (delos Heros et al., 2014). Threonine (Thr) 212 and Thr 217 on NKCC, and Thr 991 and Thr 1048 on KCC proteins have been identified as the key phosphorylation sites which determine the activity of the co-transporters. The kinases and phosphatases that regulate the phosphorylation status of these CCCs have recently been identified (Kahle et al., 2010). The kinases include; With-No-Lysine (WNK) kinases (de los Heros et al., 2006; Thastrup et al., 2012), the closely related Oxidative Stress Response Kinase 1 (OSR1) and Ste20-like Proline Alanine Rich Kinase (SPAK) (Gagnon et al., 2006; Piechotta et al., 2002), while the phosphatases are protein phosphatase 1 (PP1) and 2A (PP2A) (Franceschi et al., 2006; Gagnon and Delpire, 2010; Gusev and Agalakova, 2010). The WNK kinases are viewed as the osmosensors that detect the osmotic challenge and cell volume perturbation, while kinases SPAK and OSR1, and phosphatases PP1 and PP2A as transducers of cell volume regulation that alter the activity of the CCCs that effect the appropriate change in ion and water fluxes to restore cell volume back towards steady state values (Kahle et al., 2010). Interestingly, lenses cultured in isotonic medium were also susceptible to pharmacological inhibition of Cl\(^{-}\) channels and CCC, a result that suggested that a constitutively active Cl\(^{-}\) flux is present in the lens, which functions to regulate lens volume in the absence of osmotic challenge (Tunstall et al., 1999).

1.4.2 Lens volume regulation under isotonic conditions: a role for cation chloride cotransporter

Experiments on rat lenses organ cultured under isosmotic conditions that have utilised a variety of inhibitors (Chee et al., 2006; Merriman-Smith et al., 2002; Tunstall et al., 1999; Webb et al., 2004; Young et al., 2000), have revealed that blocking Cl\(^{-}\) transport (Figure 1.5E) induces either one of two spatially distinct tissue damage phenotypes, or on
occasions a combination of the two phenotypes. For example, culturing lens in the presence of bumetanide, which preferentially blocks NKCC (Chee et al., 2006), resulted in peripheral cell shrinkage in addition to extracellular fluid accumulation in the deeper lens as seen in response to the Cl⁻ channel inhibitor, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) (Figure 1.5B). In contrast, culturing rat lenses in isotonic AAH supplemented with a KCC inhibitor, DIOA ([(dihydronindenyl)oxy] alkanoic acid) (Figure 1.5C), demonstrated swelling of fibre cells located at the lens periphery up to 150µM from the lens capsule with some extracellular space dilations between cells in the deeper cortex of the lens (Donaldson et al., 2009).

The two distinct damage phenotypes generated by the inhibitors can be explained with reference to previous experimental measurements of lens membrane potential, and the Nernst potential for Cl⁻, $E_{Cl}$ (Mathias, 1985; Mathias and Rae, 1985). These measurements predicted that in the deeper lens the electrochemical gradient favours Cl⁻ influx from the extracellular space, while in the lens periphery it promotes Cl⁻ efflux from the cytoplasm of fibre cells into the surrounding extracellular space. Since these two regions are connected by gap junctions, a circulating current of Cl⁻ is established that maintains an appropriate balance between Cl⁻ influx and efflux that ensures steady state lens volume is achieved (Mathias et al., 2007).

Hence, pharmacological inhibition of Cl⁻ uptake in deeper fibre cells would be expected to cause an accumulation of Cl⁻ ions and water in the tortuous extracellular space and result in extracellular space dilations, while in periphery fibre cells the inhibition of the efflux of Cl⁻ ions would lead to cytosolic pooling of osmolytes and subsequent cell swelling (Donaldson et al., 2009). Based on the microcirculation model, spatially distinct regions in the lens cortex of Cl⁻ ion efflux in the lens periphery and influx in deeper fibre cells have
been identified, where an appropriate balance between Cl⁻ ion influx and efflux is required to maintain steady state lens volume (Donaldson et al., 2009) (Figure 1.5D).
Figure 1.5 Spatially distinct efflux and influx zones in the lens. (A & B) Equatorial cross-sections labelled with the membrane marker wheat germ agglutinin (WGA) of rat lenses incubated in (A) isotonic AAH, (B) AAH +10mM NPPB and (C) AAH+10mM DIOA for 18 hours. Blocking Cl⁻ channels with (B) NPPB disrupts the ordered tissue architecture of the lens and induces a localised band of tissue disorder caused by the dilation of the extracellular spaces between fibre cells (B, insert). In contrast, the major effect of KCC inhibitor DIOA on lens morphology is the swelling of fibre cells in the lens periphery (C) although some extracellular dilations are also observed in deeper cells (C, insert). (D & E) Schematic diagrams summarising the effects of inhibitors of Cl⁻ transport on fibre cell morphology of organ cultured lenses. (D) In the absence of inhibitors, the electrochemical gradient favours Cl⁻ influx in the deeper lens, while in the periphery it promotes Cl⁻ efflux. (E) Blocking Cl⁻ influx mediated by either Cl⁻ channels or NKCC with NPPB and bumetanide, respectively, results in the accumulation of Cl⁻ and water between deeper fibre cells, and the formation of extracellular space dilations. Blocking Cl⁻ channel efflux mediated by KCC with DIOA results in the intracellular accumulation of osmolytes and the swelling of peripheral fibre cells. (A-C) Reproduced from (Donaldson et al., 2009) with permission from Elsevier. (D & E) Schematics adapted from (Donaldson et al., 2009).
This relative simple view of circulating Cl⁻ fluxes derived from these morphological experiments has been confirmed and extended by a series of molecular localization, electrophysiological, and additional morphological studies (Donaldson et al., 2009). KCC1 and 4 have been identified in a human lens epithelial (HLE) B3 cell line, and in human cataractous lens epithelial cells (Misri et al., 2006). In the rat lens KCC1, 3 and 4 were identified at the transcript and protein level, and were found to be expressed in a differentiation-dependent manner (Figure 1.6B) (Chee et al., 2006). Expression of KCC1 was confined to the lens epithelium and peripheral fibre cells of the efflux zone of the outer cortex, and KCC3 localized to both zones of the outer cortex, while KCC4 was detected to be expressed throughout the entire lens, including the lens core. Immunohistochemistry showed the subcellular localisation of the three KCC isoforms was mainly cytoplasmic in nature in the cortex under isotonic conditions. However, upon exposure to hypotonic stress, KCC1 and KCC4 located in peripheral fibres cells were dynamically recruited from an inactive cytoplasmic pool to the membrane presumably to mediate RVD and restore lens volume. Interestingly, KCC3 in the cortical fibre cells was not recruited to the membrane in response to hypotonic stress (Chee et al., 2006). Although a number of studies have shown that KCC3 can play a critical role in cell volume regulation in response to hypotonic stress in a variety of other cells types, including red blood cells (Crable et al., 2005; Lauf et al., 2001; Pellegrino et al., 1998; Rust et al., 2007; Su et al., 1999), renal tubule and neuronal cells (Boettger et al., 2003), one study has claimed KCC3 expressed in NIH/3T3 cells is not activated by changes in cell volume (Shen et al., 2001). In this example, KCC3 activity was increased by growth factors, such as insulin like growth factor 1 (IGF1) and lead to the stimulation of the growth of NIH/3T3 cells (Shen et al., 2001). This led to speculation by Chee et al., that the observed lack of membrane insertion of KCC3 in response to hypotonic stress in the lens, may indicate that it is activated by a different stimulus, such as the growth factor IGF1, to promote the
increase in cell volume required to drive the massive elongation that occurs during fibre cell differentiation (Chee et al., 2006).

In the lens core, under isotonic conditions KCC4 labelling was strongly associated with the membranes, suggesting that the KCC4 is primarily active in this region (Chee et al., 2006). The initial cytoplasmic accumulation of KCC4 in the peripheral fibre cells followed by its subsequent insertion into the membrane in mature fibre cells, resembles the differentiation dependent insertion seen for other membrane transport proteins in the lens (Lim et al., 2006; Merriman–Smith et al., 1999). Since mature fibre cells lose their cellular organelles and nuclei necessary to perform de novo protein synthesis, Donaldson et al., hypothesized that young fibre cells produce a cytoplasmic pool of membranes proteins that can be inserted into the membrane at later stages in fibre cell differentiation (Donaldson et al., 2004). Unfortunately the functionality of KCC4 in the lens core remains to be determined.

Of the three Na\(^+\)-dependent chloride cotransporters, only NKCC1 and NCC have been identified at the transcript and protein levels in the lens. NKCC1 has been identified at the protein level in a human lens epithelial cell line (HLE) (Lauf et al., 2008), and in the rabbit (Alvarez et al., 2001), mouse (Bassnett et al., 2009) and rat (Chee et al., 2010) lens. NCC on the other hand has only been found in a human lens epithelial cell line (HLE) (Lauf et al., 2008) and the rat lens (Chee et al., 2010). In the rat lens, immunohistochemistry and western blotting showed that both NKCC1 and NCC were expressed in the lens cortex, but only NCC was expressed in the lens core (Figure 1.6B) (Chee et al., 2010). Under isotonic conditions, although there was some membrane labelling of NKCC1 and NCC in epithelial and cortical fibre cells, the majority of the labelling was localized to the cytoplasm, implicating that most of the NKCC1 and NCC transporters in the lens cortex reside as cytoplasmic pools of inactive transporters. In contrast, NCC labelling in the lens core was predominantly
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associated with the membrane (Chee et al., 2010), indicating that NCC, like KCC4, could be functionally active in the lens core.

Unlike KCC1 and 4, the subcellular distribution of NKCC1 and NCC in either the efflux or influx zones of the rat lens cortex was unaffected by exposure to osmotic stress (Chee et al., 2010). This was despite a previous study conducted on rabbit lenses exposed to hypertonic stress which exhibited an increase in Rb⁺ uptake which was blocked by the addition of bumetanide a NKCC specific inhibitor (Alvarez et al., 2001). Therefore, given that NKCC is activated in the lens in response to hypertonic stress in the lens, Chee et al., speculated that this observed increase in ion flux is brought about by a mechanism other than recruitment of additional transporter to the plasma membrane. Since culturing rabbit lenses under hypertonic conditions elevates cyclic AMP levels and enhances a bumetanide-sensitive Rb⁺ flux, it has been proposed that alteration of the phosphorylation status of NKCC1 modulates its activity (Russell, 2000).

Taken together, the above experiments suggest the existence of spatially segregated zones of ion efflux and influx to exist in the lens cortex which is mediated by Na⁺ and/or K⁺ dependent CCCs along with Na⁺/K⁺ATPase, K⁺ and Cl⁻ Channels. KCC, NKCC and Cl⁻ channels are constitutively active in the lens, and under isotonic conditions, KCC predominantly mediates ion efflux in the lens periphery while NKCC, KCC and Cl⁻ channels all mediate ion influx in the deeper lens (Figure 1.6A). Ultimately, all these key players work in concert to balance ion efflux and influx in the lens to maintain steady state lens volume, and therefore overall transparency. Based on this working model of lens volume regulation it is possible to speculate on how dysfunction of this system could produce the unique spatially localised damage phenotype observed in diabetic cataract.
Figure 1.6 Roles of cation chloride cotransporter in fibre cell volume regulation in spatially distinct regions of the lens. (A) A schematic of the emerging molecular model of cation chloride cotransporter and other key transport systems that facilitate net ion efflux at the periphery, and net ion influx in the deeper lens mediating steady state lens volume. (B) A schematic summary of cation chloride co-transport subcellular (cytoplasmic and/or membrane) and regional distribution in the rat lens. The data for KCC distribution is taken from (Chee et al., 2006), while NKCC1 and NCC data from (Vorontsova, 2013). Schematics adapted from (Vorontsova, 2013).

1.5 Dysfunction of cell volume regulation in the diabetic lens

Diabetic cortical cataract manifest itself as a discrete zone of tissue liquefaction in the lens cortex that is surrounded by fibre cells which appear to have a normal morphology (Bond et
al., 1996). The realisation that spatially distinct zones of ion and water influx exist in the lens cortex, and that the zone of tissue liquefaction coincides with the influx zone, offers a potential explanation for the observed damage phenotype. Indeed, Chee et al., showed that incubating rat lenses in the presence of an activator of KCC, NEM resulted in a damage phenotype (Figure 1.7A) that closely mimicked that observed in diabetic cataract (Chee et al., 2006) (Figure 1.7B). NEM has long been known to activate KCC, via oxidation of the critical thiol groups in the upstream regulatory kinases that phosphorylate KCC and reduce its activity. Since in red blood cells KCC activity has been shown to be increased by oxidative stress caused by depletion of GSH (Bassnett, 2002), and hyperglycaemia is known to deplete GSH levels in the diabetic lens, Donaldson et al., proposed that oxidative damage to these upstream regulatory kinases maybe an initiating event in the aetiology of diabetic lens cataract (Donaldson et al., 2009). Furthermore, oxidative stress has also been shown to inactivate NKCC1 in vascular endothelial cells (Elliott and Schilling, 1992), which suggest oxidative stress in the lens would increase and decrease KCC and NKCC activity, respectively. Due to the reversal of $K^+$ and $Cl^-$ gradients with distance into the lens, the activation of KCC would lead to increased ion efflux in the lens periphery, but increased ion influx deeper in the lens, to produce the peripheral cell shrinkage and localized cell swelling observed in the diabetic rat lens. Work in other tissues has recently identified the upstream kinases and phosphatases that set the phosphorylation status of KCC and NKCC enabling us to investigate not only if these regulatory proteins are expressed in the lens, but also whether their dysfunction is involved in the initiation of diabetic cataract.
Figure 1.7 Over-activated KCC in the lens mimics diabetic cataract morphology. Equatorial cross-sections of (A) rat lenses incubated in AAH + 1mM NEM for 18 hours and (B) streptozotocin-induced diabetic rat lenses labeled with membrane marker wheat germ agglutinin (WGA). Both sections demonstrate peripheral cell shrinkage and deep cortical cell swelling. (A) Reproduced from (Chee et al., 2006) and (B) from (Bond et al., 1996) with permission from ARVO.

1.5.1 The regulation of CCC activity in the lens

PP1 and PP2A upstream regulatory phophatases are known to be actively expressed in human, mouse, rat and bovine lenses (Li et al., 2006; Li et al., 2001; Liu et al., 2008; Umeda et al., 2004), but there was little knowledge about the upstream regulatory kinases. Although SPAK was identified in the mouse (Bassnett et al., 2009) and human (Wang et al., 2013) lens proteome, Vorontsova et al., was the first to identify the WNK-SPAK/OSR1-PP1/PP2A signalling pathway of CCC regulation in rat and human lens (Vorontsova et al., 2015). WNK1, WNK4, SPAK and OSR1 were identified at the transcript and protein level in the rat lens, while WNK3 due to the lack of a commercial WNK3 antibody was only detected at the transcript level. SPAK and OSR1 were detected in the epithelium, inner cortex, outer cortex and core, and were largely associated with the membrane. This finding is consistent with SPAK and OSR1 being in close proximity to the membrane where they can interact with and phosphorylate NKCC and KCC (Vorontsova et al., 2015). In the human lens, WNK1 was
also identified at the protein level in all lens regions, with a weaker signal in the lens core. SPAK and OSR1 were also detected in all regions in the human lens; however OSR1 was strongly detected in the core compared to SPAK.

Taken together the molecular and functional results (Chee et al., 2006; Chee et al., 2010; Donaldson et al., 2009; Vorontsova et al., 2015) from the MVL suggest that the cotransporter NKCC and KCC regulate fibre cell volume, and their phosphorylation status, hence their reciprocal activation is modulated by the WNK-SPAK/OSR1-PP1/PP2A pathway present in the lens (Figure 1.8). However, the co-expression of CCCs and their regulators in the lens does not prove a functional link between diabetes and dysfunction of the regulatory pathways of the cotransporter. Therefore, in my Masters project I will attempt to establish a mechanistic link between hyperglycaemia and KCC and NKCC activity.

Figure 1.8 Proposed model for the regulation of cell volume regulators in the lens. Schematics of KCC and NKCC regulation by their upstream regulators. In the normal lens, the highly ordered rows of hexagonal fibre cells are a result of the tightly regulated balance between the net ion efflux and influx maintained by appropriate KCC and NKCC upstream signalling.
Chapter 1 – Introduction

1.6 Aims and Research Design

To test our hypothesis that in diabetic cataract a mechanistic link exists between hyperglycaemia and NKCC and KCC activity, the relative change in the phosphorylation status of the cotransporters in response to hyperglycaemia/diabetes needs to be determined. To achieve this, I needed a means for detecting the phosphorylation status of the cotransporters that could be easily applied to normal and diabetic lenses. Hence, to work towards our overall goal of understanding the aetiology of diabetic cataract I will pursue the following specific aims in this study.

1) To characterize phospho-specific antibodies raised against KCC3 and NKCC1 as tools to detect changes in cotransporter phosphorylation status in response to changes in extracellular osmolarity

2) To establish an acute hyperglycaemia model of diabetic cataract in the bovine lens

3) To determine whether hyperglycaemia induces changes in the phosphorylation status of NKCC1.
Chapter 2 – Materials and Methods

2 Materials and Methods

2.1 Laboratory Health and Safety Practices

All laboratory practices complied with University of Auckland Health and Safety Guidelines. Protective clothing was worn at all times which includes laboratory coats, Promed® powder free latex examination gloves (ThermoFisher Scientific) and safety glasses. Face masks (inHealth, NZ) were worn when handling volatile liquids or substance of fine powdery texture. Fume hoods were used for preparation of toxic/noxious substances and storage of such chemicals. Corrosive, explosive and toxic chemicals were stored in specially assigned cabinets or air vented storage facilities as per specifications. Chemicals were weighed out using weigh boats and metal spatulas sanitised with 70% ethanol. All glassware was washed and autoclaved. Animal tissue was temporarily stored in labelled, transparent sealed bags in -20°C freezers assigned for biological tissue waste storage until disposed. Benches were routinely cleaned with Accel diluted in water at 1:40. These procedures ensured safety of researchers, minimised chances of contamination, and provided a hygienic and safe work space.

2.2 General Solutions

All solutions were prepared in milliQ water with a resistivity of 18.2MΩ at 25°C unless stated otherwise. pH was measured using a S20 SevenEasyTM pH meter (Mettler Toledo, USA) or pH strips of ranges 0-6, 6.0-7.5 and 4.5-10 (Sigma, St Louis, Mo, USA). pH adjustments were made using NaOH, or HCl as required. Osmolarity was measured using an Osmometer (Wesco AC061, Logan, Utah) that was calibrated with fresh 290 and 1000mOsm standards before usage. All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), unless stated otherwise.
Artificial aqueous humour (AAH) was selected as the medium for lens culture experiments as it most closely resembles the natural composition of the human aqueous humour (Lucas et al., 1986). AAH was used as the foundation for the preparation of isotonic, hypertonic, hypotonic, high glucose and high mannitol AAH (see Table 2.1). To these solutions, 1% v/v penicillin, streptomycin and neomycin were supplemented to minimise microbial growth. Solutions were stored up to three weeks at 4°C, or up to 3 months in -20°C. PBS was always used at 0.01M unless stated otherwise.

**Table 2.1 Recipes of general solutions.**

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<th>Type</th>
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<tr>
<td>PBS</td>
<td>Commercial Phosphate Saline Buffered (137mM NaCl, 2.7mM KCl, 10mM phosphate buffer, pH 7.4) Tablets, P4417 Sigma Aldrich 1 tablet in 200mL of milliQ water yielding 0.01M PBS solution, 290mOsm/kg</td>
</tr>
<tr>
<td>Isotonic AAH</td>
<td>125mM NaCl, 0.5mM MgCl2, 4.5mM KCl, 10mM NaHCO3, 2mM CaCl2, 5mM Glucose, 20mM Sucrose, 10mM HEPES, pH 7.2-7.4, 290mOsm/kg</td>
</tr>
<tr>
<td>Hypotonic AAH</td>
<td>50mM NaCl, 0.5mM MgCl2, 4.5mM KCl, 10mM NaHCO3, 2mM CaCl2, 5mM Glucose, 20mM Sucrose, 10mM HEPES, pH 7.2-7.4, 170mOsm/kg</td>
</tr>
<tr>
<td>Hypertonic AAH</td>
<td>200mM NaCl, 0.5mM MgCl2, 4.5mM KCl, 10mM NaHCO3, 2mM CaCl2, 5mM Glucose, 20mM Sucrose, 10mM HEPES, pH 7.2-7.4, 420-430mOsm/kg</td>
</tr>
<tr>
<td>High Glucose AAH</td>
<td>125mM NaCl, 0.5mM MgCl2, 4.5mM KCl, 10mM NaHCO3, 2mM CaCl2, 50mM Glucose, 20mM Sucrose, 10mM HEPES, pH 7.2-7.4, 340mOsm/kg</td>
</tr>
<tr>
<td>High Mannitol AAH</td>
<td>125mM NaCl, 0.5mM MgCl2, 4.5mM KCl, 10mM NaHCO3, 2mM CaCl2, 5mM Glucose, 20mM Sucrose, 10mM HEPES, 50mM Mannitol pH 7.2-7.4, 340mOsm/kg</td>
</tr>
</tbody>
</table>

**2.3 Bovine Lens Extraction**

Bovine eyes were obtained from the local abattoir (Auckland Meat Processing, Auckland, New Zealand) and kept at room temperature (RT) until dissection. Whole eyes were submerged in 70% ethanol for a few seconds to minimise microbial contamination. An initial incision was made into the vitreous chamber at the sclera with the blade angled downward towards the posterior of the eye to avoid piercing of the lens. Using a curved pair of scissors, starting from the point of incision a 2/3 cut was made in the sclera. The eye was inverted inside out revealing the tapetum lucidum and retina, and the posterior of the lens. The
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The vitreous body was then gently removed and the lens released by carefully cutting the zonular fibres around the lens. Lenses were subsequently handled using custom-made glass loops to prevent physical damage.

2.4 Bovine Lens Culturing

Bovine lenses were cultured under five different treatment conditions for four different incubation times (see Table 2.2). Lenses were placed in sterile 6 well culture plates (BD Falcon, NJ USA) containing 10mL of pre-warmed (37°C) isotonic AAH, hypertonic AAH, hypotonic AAH, isotonic, high glucose AAH, or high mannitol AAH for either 2, 24, 48 or 72 hours at 37°C, 5% CO₂ that were maintained in a tissue culture incubator (Thermoscientific, HERACELL 150i CO₂ incubator). Media and culture plates were replaced every 24h to further minimise microbial contamination. After incubation, lenses were assessed for transparency and weighed before being processed for either western blotting or immunohistochemical analysis.
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Table 2.2 Summary of lens incubation times and applications.

<table>
<thead>
<tr>
<th>Osmotic challenge/Treatment</th>
<th>Incubation time</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic AAH (125mM NaCl, 290mOsmol/kg)</td>
<td>2h</td>
<td>WB, IHC</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>IHC</td>
</tr>
<tr>
<td>Hypotonic AAH (50mM NaCl, 170mOsmol/kg)</td>
<td>2h</td>
<td>WB, IHC</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>IHC</td>
</tr>
<tr>
<td>Hypertonic AAH (200mM NaCl, 420-430mOsmol/kg)</td>
<td>2h</td>
<td>WB, IHC</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>IHC</td>
</tr>
<tr>
<td>High Glucose AAH (50mM Glucose, 125mM NaCl, 340mOsm/kg)</td>
<td>2h</td>
<td>WB, IHC</td>
</tr>
<tr>
<td></td>
<td>24h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>IHC</td>
</tr>
<tr>
<td>High Mannitol AAH (50mM Mannitol, 125mM NaCl, 340mOsm/kg)</td>
<td>2h</td>
<td>WB, IHC</td>
</tr>
<tr>
<td></td>
<td>48h</td>
<td>IHC</td>
</tr>
<tr>
<td></td>
<td>72h</td>
<td>IHC</td>
</tr>
</tbody>
</table>

2.5  Assessment of lens transparency

Freshly extracted or cultured bovine lenses were placed in 10mL of pre-warmed isotonic AAH in sterile 6 well culture plates and lens transparency assessed using bright-field microscopy. A 5 x 5 mm grid was also placed under the culture plate to monitor for grid distortions caused by changes to the refractive properties of the lens.

2.6  Lens mass change analysis

All lenses were weighed pre- (freshly extracted lenses) and post-incubation (after being subjected to the appropriate osmotic challenge/treatment condition) using an analytical balance (Adventurer Ohaus Discovery Balance, Carolina Scale, Inc, Sanford, NZ), with sensitivity down to 0.01mg.
2.6.1 Data presentation and statistical analysis of lens wet weight change

Analysis for statistical significance in lens weight change incubated in different media was carried out using GraphPad Prism 6.07 Software. A One-way ANOVA was first carried out on pre-incubated lens measurements to ensure no significant differences in baseline lens weights between treatment groups. No significant difference was identified on any of the pre-incubation lens weights allowing for a One-way ANOVA to be carried on lens weight differences (post incubation weight - pre incubation weight) between treatment groups (e.g. isotonic, hypertonic and hypotonic). Given that there was a statistical difference following a One-way ANOVA between treatment groups, a Tukey-Kramer post-hoc analysis was then carried out to specify between which treatment groups (e.g. isotonic and hypertonic, isotonic and hypotonic and/or hypertonic and hypotonic) there was significance. Tests were done for statistical significance at 0.05, 0.01, 0.001 confidence levels. Data has been presented as a percentage weight change with error bars representing standard error of mean (SEM) using GraphPad Prism 6.07 Software.

2.7 Immunohistochemistry

Protocols for fixing and sectioning lenses have previously been developed and optimised in the rat (Jacobs et al, 2003), mouse (Jacobs et al, 2003) and human lens (Lim et al, 2009). As a result, a series of fixation reagents, fixative concentrations and fixation times were trialled (Table 2.3) to determine the most suitable method for optimal fixation of the bovine lens. Note that the pH of all the trialled fixative solutions shown in Table 2.3 was adjusted to 7.2-7.4 using 2M NaOH.
Table 2.3 Summary of different conditions trialled for optimising bovine lens fixation.

<table>
<thead>
<tr>
<th>Fixative, concentration, solvent</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% Paraformaldehyde in PBS</td>
<td>48 hours</td>
</tr>
<tr>
<td>4% Paraformaldehyde in PBS</td>
<td>48 hours</td>
</tr>
<tr>
<td>2% Paraformaldehyde + 0.01% Glutaraldehyde in PBS</td>
<td>48 hours</td>
</tr>
<tr>
<td>4% Paraformaldehyde + 0.01% Glutaraldehyde in PBS</td>
<td>48 hours</td>
</tr>
<tr>
<td>2% Paraformaldehyde + 0.01% Glutaraldehyde in PBS</td>
<td>96 hours</td>
</tr>
<tr>
<td>4% Paraformaldehyde + 0.01% Glutaraldehyde in PBS</td>
<td>96 hours</td>
</tr>
<tr>
<td>2% Paraformaldehyde + 0.01% Glutaraldehyde in PBS</td>
<td>Fixed for 48 hours: followed by cutting the lenses in half, replacing fixative, and fixing for additional 48 hours</td>
</tr>
<tr>
<td>4% Paraformaldehyde + 0.01% Glutaraldehyde in PBS</td>
<td>Fixed for 48 hours: followed by cutting the lenses in half, replacing fixative, and fixing for additional 48 hours</td>
</tr>
<tr>
<td>2% Paraformaldehyde in PBS</td>
<td>7 days (168 hours) (Fixative was replaced every 48 hours)</td>
</tr>
<tr>
<td>4% Paraformaldehyde in PBS</td>
<td>5 days (120 hours) (Fixative was replaced every 48 hours)</td>
</tr>
<tr>
<td>2%Paraformaldehyde in 0.005M PBS + 5% (v/v) acetic acid</td>
<td>48 hours (acetic acid added at 24 hours)</td>
</tr>
</tbody>
</table>

2.7.1 Optimised Bovine Lens Fixation

Due to the sheer size of the bovine lens, its fixation is an inherently difficult process. Of the trialled fixation protocols, the 2% w/v paraformaldehyde (PFA) in 0.005M PBS, pH 7.2-7.4 (pH adjusted using 2M NaOH) for 24 hours, followed by supplementation with 5%v/v acetic acid and fixing for a further 24 hours yielded lens sections which were sufficiently sturdy and intact for histochemical analysis. There was no apparent difference observed in the fixation process between 2% PFA and 4% PFA concentrations, therefore, the lower concentration was selected. The osmolarity of 0.01M PBS is approximately 290mOsmol/kg (isotonic). Addition of PFA to 0.01M PBS inevitably raises the osmolarity of the resultant solution potentially leading to cell shrinkage. To ameliorate this, 0.005M PBS was used instead of 0.01M
PBS.PFA can cause cell shrinkage and tissue hardening, while acetic acid can cause tissues to swell; therefore combining the two gave better preservation of tissue morphology.

### 2.7.2 Cryoprotection

Following fixation, lenses were removed and washed three times in PBS for 10 minutes. Lenses were cryoprotected for 1 hour at RT in 10% sucrose, followed by 1 hour in 20% sucrose, and finally in 30% sucrose in PBS overnight at 4°C.

### 2.7.3 Sectioning

A globule of Tissue-Tek® OCT (Sakura Finetek, Torrance, CA) was applied to a chuck, onto which the bovine lens was placed anterior side facing upwards and the equator carefully aligned parallel to the chuck. This orientation enables visualization of the epithelium and the hexagonal profile of fibre cells from epithelium to the lens centre. The lens was then covered with OCT until completely submerged and then snap frozen in liquid nitrogen for 20 seconds. The chucks were placed in the cryostat (CM3050; Leica Microsystems, Wetzlar, Germany) pre-cooled to -20°C. Equatorial sections of 25-30 μm were collected onto Single Frost Microscope Slides (Fronine, 7105Wt 1-1.2 mm, Lomb Scientific Pvt Ltd, Taren Point, NSW) with PBS. The integrity of the sections were analysed under an upright light microscope (Leitz, Dialux20, Mount Holly, NJ or a Leica, DM LB Houston, TX) at 10x and 40x magnification and sections with an intact capsule, epithelium and cortex were retained. Following section collection, excess OCT was washed off by performing three 5 minute washes with PBS. Sections were then stored at 4°C in an air tight humidity box.

### 2.7.4 Immunolabelling

The cell membrane marker wheat germ agglutinin (WGA) conjugated to Alexa Fluor® 594 (Invitrogen) was diluted (1:500) in PBS and applied on to the sections for 2 hours at RT.
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Sections were then washed three times for 5 minutes in PBS and slides mounted using 20 µL of VectaShield (Vector Laboratories, Burlingame, CA) and covered with a 76 x 26 x 1 mm cover slip (Menzel Glaser, Braunschweig, Germany). Cover slips were sealed with nail polish to prevent moisture loss and stored at 4°C in a slide folder.

2.7.5 Confocal Microscopy

Labelled sections were imaged using an Olympus FV1000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) located in the Biomedical Imaging Research Unit (BIRU) at the University of Auckland. Sections were live scanned at 8µs/pixel at a resolution of 512 x 512 pixels and images of the lens cortex captured using the 40x oil objective or a 60x oil objective with digital of zooms 1 and 2 to obtain high magnification images. Laser wavelength 594 was employed for fluorophore excitation and specific emission filters were used to minimise bleed-through fluorescence. Merging and processing of images obtained from separate channels was carried out using Adobe Photoshop CS5.1.

2.8 Protein preparation

Using sharpened tweezers, the lens capsule was gently pulled away from the fibre cell mass in PBS. The outer cortex, which is very transparent and jelly-like, was then gently peeled away from the rest of the lens. This dissected tissue was then immediately transferred to pre-cooled 2mL Eppendorf tubes containing 350µL of ice cold homogenizing solution (5mM EDTA, 5mM EGTA, 5mM Tris-HCl, pH 8.0) containing phosphatase inhibitor tablets (Phos-STOP phosphatase inhibitors, Roche) and protease inhibitors (Protease inhibitor cocktail tablets, Roche) and homogenized using a Metabo BE 10R Electric Drill (Vollwellenelektronik). A further 400µL of homogenizing solution was added to the partially homogenized tissue, and further homogenized (at speed setting 6) until a uniform mixture was obtained. Homogenates were centrifuged at 12,000rpm at 4°C for 20 minutes, and the
resultant supernatant carefully removed without disturbing the pellet. This supernatant was retained and represents the lens soluble (LS) fraction of the outer cortex. The pellet was then washed in 1mL of storage solution (2mM EDTA, 2mM EGTA, 100mM NaCl, 5mM Tris-HCl, pH 8.0 with phosphatase and protease inhibitors) and centrifuged at 12,000rpm at 4°C for 20 minutes. The pellet was suspended in storage solution and centrifuged two more times. Finally, the pellet was re-suspended in 300-700µL of storage solution and retained as the lens membrane (LM) fraction of the outer cortex crude. The LS and LM outer cortex preparations were stored at -80°C until further use.

2.9 Protein concentration determination

Protein concentration was measured using the Direct Detect® spectrometer (Merck Millipore) an infrared based protein quantitation system. Each sample was measured 3 times and the average protein concentration calculated.

2.10 Western Blotting

90-120µg of protein samples were incubated with SDS loading dye containing beta-mercaptoethanol. Samples were loaded into the wells of Mini-PROTEAN TGX Precast Gels gradient 4-15% gels (Bio-Rad) using the Mini Protean Tetra cell (Bio-Rad), along with 10µL of Precision Plus Protein TM Standards Dual Color Ladder (Bio-Rad). Gels were electrophoresed in SDS-PAGE buffer (Table 2.4) at 90-150V for 1-4 hours with the tank submerged in an ice bath to prevent overheating. The current was maintained below 30mA per gel using a EPS301 (Amersham Pharmacia Biotech Inc. Piscataway, NJ) power supply unit. A PVDF membrane (Biorad ImmunBlot PVDF) was pre-incubated in 100% methanol (Merck) for 2-5 minutes, and then left in transfer buffer (Table 2.4). For each gel, two scotch pads and two pieces of filter paper (WHB30306188, Global Science) were also soaked in transfer buffer. Once the electrophoresis was complete, the two gel plates were separated and
the stacking gel trimmed off from the rest of the gel. The gel was then gently lifted over the back plate and laid on top of the PVDF membrane. The western blot was arranged like a sandwich in the following order: scotch pad, filter paper, membrane, gel, filter paper and scotch pad. A light rolling pin was used to push out any air trapped between the gel and membrane. The sandwich was then secured into a cassette and submerged in a tank containing transfer buffer. Proteins were transferred from the gel to the PVDF membrane at 85mA for 2 hours in an iced bath.

The PVDF membrane was then quickly rinsed twice in TBS-Tween 20 (TBS-T) (Table 2.4) and then blocked in 3-5% (w/v) BSA in TBS-T (for phospho-antibodies), or 5% w/v milk in TBS-T (for non-phospho-antibodies) to block non-specific binding for 1 hour at RT. The membrane was given two quick rinses in TBS-T followed by three 10 minute washes in TBS-T on a rocker. Membranes were then placed in 50mL falcon tubes (BD Falcon) containing the primary antibody diluted in 1-2mL of antibody dilution solution or TBS-T (Table 2.5) and incubated overnight at 4°C on a tube roller. Antigenic peptide competition studies were carried out when control peptides were available for the antibodies. For this, antibodies were first diluted at 1:500 in antibody dilution solution and incubated with 5X excess of the corresponding antigenic peptide at 37°C for 2 hours, followed by overnight incubation at 4°C. This mixture was then used to incubate the membranes overnight at 4°C as usual. The membrane was then given two quick rinses in TBS-T followed by three 10 minute washes in TBS-T with gentle rocking. The membrane was then incubated with the appropriate secondary antibody diluted in 10mL of antibody dilution solution (Table 2.5) at RT for 1 hour. Labelled membranes were then given two quick rinses in TBS-T followed by three 10 minute washes in TBS-T.

PVDF membranes were developed using Amersham ECLTM Select or Prime (GE Healthcare) following the manufacturer’s instructions. The membranes were incubated in the
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ECL mixture for 5 minutes, blotted to drain excess ECL, placed between two acetate sheets and the protein ladder marked for reference. The membrane was developed using the Fujifilm LAS 4000 imager with chemiluminescence (Life Science, USA), while digital exposure was used for to image the ladder.

Membranes were stripped and re-probed with other antibodies (non-phosphorylated/total protein or beta-actin) by performing two quick washes in TBS-T followed by three 10 minute washes in TBS-T. The membranes were incubated in 100% methanol for 5 minutes and then incubated with pre-warmed stripping buffer (Table 2.4) for 30 minutes at 50°C with shaking. Membranes were then washed again in TBS-T, and then blocked with the appropriate blocking solution and labelled as per standard protocols described above.

<table>
<thead>
<tr>
<th>Table 2.4 Buffers used for western blotting.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SDS PAGE (Polyacrylamide gel electrophoresis) Buffer</strong></td>
</tr>
<tr>
<td><strong>Transfer Buffer</strong></td>
</tr>
<tr>
<td><strong>TBS-T (Tris buffered saline-Tween 20)</strong></td>
</tr>
<tr>
<td><strong>Blocking solution</strong></td>
</tr>
<tr>
<td><strong>Antibody Dilution Solution</strong></td>
</tr>
<tr>
<td><strong>Stripping Buffer</strong></td>
</tr>
</tbody>
</table>
Table 2.5 Primary antibodies and corresponding secondary antibodies utilized for western blotting.

<table>
<thead>
<tr>
<th>Antibody, Source, Concentration</th>
<th>Primary Antibody Details</th>
<th>Corresponding Secondary Antibody Details</th>
<th>Membrane Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;R5&quot; anti-phospho NKCC1, Yale University (Flemmer et al., 2002)</td>
<td>Diphospho-peptide of Human NKCC1 N-terminus containing Thr212 and Thr217, Sequence: YYLRT<em>FGHNT</em>M DAVPR</td>
<td>Rabbit IgG (whole antibody)</td>
<td>3% (w/v) BSA in TBS-T</td>
</tr>
<tr>
<td>Anti-phospho KCC3(A) Thr 991 (same as Thr 982) (S959C), Dundee University Phosphorylation Unit, 1μg/mL</td>
<td>Against the phosphopeptide residues 984-1000 of Human KCC3 containing Thr 991, Sequence: SAYTYERT*LMM EQRSRR</td>
<td>Anti-sheep IgG secondary antibody HRP conjugate (81-8620), Invitrogen, 1.5mg/mL</td>
<td></td>
</tr>
</tbody>
</table>

"R5" anti-phospho NKCC1
Diphospho-peptide of Human NKCC1 N-terminus containing Thr212 and Thr217, Sequence: YYLRT*FGHNT*M DAVPR
Anti-rabbit IgG secondary antibody HRP conjugate (NA934) GE Healthcare Life Sciences

Rabbit IgG (whole antibody)

Host: rabbit, Reactivity: human, rat, shark, bovine
Affinity purified, Polyclonal
Applications: WB and IF

Host: donkey, Reactivity: rabbit, Affinity purified, Polyclonal, Application: WB

1:500 in antibody dilution solution
1:10K in antibody dilution solution

3% (w/v) BSA in TBS-T

Anti-phospho KCC3(A) Thr 991 (same as Thr 982) (S959C), Dundee University Phosphorylation Unit, 1μg/mL
Against the phosphopeptide residues 984-1000 of Human KCC3 containing Thr 991, Sequence: SAYTYERT*LMM EQRSRR
Anti-sheep IgG secondary antibody HRP conjugate (81-8620), Invitrogen, 1.5mg/mL

Sheep IgG (H+L)

Host: sheep, Reactivity: human, bovine
Affinity purified against phospho-specific peptide, Polyclonal, Applications: WB

Host: rabbit Reactivity: sheep, Affinity purified, Polyclonal, Applications: WB, IF, IHC, ELISA, IP

1μg/mL in TBS-T
1:10K in TBS-T
5% (w/v) BSA in TBS-T
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary Antibody Details</th>
<th>Corresponding Secondary Antibody Details</th>
<th>Dilution of Antibodies</th>
<th>Membrane Blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody, Source, Concentration</strong></td>
<td><strong>Epitope</strong></td>
<td><strong>Host Species, Reactivity, Purity, Clonality, Applications</strong></td>
<td><strong>Antibody, Source, Concentration</strong></td>
<td><strong>Epitope</strong></td>
</tr>
<tr>
<td>Anti- phospho KCC3(A) Thr 1048 (S959C) Dundee University Phosphorylation Unit 1μg/mL</td>
<td>Phosphopeptide of Human KCC3 residues 1041-1055 containing Thr 1048, Sequence: CYQEKVHMT*WT KDKYM</td>
<td>Host: sheep, Reactivity: human, bovine Affinity purified against phospho-specific peptide, Polyclonal, Applications: WB</td>
<td>Anti-sheep IgG secondary antibody HRP conjugate (81-8620), Invitrogen, 1.5mg/mL</td>
<td>Sheep IgG (H+L)</td>
</tr>
<tr>
<td>Anti-NKCC1 (sc-21545) Santa Cruz 0.2μg/μL, sc-21545 Peptide 0.2μg/μL</td>
<td>Human NKCC1 N-terminus (N-16)</td>
<td>Host: goat Reactivity: human, rat mouse, bovine, porcine Affinity purified: Yes Polyclonal: Yes Applications: ELISA, WB, IF, IHC, IP</td>
<td>Anti-goat IgG secondary antibody HRP conjugate (81-8620), Invitrogen, 1.5mg/mL</td>
<td>Goat IgG (H+L)</td>
</tr>
<tr>
<td>Primary Antibody Details</td>
<td>Corresponding Secondary Antibody Details</td>
<td>Dilution of Antibodies</td>
<td>Membrane Blocking</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------------------------------------</td>
<td>------------------------</td>
<td>-------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Anti-KCC3(A)</strong> (S701C) Dundee University Phosphorylation Unit 1μg/mL</td>
<td>Amino acids 1-175 of human KCC3 N-terminus</td>
<td>Anti-sheep IgG secondary antibody HRP conjugate (81-8620), Invitrogen, 1.5mg/mL</td>
<td>1μg/mL in TBS-T</td>
<td>5% (w/v) milk in TBS-T</td>
</tr>
<tr>
<td><strong>Anti-Beta-Actin antibody</strong> (ab8227), Abcam, 0.6mg/mL</td>
<td>Human beta-actin amino acids 1-100</td>
<td>Sheep IgG (H+L)</td>
<td>1:10K in antibody dilution solution</td>
<td>1:10K in antibody dilution solution</td>
</tr>
</tbody>
</table>
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2.11 Quantification of the phosphorylation signal

Signal intensities for the NKCC1 bands obtained using the phospho-specific and total protein antibodies were quantified using the ‘gel analysis’ tool in ImageJ software (1.48v, Wayne Rasband, NIH, USA). The area under the intensity curve obtained for phosphorylated NKCC1 was divided by the corresponding total protein band intensity to yield a ratio of normalised intensity. Each treatment group had four repetitions. The intensity ratios between treatment groups were found to be normally distributed. This allowed a One-way ANOVA to be carried out. Given that there was a statistical difference between treatment groups (e.g. isotonic, hypertonic and hypotonic) following a One-way ANOVA, a Tukey-Kramer post-hoc analysis was carried out to specify between which treatment groups (e.g. between isotonic and hypertonic, isotonic and hypotonic and/or hypertonic and hypertonic) there was significance. Tests were done for statistical significance at 0.05, 0.01 and 0.001 confidence levels. Data was presented as intensity ratios between the phospho- and total signals with error bars representing standard error of the mean (SEM) using the GraphPad Prism 6.07 Software.
Chapter 3 – Characterisation of the phosphorylation status of cation chloride co transporters in the bovine lens

3 Characterisation of the phosphorylation status of cation-chloride cotransporter in the bovine lens

Previous work in the Molecular Vision Laboratory has led to the hypothesis that diabetic lens cataract is initiated by a failure in fibre cells to regulate their volume regulation (Donaldson et al., 2009). This work has identified the Cation Chloride Cotransporters, NKCC1 and KCC, as key effectors of fibre cell volume regulation (Chee et al., 2006; Chee et al., 2010; Vorontsova et al., 2015). Since others have shown that the activity of CCCs are regulated via their phosphorylation status (Delpire and Gagnon, 2008; Kahle et al., 2006), I wanted to first determine if antibodies raised against the phosphorylated forms of KCC3 or NKCC1 could be used to detect changes to the phosphorylation status of the two transporters in bovine lenses exposed to osmotic challenge. These antibodies could then be used as tools to determine the relationship between CCC phosphorylation status and the onset of diabetic lens cataract (see Chapter 4). In this chapter, a review of the different phosphorylation sites shown to be involved in the modulation of transporter activity is first presented to provide a background for the selection of the phospho-specific antibodies subsequently used in this study. This is followed by a characterisation of the utility of selected phospho-specific antibodies to determine the phosphorylation status, and therefore the activity of KCC3 and NKCC1, in the bovine lens.

3.1 Phospho-regulation of NKCC and KCC via the WNK-SPAK/OSR1 signalling mediated pathway.

As discussed previously, in response to a change in cell volume, WNK kinases act as osmosensors, that modulate the activity of SPAK/OSR1 and the protein phosphatases PP1/PP2A that work as transducers to reciprocally regulate the effectors NKCC and KCC,
which elicit RVI and RVD, respectively to maintain cell volume (Delpire and Gagnon, 2008; Kahle et al., 2006). This pathway utilises changes in protein phosphorylation to effect changes in protein function. For example, hyperosmotic stress and the resultant cell shrinkage is initially sensed by changes to the phosphorylation of critical regulatory sites on WNK1. Hyperosmotic challenge, whether it be via high extracellular NaCl, KCl or sorbitol, rapidly increases the phosphorylation of WNK1 at amino acid Ser382 located within the T-loop of its kinase domain (Lenertz et al., 2005; Xu et al., 2000; Zagorska et al., 2007), a site found to be conserved among all WNK isoforms. Although the exact mechanism by which Ser382 is phosphorylated in response to cell shrinkage is still not fully understood, the data to date suggests that in response to hypertonic stress WNK1 undergoes a trans-autophosphorylation (Thastrup et al., 2012; Zagorska et al., 2007), which supports the role of WNK1 as an osmosensor.

SPAK/OSR1 kinases are the key downstream substrates of WNK kinases (Zagorska et al., 2007). WNK kinases phosphorylate SPAK/OSR1 at conserved regulatory threonine and serine residues within the T-loop (Thr233 for human SPAK) and S-motif (Ser373 for human SPAK). In vitro studies have shown that these sites are consistently phosphorylated by WNK1, and the genetic ablation of WNK1 leads to a decrease in phosphorylation and subsequent activation of SPAK/OSR1 in response to sorbitol induced hyperosmotic challenge. Although phosphorylation of the T-loop triggers activation of SPAK and OSR1 and is essential for their ability to physically interact with their downstream substrates NKCCs and KCCs, it is unclear what purpose phosphorylation of the S-motif serves, as mutation of the conserved Ser373 in the S-motif does not affect the activation of SPAK and OSR1 kinases (Thastrup et al., 2012; Vitari et al., 2005).
Once activated, SPAK/OSR1 has been shown to phosphorylate conserved threonine residues at several sites located in the cytosolic N terminal domain of NKCC1 in different of species (Figure 3.1), although only certain sites are critical for the activation of NKCC1 in response to hypertonic stress (Darman and Forbush, 2002; Flemmer et al., 2002; Gagnon et al., 2007). Furthermore, physical docking of SPAK/OSR1 to NKCC1 was found to be necessary for cotransporter activity under both baseline and hyperosmotic conditions (Gagnon et al., 2007).

Similarly, several phosphorylation sites were identified in the N and C termini of human KCC 3 (Rinehart et al., 2009). Of these sites, phosphorylation of Thr991 and Thr1048 in the N terminus, in response to hypertonic stress, caused inactivation of KCC3 in HEK293 cells and mature human erythrocytes (Figure 3.2). In response to hypotonic challenge, sites Thr991 and Thr1048 of KCC3 were also shown to undergo a rapid dephosphorylation that lead to activation of the cotransporter (Rinehart et al., 2009). Taken together these studies have identified the specific residues that determine the phosphorylation status and therefore activity of the CCC isoforms in response to osmotic challenge. Therefore monitoring the changes to the phosphorylation of these key residues can act as indicators of CCC functionality.
Figure 3.1 Phosphorylation ‘hot spots’ on NKCC1 protein. Membrane topology plot of NKCC1 containing the amino acid sequence alignments of a small portion of the cytosolic N-terminus in shark, human and mouse NKCC1 proteins. Residues coloured in red are phosho-regulatory sites that are critical for activation of NKCC1, and coloured in green are other threonine sites which were found to be phosphorylated in this region identified by (a) (Darman and Forbush, 2002), (b) (Vitari et al., 2006) and (c) (Flemmer et al., 2002), (d) (Gagnon et al., 2007).
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**Figure 3.2 Phosphorylation sites in KCC3 protein.** Membrane topology plot of human KCC3 showing regions of amino acid sequence in the C and N-terminal regions of the protein that contain the phosphorylation sites at Thr991, Ser1023, Ser1029 and Thr1048 identified by (Rinehart et al., 2009). Of these sites Thr991 and Thr1048 (coloured in red) were found to be critical phospho-regulatory sites for modulation of KCC3 activity.
3.2 Phospho-specific antibodies – a functional tool to assess CCC activation in response to osmotic challenge

In this section, I have evaluated a number of antibodies raised against the key serine/threonine residues in KCC3 or NKCC1 proteins that have been shown to change their phosphorylation status in response to osmotic challenges with the goal to determine which of these antibodies would be most effective to trial in the bovine lens.

Thr1048 and Thr991 are conserved in all four KCC isoforms and since they are rapidly dephosphorylated in response to hypotonic stress and cell swelling (Rinehart et al., 2009), present as good candidates for determining the activity of KCC in the bovine lens. Two anti-phospho human KCC3 antibodies targeting human KCC3 Thr1048 and Thr991 (Figure 3.2) sites were purchased from the University of Dundee in Scotland. Thr1048 and Thr991 are conserved in bovine tissue (see sequence alignments in Figure 3.3). The two anti-phospho KCC3 antibodies used in this study will be addressed as KCC3-P Thr991 and KCC3-P Thr1048, respectively.

It has been shown that Thr212 and Thr217 sites in human NKCC1 undergo phosphorylation in response to hypertonic stress (Figure 3.1) and are good indicators of NKCC1 activity (Flemmer et al., 2002). Furthermore, Thr212 and Thr217 are conserved in bovine tissue (see sequence alignments in Figure 3.4). An affinity purified rabbit phospho-specific NKCC1 antibody (Flemmer et al., 2002) which targets a diphospho-sequence in human NKCC1 containing the key residues Thr212 and Thr217 (named R5 by the producers) was obtained from the Forbush laboratory (Yale University, New Haven, CT) and used to examine the activity of NKCC1 in the bovine lens. The phospho-specific NKCC1 antibody will be addressed as NKCC1-P in this study. These phospho-specific antibodies can now be tested
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for their utility to detect the activation of KCC3 and NKCC1 in bovine lenses exposed to osmotic challenge by Western blotting.
Figure 3.3 Binding sites of KCC3 antibodies are preserved amongst different species. Membrane topology of KCC3 showing the location of antibody binding sites and the conservation of binding sites in the human (hKCC3), bovine (bKCC3), rat (rKCC3) and mouse (mKCC3) in the C-terminus of KCC3 that contain the key residues Thr991 and Thr1048. The large orange circle illustrates the binding site of the total KCC3 antibody (from University of Dundee) in the N-terminus of KCC3. Sequence conservation between hKCC3 and bKCC3 is shown in Appendix Table A3.
Figure 3.4 Binding sites of NKCC1 antibodies are preserved amongst species. Membrane topology of NKCC1 showing the location of antibody binding sites and the conservation of binding sites in the N terminus of human (hNKCC1), bovine (bNKCC1), rat (rNKCC1) and mouse (mNKCC1) in NKCC1 that contain the key residues Thr212 and Thr217. The green circle depicts the binding site of the NKCC1 P antibody which targets the diphospho sequence containing phospho threonine sites Thr212 and Thr217, the purple circle illustrates the binding site of the total NKCC1 antibody (Santa Cruz Biotechnology). Sequence conservation between hNKCC1 and bNKCC1 is shown in Appendix Table A4.
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3.3 Identification of KCC3-P and NKCC1-P

Since it was previously established that NKCC1 and KCC3 are expressed predominantly in the outer cortical regions of the bovine lens (Vorontsova, 2013), this region was dissected from a fresh bovine lens, homogenised and centrifuged. The supernatant contained the fibre soluble protein (FS) fraction, while the pellet, which represents the fibre membrane protein (FM) fraction, was retained and analysed by western blotting to detect the phosphorylated and total forms of NKCC1 and KCC3.

Before proceeding to the detection of phosphorylated NKCC1 (NKCC1-P), I first wanted to make sure I was able to detect total NKCC1 (NKCC1-T) protein in the FM fraction. An antibody that captures NKCC1-T from Santa Cruz Biotechnology identified multiple bands, including a band at 130kDa which is consistent with the predicted molecular weight for bovine NKCC1 (Figure 3.5, left panel). The pre-absorption of the NKCC1-T antibody with its corresponding antigenic peptide completely knocked down the 130kDa band (Figure 3.5, right panel). Beta actin levels were also similar between the two lanes indicating equal protein loading (Figure 3.5). This confirms the specificity of the 130kDa band for NKCC1.
I next proceeded to the detection of NKCC1-P utilizing an antibody that recognises phospho-threonine 212 and 217 in the NKCC1 protein. Detecting phosphorylated forms of NKCC1 and KC3 is an inherently difficult process as these cotransporters are relatively low abundant proteins, of which only a small fraction of the total protein is phosphorylated. Furthermore, phospho-signals are easily dephosphorylated by naturally occurring phosphatases in the lens (Li et al., 2001). Therefore, before starting my experiments, I had to include several steps to ensure the preservation of this phospho-signal. This included the addition of commercial phosphatase inhibitors during the preparation of my lens protein fractions and avoiding freeze-thaw cycles of my protein samples. In addition, I found through
several trial experiments that in order to achieve a visible phospho-band from fresh bovine lenses, a large quantity (90μg) of the protein preparation needed to be used. However, this had to be balanced with not over loading the gel to ensure that protein bands were well separated. I also found that the selection of a blocking solution was important so that instead of the 5% non-fat milk solution commonly used in the laboratory for blocking non-specific binding to the PVDF membrane, 5% bovine serum albumin (BSA) was trialled, since milk contains casein which could result in pseudo-signals. However, the expected 130kDa band for NKCC1-P at 130kDa was very faint suggesting that 5% BSA was not a suitable blocking agent (Figure 3.6A). Instead, I found that 3% BSA noticeably increased the signal of the 130kDa band (Figure 3.6B). I also discovered that I was able to further improve NKCC1-P detection using pre-cast 4-15% gradient SDS-polyacrylamide gels, separated at 90V for 3.5 hours rather than home-made fixed 8% or 10% acrylamide gels separated at 150V for 1 hour. The combination of using all these optimisation conditions resulted in the detection of a strong band at 130kDa (Figure 3.6C). Stripping of the NKCC1-P antibody from this blot and re-probing with the total NKCC1 antibody revealed a band at 130kDa, which aligned with the 130kDa band detected with the NKCC1-P antibody (Figure 3.6D). This strong, single band for total NKCC1 was a much improved and cleaner signal than what I had previously obtained in Figure 3.5, suggesting that my protocols for optimising the NKCC1-P band had inadvertently improved the total NKCC1 band. Overall, these findings indicate that I was successful in detecting the phosphorylated form of NKCC1 in the outer cortex of the bovine lens.
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Figure 3.6 Detection of NKCC1-P in the bovine lens outer cortex. Optimisation of western blotting protocol for detection of NKCC1-P using either 5% BSA (A) or 3% BSA (B) blocking solution. (C) Optimisation of western blotting protocol for detection of NKCC1-P antibody using a gradient SDS PAGE gel and 3% BSA reveals a strong band at 130kDa (indicated by the *). (D) Stripped blot from (C) labelled with the NKCC-T antibody revealing a single band at 130kDa that aligns with the 130kDa from (C).

Having optimised the conditions required to detect phosphorylated NKCC1 in the bovine lens outer cortex, I next wanted to detect phosphorylated KCC3 (KCC3-P). For KCC3, two KCC3-P antibodies were commercially available; anti-KCC3-P Thr991 and anti-KCC3-P Thr1048. Detection of the phosphorylated form of KCC3, utilizing an antibody that recognises phospho-threonine 991 in the KCC3 protein, revealed a single band at 75kDa (Figure 3.7A, left panel). Stripping of the KCC3-P antibody from this blot and re-probing with the total KCC3 antibody (Dundee University), revealed a band at ~90kDa, and a faint band at ~75kDa (Figure 3.7A, right panel). While the predicted molecular size of the KCC3 protein is 130kDa, other members of our laboratory have shown in rat lenses that using a total KCC3 antibody from Santa Cruz, two bands are detected at 75kDa and 130kDa, of which
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only the 75kDa protein is specific for KCC3 by control peptide studies (Vorontsova, 2013). Unfortunately, in my study, I was not able to confirm the specificity of these bands for KCC3, due to the unavailability of an antigenic peptide. However, it appears that in the bovine lens, KCC3 may exist also at a molecular weight of 75kDa. Comparison of the KCC3-P band with the bands for total-KCC3 revealed that the 75kDa bands aligned. The strong signal intensity for the 75kDa KCC3-P band, relative to the total-KCC3 band at 75kDa was intriguing. However, a similar finding was also found utilizing an antibody that recognizes phosphothreonine 1048 in the KCC3 protein (Figure 3.7B, left panel). Here, two prominent bands at 75kDa and 130kDa were detected. Re-probing with the total KCC3 antibody identified two bands at 75kDa and 90kDa (Figure 3.7B, right panel), of which the 75kDa band aligned with a strong band at 75kDa for the KCC3-P Thr1048 antibody indicating that I was potentially able to detect the phosphorylated form of KCC3.
3.4 Effects of osmotic stress on lens transparency, lens wet weight and fibre cell morphology

Having shown that our panel of antibodies are able to detect the phosphorylated forms of NKCC1 and KCC3 in fresh bovine lenses, I next wanted to determine the effects of osmotic stress on the phosphorylation status of these transporters. Before attempting to detect changes in phosphorylation of these cotransporter, I first examined the effects of lens wet weight, lens transparency and fibre cell morphology in response to osmotic stress. Bovine lenses were cultured in isotonic artificial aqueous humour (AAH); 290mOs/kg hypotonic AAH; 170mOs/kg and hypertonic AAH; 420-430mOsm/kg for a period of 2 hours after which time,
lenses were assessed for three parameters; lens wet weight, lens transparency and fibre cell morphology.

### 3.4.1 Wet weight

Wet weight measurements were obtained prior to incubation and 2 hours post incubation and the difference between the two expressed as % wet weight change (Figure 3.8). It can be seen that at 2 hours, lenses exhibit changes in their wet weight, with lenses incubated under hypotonic conditions exhibiting an increase in wet weight of 7.08% relative to isotonic lenses (p<0.001) and lenses incubated under hypertonic conditions exhibiting a decrease in weight of 2.61% relative to isotonic lenses (p<0.001). This shows that at 2 hours, in response to changes in the osmolarity of the media, the lens exhibits changes in its wet weight.

![Figure 3.8 Effect of osmotic stress on lens wet weight.](image)

**Figure 3.8 Effect of osmotic stress on lens wet weight.** Percentage change (± SEM) in wet weight between pre- and post-cultured lenses in isotonic (Iso) (n=17), hypotonic (Hypo) (n=9) and hypertonic (Hyper) (n=9) for 2 hours. (*** denotes p<0.001. Refer to Appendix Table A2 for raw data of lens wet weight changes.

### 3.4.2 Lens transparency

To assess the transparency of the lens, a grid was placed underneath the lens and images captured by bright field microscopy. Lenses were examined to determine if they were clear or opaque and if any distortions in the grid lines were evident, an indicator of changes in the
refractive properties of the lens. Incubation of bovine lenses in isotonic AAH (Figure 3.9A) for 2 hours resulted in a clear lens with no signs of grid distortions. Under hypotonic conditions (Figure 3.9B), lenses appeared slightly opaque with no signs of grid distortions, while under hypertonic conditions, lenses were completely opaque so that the grid lines were not visible (Figure 3.9C).

![Figure 3.9 Effect of osmotic stress on lens transparency.](image)

**Figure 3.9 Effect of osmotic stress on lens transparency.** Bright-field shots of lenses cultured in isotonic (A), hypotonic (B) and hypertonic (C) AAH solutions for 2 hours. Grid size: 2mmx2mm.

### 3.4.3 Fibre cell morphology

To determine whether fibre cell morphology in the lens cortex was maintained under hypotonic or hypertonic conditions, lenses were fixed and cryosectioned in an equatorial orientation. Sections were then labelled with the membrane marker, WGA, and morphology assessed using confocal microscopy. Sections from lenses incubated in isotonic AAH (Figure 3.10A) for 2 hours showed normal fibre cell morphology with hexagonal shaped cells in a columnar arrangement. Sections from hypotonic lenses (Figure 3.10B) showed evidence of cell swelling in the lens periphery with cells appearing rounder in structure. In contrast, hypertonic stress resulted in cells that appear to be more packed together indicating that fibre cell shrinkage had occurred (Figure 3.10C).
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Overall, these findings indicate that in comparison to isotonic conditions, incubation of lenses under hypotonic conditions results in a gain in lens wet weight, slightly opaque lenses and the initiation of signs of fibre cell swelling in the lens cortex. On the other hand, under hypertonic conditions, lenses exhibit a decrease in wet weight, become very opaque, with signs of fibre shrinkage. Having established that the lens responds differently to hypotonic vs hypertonic conditions, I was now confident to test what effects these culturing conditions had on the phosphorylation status of KCC3 and NKCC1.

3.5 Changes in the phosphorylation status of NKCC1 and KCC3 in response to osmotic stress

Bovine lenses were cultured under isotonic, hypotonic or hypertonic conditions for 2 hours. The outer cortex region was dissected, homogenised and then centrifuged to obtain the FM fraction. The FM fractions for each condition were then analysed for KCC3-P and total KCC3, and NKCC1-P–and total NKCC1 by western blotting.

Using the KCC3-P Thr991 antibody, a strong band at 75kDa was detected under isotonic, hypotonic and hypertonic conditions, which did not appear to change in intensity between the three conditions (Figure 3.11A, top panel). Stripping of this blot and re-probing with the KCC3 total antibody revealed a very faint band at 75kDa (Figure 3.11A, middle panel). Beta
actin levels were relatively similar between all three lanes (Figure 3.11A, *bottom panel*), but perhaps slightly lower for the hypertonic sample, and demonstrates relative equal loading of protein between the three conditions and that proteins were retained on the blot post-stripping.

A similar finding was also observed with the KCC3-P Thr1048 antibody (Figure 3.11B, *top panel*) and the KCC3 total antibody (Figure 3.11B, *middle panel*), with the signal intensity for KCC3-P being stronger than for the total KCC3 signal. Beta actin levels were similar between all three conditions (Figure 3.11B, *bottom panel*). It was strange that with both phospho-antibodies, the KCC3-P signal was more intense than the total KCC3 signal. Possible explanations for this will be discussed in the Summary section.

![Figure 3.11 Effect of osmotic stress on phosphorylation of KCC3. (A) & (B) Top panel: Band obtained with the KCC3-P Thr991 (A) or KCC3-P Thr1048 (B) antibodies following culturing of lenses in either isotonic (Iso), hypotonic (Hypo) or hyper (Hyper) AAH solutions for 2 hours. Middle panel: Band obtained using the KCC3-T antibody following stripping off the KCC3-P antibody. Bottom panel: Band obtained with the actin antibody after stripping off the KCC3-T antibody.](image)

The signal intensity of the bands obtained with the NKCC1-P antibody differed between isotonic, hypotonic and hypertonic conditions (Figure 3.12A, *top panel*). Stripping of this blot and re-probing with the NKCC1-total antibody revealed a single, strong band at 130kDa with
signal intensity appearing similar between each of the three conditions (Figure 3.12A, middle panel). Beta actin levels were also similar between isotonic, hypotonic and hypertonic conditions (Figure 3.12A, bottom panel). To obtain quantitative information of the NKCC1 phosphorylation status, we measured the signal intensity of the NKCC1-P band relative to signal intensity from the corresponding total NKCC1 band, for each condition, and expressed this as the NKCC1-P/NKCC1-total intensity ratio (Figure 3.12B). The NKCC1-P/NKCC1-total intensity ratio confirmed that NKCC1 phosphorylation was significantly increased under hypertonic conditions (p<0.001), with a 2 fold increase in NKCC1-P under hypertonic conditions relative to isotonic conditions. In addition, NKCC1 phosphorylation was significantly decreased under hypotonic conditions (p<0.05), with a 2.4 fold decrease in NKCC1-P under hypotonic conditions relative to isotonic conditions. Overall, these findings demonstrate that under hypertonic conditions, NKCC1 is phosphorylated and presumably activated to affect an increase in ion influx to attenuate lens shrinkage. In contrast, under hypotonic conditions, NKCC1 phosphorylation is decreased, resulting in less NKCC1 activity to attenuate lens swelling.
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Figure 3.12 Effect of osmotic stress on phosphorylation of NKCC1. (A) Top panel: Band obtained with NKCC1-P antibody following culturing of lenses in either isotonic (Iso), hypotonic (Hypo) or hyper (Hyper) AAH solutions for 2 hours. Middle panel: Band obtained using the NKCC1-T antibody following stripping off the NKCC1-P antibody. Bottom panel: Band obtained with the actin antibody after stripping off the NKCC1-T antibody. (B) represents the quantitative analysis of the intensity ratios of NKCC1-P/ NKCC1-T (+ SEM) for the three different conditions at 2 hours. Number (n) of lanes analysed per treatment group: n= 4 Iso, 4 Hypo, 4 Hyper. (*) denotes p<0.05, (****) denotes p<0.001. Refer to Appendix Table A.1 for raw data.

3.6 Summary

Previously, we have shown that in the rat, KCCs and NKCC1 are involved in the control of steady state lens volume and the maintenance of lens transparency (Donaldson et al., 2009). Using an antibody that detects the phosphorylated form of NKCC1, I have shown that the phosphorylation status of NKCC1 is modulated by the osmolarity of the extracellular bathing medium, and is significantly increased by hypertonic challenge. This increase in NKCC-P in turn implies that the NKCC1 activity is increased, which would result in an influx in ions to counteract lens shrinkage. Conversely, under hypotonic conditions, NKCC1 phosphorylation is decreased, which suggest a reduction in NKCC1 mediated ion influx that would help to attenuate the lens swelling induced by the hypotonic stress. This demonstrates for the first time that the phosphorylation status of NKCC1 can be dynamically regulated in response to osmotic stress to maintain lens volume regulation.
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Unfortunately, we were not able to examine the phosphorylation status for KCC3, due in part to poor labelling for total KCC3 with a KCC3 antibody purchased from Dundee University. There are at least two reasons that might explain this. First, I always probed with the KCC3-P antibody first, stripped the blot and then re-probed with total KCC3 antibody. This was because the detection of phosphorylated KCC3 is only a proportion of the total KCC3 signal and I thought I would have a better chance of detecting P-KCC3 from a new blot as opposed to a stripped blot. However, in fact the signal for KCC3-P was substantially stronger than the total KCC3 signal, and if time permitted, I would have tried to see if I could get a better signal for total KCC3 by probing with the total KCC3 antibody first and then subsequently the P-KCC3 antibody. Secondly, the total KCC3 antibody may not be the best antibody for detecting total KCC3 protein. I decided to use this antibody since it was from the same place that provided me with the KCC3-P antibodies. In previous studies, the laboratory has used a total KCC3 antibody from Santa Cruz. With this antibody, strong bands for total KCC3 have been detected (Vorontsova, 2013). It would be interesting to see if using this antibody on my samples would give me a more robust signal for total KCC3.

In conclusion, we have shown that NKCC1 is dynamically regulated in the bovine lens through alteration of the phosphorylation of its key regulatory threonine residues to either increase or decrease NKCC1 activity in response to changes in osmotic stress. While, we were only able to demonstrate this for NKCC1, the response of the lens to maintain lens volume homeostasis, also likely involves KCCs. However, the investigation of this was hampered by a total KCC3 antibody which did not appear to work as well as the KCC3-P antibodies. Given the ability of our NKCC1-P and total NKCC1 antibodies to detect changes in the phosphorylation status of NKCC1 under osmotic stress, in the next chapter, I will focus on NKCC1 and the effects of hyperglycemia on NKCC1 phosphorylation status and activity,
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since hyperglycemia is known to elicit changes in fibre cell volume that ultimately manifest as diabetic cortical cataract.
4 Hyperglycaemia cataract model

Having validated the utility of the NKCC1 phospho-specific antibody to detect changes in the phosphorylation activity of NKCC1, I now wanted to use this tool to investigate links between CCC phosphorylation activity and the onset of diabetic lens cataract. To achieve this goal, I first needed to establish a model of diabetic cataract that can be applied to the bovine lens. In this chapter, I describe the effects of organ culturing bovine lenses under hyperglycaemic conditions designed to mimic the high levels observed in uncontrolled diabetes. I describe the effects of hyperglycaemia on lens transparency, wet weight, fibre cell morphology and the associated changes in the phosphorylation status of NKCC1. Since my results show that lenses exposed to hyperglycaemia undergo peripheral cell swelling, I have compared my results to those obtained from lenses incubated in hypotonic solutions. However, I begin this section with a review of effects of hyperglycaemia on the lens to aid the evaluation of the merits of organ culturing lenses in high glucose as an in vitro model to mimic diabetic cataract.

4.1 Diabetic lens cataract

Diabetes mellitus (DM) is a heterogeneous group of disorders mainly characterized by elevated blood glucose levels (hyperglycaemia), and may result from impaired insulin production by the pancreas, or ineffective insulin action on target cells (King, 2012). Tissues that function independently of insulin activity in their glucose uptake, such as the lens, are primary targets of hyperglycaemic assault (Pokupec et al., 2003). These tissues are incapable of down-regulating the cellular uptake of glucose in response to rising levels of extracellular glucose concentration and are therefore exposed to the deleterious effects of hyperglycaemia (Obrosova et al., 2010a). In this section, I first describe the damage phenotypes associated with diabetic cataract in humans and animals models, before focussing on the potential
cellular mechanisms by which exposure to hyperglycaemia causes the observed pathology associated with diabetic cataract.

4.1.1 The diabetic cataract damage phenotype

The effects of diabetes most frequently manifest themselves as cortical and/or posterior subcapsular cataracts (Bron et al., 1993; Falck and Laatikainen, 1998; Obrosova et al., 2010a). These are characterised by multiple chalky-white deposits in the cortical region that resemble snowflakes and are followed by the formation of vacuoles in the lens capsule and clefts in the underlying lens cortex. With time, the cataract matures and the entire lens becomes opaque. This phenotype is observed in patients with type 1 DM in which cataracts development is rapid. Adult onset diabetic cataract, also known as “senescent” cataract is prevalent in subjects with type 2 DM and is so called since cataract development occurs slower (~10-20 years) than cataract development in type 1 DM patients. These are typically cortical and/or subcapsular in nature. However adult-onset diabetic cataracts can also often present as nuclear opacities that are indistinguishable from the typical age-related cataracts which occur in the absence of diabetes mellitus (Bron et al., 1993). However, adult onset diabetic cataracts progress more rapidly than age-related cataracts and develop age-related lens changes at a much younger age compared to subjects without diabetes (Bron et al., 1993).

Morphological analysis of human diabetic cataract lenses reveal a discrete zone of major cellular damage that is typically observed in the deeper lens cortex and which is surrounded by relatively undamaged cells in the peripheral cortex and nucleus (Obrosova et al., 2010b). In addition, Morganinan globules, intracellular globules containing α, β, and γ-crystallin and actin (Creighton et al., 1978), breakdown of lens fibres, swelling of broken ends of cortical fibres, and undulating and folded fibres at the cortical-nuclear interface (Michael et al., 2008)
Chapter 4 – Hyperglycaemia cataract model

have also been reported. Ultra-structural studies also reveal lenses from adult-onset diabetes to exhibit a reduction in the epithelial cell density (Takamura et al., 2000; Tkachov et al., 2006). Research with human donor tissue offers the best platform to study the underlying mechanisms of diabetic cataractogenesis (as well as other cataract types in humans) which form the foundation for the identification of targets for potential preventive therapeutics that are effective in humans (Chan, 2016). However, working with human donor tissue is difficult for many reasons: (1) the availability of human donor lenses and intact diabetic cataract lenses are very limited, (2) narrow age range, (3) inherent differences between donors in terms of genetics, life style, exposure to environmental risk factors, other diseases and cause of death, and (4) post-mortem delay between death and tissue processing. These limitations have lead researchers to develop suitable animal models to mimic diabetic cataract in humans to study the pathways of diabetic cataract formation and identify therapeutic options.

Diabetes mellitus has been induced in number of animal models that utilise rodents, non-human primates, and dogs using a variety of methods (Chan, 2016). Intraperitoneal injection of pharmacological agents such as streptozotocin and alloxan that selectively damage the insulin-producing islets of Langerhans in the pancreas into rodents is perhaps the most commonly used method (Pollreisz and Schmidt-Erfurth, 2010). Rats in particular have been the preferred model to study diabetic cataract, since diabetes can be easily induced either with a single dose of streptozotocin, or by feeding them galactose. A single dose of streptozotocin can lead to a high accumulation (approximately 30nmol/g lens) of sorbitol in the rat lens, resulting in “fast” sugar lens opacities (Chan, 2016; Obrosova et al., 2010a). In the diabetic rat lens, formation of cortical opacities has been linked to a significant increase in glucose uptake and subsequent accumulation of sorbitol (Gould and Holman, 1993; Lee et al., 1995).
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The MVL has previously utilised two models of hyperglycaemia to mimic diabetic cataract in the rat lens. Bond et al., (1996) (Figure 4.1A-C) utilised a single dose of streptozotocin to induce diabetes in the rat (STZ rat) *in vivo*, while Merriman-Smith et al., (2003) cultured rat lenses *in vitro* in the presence of high glucose (50mM) (Figure 4.1D-F). In both models, the initial tissue damage was observed as a localized zone of fibre cell swelling in the lens cortex. However, with prolonged exposure to hyperglycaemia, cell swelling develops into more extensive tissue breakdown and formation of large fluid spaces. This damage phenotype closely resembles the damage seen in human diabetic cataracts (Bond et al., 1996). The observed fibre cell damage and liquefaction of cortical tissue in the diabetic rat and human lenses have been associated with changes in ion homeostasis and the loss of ability to regulate fibre cell volume (Duncan and Bushell, 1975; Jacob, 1999).
Figure 4.1 Progression of fibre cell damage in the diabetic lens. (A-C) Equatorial cross-sections demonstrating fibre cell damage in the outer cortex of lenses from diabetic rats after (A) 1 week, (B) 2 weeks and (C) 3 weeks following a single streptozotocin injection. (D-F) Equatorial cross-sections demonstrating localised swelling (D and E) leading to fibre cell damage (F) following culturing of rat lenses in high glucose for (D) 2 days, (E) 4 days and (F) 8 days. Inserts: Monitoring of lens transparency show (D & E, inserts) opacities to initially manifest in the lens cortex and then extend to the (F, insert) lens nucleus. A-C Reproduced from (Bond et al., 1996) and D-F reproduced from (Merriman-Smith et al., 2003) with permission from ARVO.

4.1.2 Effects of hyperglycaemia on the lens

The energy requirements for lens growth and maintenance of transparency are derived predominantly from glucose taken up from the aqueous humour where glucose levels mirror that of blood (Berman, 2013). Hence, the changes in blood glucose levels experienced by diabetic patients will be mirrored by changes in glucose levels in the aqueous. Since the innermost fibre cells comprising the nucleus of the lens are free of organelles, and show limited metabolic activity, it is not surprising that the effects of diabetic cataract are primarily observed in fibre cells of the outer cortex which exhibit higher rates of metabolism. Under normal physiological conditions, the enzyme hexokinase phosphorylates 90% of the glucose in the lens to glucose-6-phosphate which is then metabolized by anaerobic glycolysis and the
pentose phosphate pathway to yield ATP and NADH which provides ~70% of the energy requirements of the lens. As little as 3% of the glucose is metabolised via the aerobic tricarboxylic acid cycle, but this provides ~25% of the energy demands of the lens (Yanoff and Duker, 2014). Exposure of lenses to hyperglycaemia has been shown to alter this balance of glucose metabolism to produce changes, which have been proposed to initiate diabetic cataract formation. The three pathways that have been proposed to contribute to the observed diabetic cataract pathology are outlined below.

**Sorbitol-induced osmotic stress:** Typically in diabetes, when glucose levels are above normal levels (>70-110mg/dL), excess glucose is fed into the polyol pathway (Williamson et al., 1993). The percentage of glucose that enters the polyol pathway under euglycaemic and hyperglycaemic conditions are 3% and 30% respectively (Cheng and González, 1986; Snow et al., 2015). The polyol pathway is a two-step metabolic pathway. In the first rate limiting step, aldose reductase (AR) catalyses the reduction of glucose to the sugar alcohol sorbitol and this reaction is coupled with the oxidation of the co-factor NADPH to NADP⁺. While in the second step sorbitol is oxidised to fructose by sorbitol dehydrogenase (SDH), which is coupled to the reduction of the co-factor NAD⁺ to NADH. The affinity of AR for glucose is low at normal blood glucose levels, but its affinity sharply increases with rising blood glucose levels, consequently activating the polyol pathway. Furthermore, under hyperglycaemic conditions, SDH activity in the lens is very low. Consequently, glucose is reduced to sorbitol by AR is faster than the rate of oxidation of sorbitol to fructose by SDH, ultimately leading to the formation of high amounts of sorbitol (Obrosova et al., 2010a). As sorbitol is highly polar in nature, it does not readily diffuse through the membrane causing it to be retained in the cytoplasm. This intracellular accumulation of sorbitol in turn increases intracellular osmotic pressure leading to increased influx of fluid into cells (Kinoshita, 1974). The long standing view is that high levels of sorbitol induce osmotic stress and changes in
lens hydration that result in fibre cell swelling and tissue liquefaction. However, this view is largely based on animal models of diabetic cataract (Burg and Kador, 1988; Kawakubo et al., 2012; Kyselova et al., 2004) and so it is unclear whether this aetiology also applies in the human lens. There are distinct biochemical differences between human and animal models used to study diabetic cataract with respect to AR activity and sorbitol accumulation. Rodents in particular demonstrate very high levels of AR activity and low levels of SDH activity (Varma and Kinoshita, 1974) contrary to that seen in human lenses, indicating that the dynamics of the sorbitol pathway in the human lens is different from that in animal lenses. Furthermore, the AR activity in human diabetic lenses was not statistically higher than that of non-diabetic lenses (Jedziniak et al., 1981). It is therefore unlikely that accumulation of sorbitol in the adult human diabetic lens will initiate osmotic stress that affects the whole lens. However, the localised accumulation of sorbitol coupled with the high levels of expression of AR in the epithelium and cortex (Jedziniak et al., 1981) most likely results in localised osmotic swelling and damage stress (Kador et al., 2016).

Non-enzymatic glycation: Under hyperglycaemic conditions, there is an increased physiological rate of glycation, where excess glucose reacts non-enzymatically with free amino groups of proteins via an Amadori rearrangement rendering the protein potentially non-functional (Brownlee, 1996). With time, the Amadori products undergo a process known as the Maillard reaction characterised by dehydration and cleavage reactions. Consequently, this generates reactive oxygen species such as superoxide radicals and advanced glycation end products (AGEs) leading to oxidative stress (Obrosova et al., 2010a). The lens specific crystallins (alpha, beta and gamma crystallins) account for nearly 90% (Boscia et al., 2000) of the total protein content of the lens and are amongst the longest lived proteins in the body since they undergo little to no protein turnover. As a result, lens crystallins are particularly susceptible to AGE modification. AGE modification of lens crystallins is presumed to be
associated with cross-linking associated aggregation, insolubilization, and brown coloration with a resultant loss of accommodation and increased light scattering (Franke et al., 2003). A significant increase in AGEs have been detected in cataractous lenses of diabetic human subjects (Franke et al., 2003; Mota et al., 1994). Furthermore, accumulation of glycated and AGE-modified beta and gamma crystallins have been detected in streptozotocin diabetic rats (Monnier et al., 1979; Nakayama et al., 1993; Ranjan et al., 2006).

Oxidative stress: Oxidative stress is frequently observed in the human diabetic lens and manifests early on as a reduction in glutathione (GSH), a potent antioxidant highly abundant in the lens that protects the lens from oxidative damage (Özmen et al., 1997). Increased activity of the polyol pathway leads to a decrease of the NADPH: NADP$^+$ ratio (Bron et al., 1993), leading to oxidative stress and the subsequent depletion of NADPH required for the regeneration of GSH from GSSG (oxidized GSH) by glutathione reductase (Kyselova et al., 2004). Diabetic lenses also demonstrate decreased activity of glucose-6-phosphate dehydrogenase, the key regulatory enzyme of the pentose-phosphate pathway. This process further reinforces the premise for reduced regeneration of NADPH, implicating that under hyperglycaemic conditions the pentose-phosphate pathway, the main regenerative source of NADPH could be impaired (Donma et al., 2002). Other signs of oxidative stress in the diabetic lens include an increase in lipid peroxidation products (Altomare et al., 1995) and reduced levels of GSH to drive the reduction of oxidised ascorbate (Anthrayose and Shashidhar, 2004). Glycation and subsequent AGE modification leading to structural changes, and/or decreased availability of GSH and/or NADPH (Shin et al., 2006) have been suggested to reduce the efficiency of antioxidant defence enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidise in the lenses of diabetic patients (Donma et al., 2002; Özmen et al., 2002). Although these findings strongly support oxidative stress induced damage in diabetic cataract in human lenses, the source of this oxidative stress
is not known. Autoxidation and non-enzymatic glycation have been implicated in contributing to the pool of free radicals in the diabetic lens (Ansari et al., 1996; Nagaraj et al., 1991; Wolff and Dean, 1987) which in turn further increases glycation (Loske et al., 2000). SDH which catalyses the conversion of sorbitol to fructose results in an increase the cytosolic ratio of free NADH:NAD. NADH in turn is oxidised by NADH oxidase further contributing to the pool of reactive oxygen species (Rose and Warms, 1966). Finally, fructose produced by the polyol pathway can be phosphorylated to fructose-3-phosphate, which is further broken down to 3-deoxyglucosone. Both fructose-3-phosphate and 3-deoxyglucosone are stronger non-enzymatic glycating agents than glucose establishing another source of AGEs (Stitt, 2005; Tang et al., 2012).

In summary, the morphological and biochemical changes in response to hyperglycaemia show that osmotic stress alone does not account for the slow development of cataract typically seen in the majority of adult diabetic patients (Chan et al., 2008). Rather, the emerging view is that hyperglycaemia stimulates polyol activity, which in turn generates osmotic stress as well as oxidative stress in the diabetic lens (Chung and Chung, 2003). Although the initiating mechanism of diabetic cataractogenesis results in osmotic stress, the lens demonstrates the ability to regulate its volume against small changes in osmotic pressure by activating volume regulatory mechanisms (Cammarata et al., 2002). However, over time the ability of the lens to actively regulate its volume becomes impaired (Chan et al., 2008), and coupled with oxidative stress, these two pathways converge and contribute to diabetic cataract formation.

An appropriate animal model is required to replicate key features of diabetic cataract in humans that can be used to identify appropriate targets which can potentially slow down the progression of diabetic cataract. The frequently utilised rodent models of diabetic cataract due to high aldose reductase activity are ‘acute models’ that replicate the fast development of
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cataract. Like the human lens, bovine lenses exhibit low AR activity (Srivastava and Petrash, 1982), and may be a better model than the rat lens to study the onset of diabetic lens cataract. Hence, in this study I will investigate whether incubating bovine lenses under hyperglycaemic conditions can serve as a model to mimic the early changes in cell volume associated with diabetic cataract in humans.

4.2 Effects of hyperglycemia on lens transparency, lens wet weight and cell morphology

A hyperglycaemic environment was mimicked by supplementing isotonic AAH with 50mM D-glucose resulting in a solution with a final osmolarity of 340mOsm/kg. Lenses were also incubated in two types of “control” culture media: (1) isotonic AAH with an osmolarity of 285-290mOsmol/kg, and (2) isotonic AAH supplemented with 50mM D-Mannitol, a membrane impermeable non-hydrolysable sugar that produced a solution with an osmolarity of 340mOsm/kg. The isotonic AAH was used as a control to mimic the ‘normal’ aqueous humour environment, while high mannitol AAH was used as a control to rule out the effects of osmolarity when comparing the effects induced by high glucose AAH. Bovine lenses were cultured in either isotonic AAH, high glucose AAH or high mannitol AAH for 2, 24, 48 or 72 hours. These time points were selected to enable me to develop a time course of changes to lens wet weight, lens transparency and cell morphology as a result of culturing lenses under hyperglycaemic conditions.

4.2.1 Wet weight

Wet weight measurements were obtained prior to incubation and post incubation and the difference between the two expressed as % change in wet weight (Figure 4.2). In the presence of high glucose, the wet weight of lenses steadily increases over the course of 72 hours, with the steepest increase between 48 and 72 hours. At 72 hours, the wet weight of lenses cultured...
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in high glucose AAH significantly increased by 1.8 fold (p< 0.05) relative to isotonic lenses. In the presence of high mannitol, the wet weight of the lenses significantly decreased (p< 0.01) over the course of 48 hours, relative to isotonic lenses. However, after 48 hours, the wet weight of the lens underwent a dramatic increase indicating pathological swelling of the lens.

![Figure 4.2](image)

**Figure 4.2 Time course of effects of high glucose on bovine lens wet weight.** Percentage change (±SEM) in wet weight between pre- and post-cultured lenses in isotonic AAH (Iso), high glucose AAH (Gluc) and high mannitol for 2 hrs, 24 hours, 48 hours or 72 hours. At 2 hours; n= 17 Iso, 15 Gluc, 9 Mann, at 24 hours; n=9 Iso, 12 Gluc, 6 Mann, at 48 hours n= 9 Iso, 12 Gluc, 6 Man and at 72 hours n= 9 Iso, 12 Gluc, 5 Mann. Significance values are given for comparisons of means between isotonic and mannitol/glucose of matched time points. (*) denotes p<0.05, (**) denotes p<0.01, (***) denotes p<0.001.

### 4.2.2 Lens transparency

Under isotonic conditions, lenses remained clear and transparent with no signs of grid distortion from 2 hours right through to 72 hours of culturing (Figure 4.3). Lenses cultured in high glucose remained relatively clear, with a slightly hazy appearance apparent at 48 hours and 72 hours, but with no signs of grid distortions. In contrast, lenses cultured in high mannitol were cloudy. At 2 hours, the outer cortex region appears cloudy, but by 72 hours, the whole lens is cloudy. However, the grid lines are still visible at this stage and there appears to be no obvious grid distortion.
Figure 4.3 Time-course of effects of high glucose on bovine lens transparency. Brightfield images of lenses following culturing in isotonic AAH (A-D), high glucose AAH (E-H) and high mannitol AAH (I-L) for 2, 24, 48 and 72 hours. Grid size underneath the lenses: 2mmx2mm.

4.2.3 Fibre cell morphology

In order to assess the effect on fibre cell morphology as a result of exposure to high glucose, following incubation, lenses were fixed and cryosectioned in an equatorial orientation. Sections were then labelled with WGA and morphology assessed using confocal microscopy (Figure 4.4).

Sections from lenses incubated in isotonic AAH for each of the four time points displayed normal cell morphology with hexagonal shaped cells arranged in a columnar fashion (Figure 4.4A-D). In contrast, sections from lenses cultured in high glucose showed peripheral cell swelling at 24 and 48 hours with extensive cell damage seen at 72 hours, in spite of these
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lenses remaining relatively clear (Figure 4.4E-H). Unfortunately, due to time pressures, we
were only able to capture images of sections from high mannitol lenses at 2 hours and 48
hours (Figure 4.4I&J). However, it can be seen that despite the similarity in osmolarity
between high glucose and high mannitol AAH, sections from lenses cultured in high mannitol
AAH for 48 hours resulted in cell shrinkage in the lens outer cortex region, while lenses
cultured in high glucose AAH at this same time point revealed peripheral cell swelling.
Taken together, these results show that while both high mannitol and high glucose AAH
solutions were hyperosmotic, it was only lenses cultured in high mannitol that exhibited cell
shrinkage. On the other hand, lenses cultured in high glucose resulted in peripheral cell
swelling, reminiscent of lenses cultured under hypotonic conditions (see Chapter 3). This
suggests that it was the effects of high glucose and not the osmolarity that resulted in cell
swelling.
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4.3 Comparison between the effects of high glucose and hypotonic challenge

Given that high glucose appears to generate peripheral cell swelling similar to that seen in lenses exposed to hypotonic challenge, I decided to take a closer look at the effects induced by hyperglycaemia and compare this to the effects induced by hypotonic stress. I compared the wet weight of lenses cultured in high glucose or hypotonic solutions and showed that while both conditions result in an increase in wet weight over time, the weight increase is significantly greater \((p<0.001)\) as a result of hypotonic stress relative to high glucose (Figure 4.5). Examination of the morphology of cells at 2 hours and 48 hours for both groups revealed that while high glucose lenses appear relatively clear (Figure 4.6A) and cell

![Figure 4.4 Time-course evaluation of effects of high glucose on fibre cell morphology of the bovine lens outer cortex. Equatorial sections labelled with WGA from bovine lenses cultured in isotonic AAH (A-D), high glucose AAH (E-H) or high mannitol (AAH) for 2, 24, 48 or 72 hours.](image)
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morphology is relatively unaltered at 2 hours (Figure 4.6B), hypotonic lenses are cloudy (Figure 4.6C) with evidence of peripheral cell swelling apparent (Figure 4.6D). At 48 hours, high glucose lenses appear slightly hazy (Figure 4.6E) with peripheral cell swelling evident (Figure 4.6F). However, hypotonic lenses are opaque (Figure 4.6G) with major cell damage apparent in the lens cortex (Figure 4.6H). This extent of damage seen for hypotonic lenses at 48 hours is similar to that observed for the damage seen in high glucose lenses at 72 hours (Figure 4.4). Overall, it appears that while high glucose AAH is a hyperosmotic solution, the effects on the lens are more comparable to those seen for lenses exposed to hyposomotic solutions. However, changes to lens wet weight, transparency and cell damage occur much more rapidly under hypotonic conditions compared to the effects on the lens induced by high glucose.

Figure 4.5 Comparison of time-course changes in wet weight between high glucose and hypotonic lenses. Percentage change (+ SEM) in wet weight between pre- and post-cultured lenses in high glucose AAH and hypotonic AAH for 2, 24, 48 or 72 hours. At 2 hours, n= 15 Gluc, 9 Hypo, at 24 hours, n=12 Gluc, 3 Hypo, at 48 hours n=12 Gluc, 3 Hypo and at 72 hours, n=12 Gluc, 3 Hypo.
Figure 4.6 Comparison of lens transparency and fibre cell morphology of lenses exposed to high glucose and hypotonic AAH solutions. (A, C, E, G). Bright-field images of lenses cultured in high glucose (A & E) for 2 hours (A) or 48 hours (E) or cultured under hypotonic conditions (C & G) for 2 hours (C) or 48 hrs (G). (B, D, F, H) Equatorial sections labelled with WGA from lenses cultured in high glucose (B & F) for 2 hours (B) or 48 hours (F) or cultured under hypotonic conditions (D & H) for 2 hours (D) or 48 hours (H).

4.4 Changes in the phosphorylation status of NKCC1 in response to hyperglycemia

Having established a time course of changes to lens wet weight, transparency and cell morphology as a result of hyperglycaemia over a 72 hour period, I next wanted to determine the effects of hyperglycaemia on the phosphorylation status of NKCC1. To do this, I decided to focus on the 2 hour time point where we had previously shown in Chapter 3 that changes in the phosphorylation status could be detected following osmotic stress.

However, before examining changes in the phosphorylation status of NKCC1, I first compared lens wet weight, transparency and cell morphology of isotonic, high mannitol, high glucose and hypotonic cultured lenses for this time point (Figure 4.7). Here it can be seen that relative to isotonic lenses, lenses cultured in high mannitol exhibited a decrease (p<0.001) in lens weight, lenses cultured in glucose showed no major changes in lens weight, while hypotonic lenses displayed a significant increase (p<0.001) in lens weight.
Figure 4.7 Comparison of change in wet weight of lenses exposed to high glucose AAH with isotonic, high mannitol and hypotonic AAH solutions. Percentage change (+ SEM) in wet weight of lenses cultured in isotonic AAH (Iso) (n=17), high mannitol (Man) (n=9), high glucose (Gluc) (n=15) and hypotonic (Hypo) AAH (n=9) solutions at 2 hours. (***$^{*}$) denotes p<0.001. Refer to Appendix Table A2 for raw data of lens wet weight changes.

With regard to changes in lens transparency, it can be seen that lenses were clear and transparent at 2 hours under isotonic conditions (Figure 4.8A), lenses were cloudy in the lens cortex in the presence of high mannitol (Figure 4.8B), lenses were relatively clear in the presence of high glucose (Figure 4.8C) and the entire lens was cloudy as a result of hypotonic stress (Figure 4.8D).

Figure 4.8 Comparing the effects of high glucose on lens transparency with that of lenses cultured in isotonic, high mannitol and hypotonic AAH solutions. Bright-field shots of lenses cultured in isotonic (A), high mannitol (B), high glucose (C) and hypotonic (D) AAH solutions for 2 hours. Grid size underneath the lenses: 2mmx2mm.

In terms of cell morphology, cells appeared normal at 2 hours for isotonic AAH (Figure 4.9A) cultured lenses. Fibre cells appeared visibly shrunken in high mannitol
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(Figure 4.9B) cultured lenses and slightly swollen in high glucose (Figure 4.9C) lenses at 2 hours. However at 2 hours, evidence of prominent peripheral cell swelling was observed for hypotonic (Figure 4.9D) lenses.

![Figure 4.9 Comparing effects of high glucose on outer cortical fibre cell morphology with that of lenses cultured in isotonic, high mannitol and hypotonic AAH solutions. WGA labelling of equatorial sections of bovine lenses cultured in isotonic (A), high mannitol (B), high glucose (C) and hypotonic (D) AAH solutions for 2 hours.](image)

In my final set of experiments, I compared the phosphorylation status of NKCC1 from FM fractions of the outer cortex from bovine lenses cultured under isotonic, high glucose, high mannitol or hypotonic conditions for 2 hours. The FM fractions for each condition were then analysed for NKCC1-P and total NKCC1 by western blotting (Figure 4.10A). The signal intensity of the bands obtained with the NKCC1-P antibody differed between isotonic, high mannitol, high glucose and hypotonic conditions. Stripping of this blot and re-probing with the NKCC1-total antibody revealed a single, strong band at 130kDa. Beta actin levels were similar between isotonic, high glucose and hypotonic samples, but was comparatively less in high mannitol. To obtain quantitative information of the NKCC1 phosphorylation status, I measured the signal intensity of the NKCC1-P band relative to signal intensity from the corresponding total NKCC1 band, for each condition, and expressed this as the NKCC1-P/NKCC1-total intensity ratio (Figure 4.10B). The NKCC1-P/NKCC1-total intensity ratio showed that NKCC1 phosphorylation was significantly increased in the presence of high mannitol, with a 2 fold increase (p<0.01) in NKCC1-P in the presence of high mannitol relative to isotonic conditions. Under high glucose conditions, NKCC1
phosphorylation was slightly decreased relative to isotonic conditions, although this was not statistically significant. Under hypotonic conditions, NKCC1-P was significant decreased (p<0.05), with a 2.5 fold decrease in NKCC1-P under hypotonic conditions relative to isotonic conditions. Overall, these findings demonstrate that in the presence of mannitol, NKCC1 is phosphorylated and activated to affect an increase in ion influx to attenuate lens shrinkage. In the presence of high glucose, NKCC1-P decreased slightly relative to isotonic lenses, suggestive of reduced NKCC1 activity and a decrease in ion influx which precedes any major changes to lens weight or cell damage. Under hypotonic conditions, NKCC1 phosphorylation is decreased, resulting in less NKCC1 activity to attenuate lens swelling.

Figure 4.10 Change in NKCC1 phosphorylation at 2 hours of lenses exposed to high glucose AAH compared with lenses exposed to isotonic, high mannitol and hypotonic AAH solutions. (A) Top panel: Band obtained with NKCC1-P antibody following culturing of lenses in isotonic (Iso), mannitol (Mann), glucose (Gluc) or hyper (Hyper) AAH solutions for 2 hours. Middle panel: Band obtained using the NKCC1-T antibody following stripping off the NKCC1-P antibody. Bottom panel: Band obtained with the actin antibody after stripping off the NKCC1-T antibody. (B) represents the quantitative analysis of the intensity ratios of NKCC1-P/ NKCC1-T (+ SEM) for the three different conditions at 2 hours. Number (n) of lanes analysed per treatment group: n= 4 Iso, 4 Man, 4 Gluc, and 4 Hypo. (*) denotes p<0.05, (**) denotes p<0.01. Refer to Appendix Table A1 for raw data.
4.5 Summary

In this section, I have shown that lenses cultured in high glucose exhibited an increase in lens wet weight after 24 hours with changes to cell morphology that presented as peripheral cell swelling and eventually resulting in major cell damage by 72 hours. At 2 hours, NKCC1-P appeared to decrease although this was not significant, indicating that under this condition, a longer time point may have been required to see a significant decrease in NKCC1-P. However, in the presence of high mannitol at 2 hours, I was able to detect a significant increase in NKCC1-P, relative to isotonic lenses, demonstrating the NKCC1-P antibody to be an excellent tool with which to assess changes in NKCC1 phosphorylation status in response to exposure to high levels of sugar.

A number of interesting observations were made when comparing lens parameters between high glucose and high mannitol cultured lenses. The peripheral cell swelling observed in the presence of high glucose was in contrast to the cell shrinkage seen in the outer cortex region of lenses cultured in high mannitol. Both high glucose and high mannitol are hyperosmotic solutions. However, there is a major difference in the ways in which these sugars are processed in the lens. Mannitol is a membrane impermeable non-hydrolysable sugar that is not accumulated by cells (Madonna et al., 2016). As a result, water leaves the cell resulting in cell shrinkage. In contrast, glucose is able to be transported across the cell membrane via glucose transporters (Merriman-Smith et al., 2003; Merriman–Smith et al., 1999), with accumulation of glucose resulting in the formation of sorbitol via the polyol pathway (Kinoshita, 1974). Sorbitol however is itself impermeable and as a result this osmolyte attracts water into the cell resulting in cell swelling. As a result, cell swelling in these lenses appears similar to the cell swelling induced under hypotonic conditions.
A close examination of lens parameters between high glucose and hypotonic treated lenses, however, reveals that while lenses swell under both conditions, the time course of cell swelling and damage is different. Under hypotonic conditions, cell swelling occurs rapidly and is detected as early as 2 hours with major cell damage observed by 48 hours. In contrast in high glucose lenses, cells look relatively normal at 2 hours with cell swelling evident at 24 hours and major cell damage apparent at 72 hours. The longer time taken for cell swelling and cell damage to occur in high glucose lenses is likely to be related to differences observed in the increase in wet weight of these lenses under high glucose vs hypotonic conditions. While both conditions resulted in an increase in lens wet weight over a 72 hour period, the gain in wet weight was markedly greater for hypotonic lenses than for high glucose lenses. The steady weight gain exhibited by high glucose lenses is most likely a reflection of rate of metabolism of glucose to sorbitol by the enzyme aldose reductase (Hegde and Varma, 2005). Aldose reductase activity is known to differ among species with activity highest in rats (Jedziniak et al., 1981; Varma and Kinoshita, 1974) and low in mice (Varma and Kinoshita, 1974) and humans (Jedziniak et al., 1981; Varma and Kinoshita, 1974). Since in the bovine lens, aldose reductase activity is even lower, ~ 1/8th the levels measured in humans lenses (Srivastava et al., 1982), this could explain the lack of a marked rapid weight gain by high glucose lenses. Another interesting difference between the two conditions was the effects of these two conditions on lens transparency. While hypotonic lenses were cloudy at each time point, it was interesting that lenses cultured in high glucose were relatively clear even at 72 hours, despite major cell damage being observed. It would be interesting in the future to determine at what time point high glucose lenses develop opacities and to correlate this with morphological analysis. The longer period of time to develop opacities despite cell damage may indicate that for high glucose lenses, other factors may contribute to loss of lens transparency.
Overall, my findings indicate that the lens is a dynamically active tissue that responds to the addition of sugars in the extracellular media by alterations to the phosphorylation status of NKCC1. In the presence of high glucose, NKCC1 phosphorylation decreases which leads to reduced ion influx to reduce cell swelling. However, with increased periods of exposure to high glucose, cells lose their ability to regulate their volume resulting in cell swelling and cell damage. While this type of damage is reminiscent of that seen in diabetic cataract, the location of this damage in human diabetic cataract is typically observed in a localised zone in the deeper lens cortex, surrounded by relatively undamaged cells in the superficial cortex and nucleus (Obrosova et al., 2010a). In my study, the major cellular damage was observed in the lens periphery and not the deeper lens cortex. This may reflect the acute nature of high glucose exposure in this model and implications of this will be raised in the next section.


5 Conclusions, significance and future work

The overall goal of the MVL is to design novel therapies to delay or prevent the onset of lens cataract. To achieve this they are actively researching at the molecular, cellular and whole lens levels how ion channels and transporters contribute to the maintenance of lens transparency in both the normal and cataractic lens. In this project, I have attempted to contribute to this ongoing body of work by studying the role of CCCs in the initiation of diabetic cataract. In this final section, I will first summarise the major conclusions of my project before discussing the significance of my findings to the aetiology of diabetic cataract and highlighting where further future work is required.

5.1 Major Conclusions

5.1.1 Chapter 3 conclusions: Characterisation of the phosphorylation status of cation-chloride cotransporters in the bovine lens.

The goal of this chapter was to determine whether antibodies raised against the phosphorylated and non-phosphorylated forms of NKCC1 and KCC3 could be used to detect changes in the phosphorylation status and therefore activity of the two cotransporters. I found that:

1. Antibodies raised against the phosphorylated form of NKCC1, but not KCC3, could be used to monitor the phosphorylation status and hence activity of NKCC1

2. NKCC1 phosphorylation was significantly increased in response to hypertonic stress, indicative of increased NKCC1 transporter activity

3. NKCC1 phosphorylation was significantly decreased in response to hypotonic stress, indicative of decreased NKCC1 transporter activity.
5.1.2 Chapter 4 conclusions: Characterisation of the phosphorylation status of NKCC1 in a bovine model of diabetic cataract

In this chapter, I wanted to determine whether the activity of NKCC1 was altered in the diabetic lens. To achieve this, I exposed organ cultured bovine lenses to hyperglycaemia in an attempt to mimic the damage phenotype observed in rat and human diabetic lenses and analysed the effect of hyperglycaemia on the phosphorylation status of NKCC1. I found that:

1. Bovine lenses exposed to high glucose exhibit peripheral cell swelling by 24 hours, and with prolonged exposure to hyperglycaemia major cell damage is evident by 72 hours.

2. This damage phenotype appeared similar to that observed in lenses cultured under hypotonic conditions. However, increases in lens wet weight and cell damage occurred much more rapidly under hypotonic conditions compared to high glucose conditions.

3. Lenses cultured in high glucose were clear and transparent

4. After a 2 hour exposure to hyperglycaemia no significant decrease in the phosphorylation status of NKCC1 was observed.

5.2 Significance and future work

5.2.1 Signalling pathways in the lens that regulate lens volume

In previous work by the MVL, CCCs such as NKCC1 and KCC3 have been demonstrated to be key effectors of fibre cell volume regulation (Donaldson et al., 2009). In other tissues the activity of NKCC1 and KCC3 has been shown to be reciprocally regulated by the WNK-SPAK/OSR1-PP1/PP2A signalling pathway via the modulation of the phosphorylation status of the two cotransporters (Kahle et al., 2010) Vorontsova et al 2015 has shown that this signalling pathway is expressed in the lens (Vorontsova et al., 2015), and now I have utilised an antibody raised against the phosphorylated forms of NKCC1, to detect changes to the
Chapter 5 – Conclusions and Significance

phosphorylation of key threonine (212 and 217) residues of NKCC1 in bovine lenses exposed to osmotic challenge (Figure 3.12). Unfortunately, my results for KCC3 (Figure 3.11) were not as clear cut as those obtained for NKCC1 since the antibodies used to study KCC3 needed further optimisation. If time permitted I would have used the phospho-KCC3 antibody in combination with a different total KCC3 antibody so that changes in KCC3 and NKCC1 phosphorylation in response to changes in osmotic stress and hyperglycaemia could have been assessed in parallel.

While the results obtained for NKCC1 confirmed that its activity in the lens is regulated by changes in osmolality, my results do not directly link the change in phosphorylation status of NKCC1 to activation of the WNK-SPAK/OSR1-PP1/PP2A signalling pathway. However, a similar antibody based approach can be used in future experiments to test for the involvement of this signalling pathway since the kinases are them self-activated by changes to their phosphorylation status (Kahle et al., 2008). The MVL has also recently acquired phospho-antibodies for WNK kinase, the osmosensor that detects osmotic challenge and SPAK/OSR1, the transducer of cell volume regulation that directly phosphorylates and thus alters the activity of the CCCs. In future work, these phospho-antibodies will be used to assess the phosphorylation status of these regulatory kinases in order to provide a more complete understanding of the phospho-regulatory pathway that controls CCC activity and therefore lens volume. Furthermore, it is hoped that this antibody toolkit can be used as tools to investigate CCC functionality in human lenses obtained from donors with and without diabetes so that we can ensure that my animal findings are translatable to humans.

5.2.2 The aetiology of diabetic lens cataract

Work from the MVL (Donaldson et al., 2009) and other laboratories (Jacob, 1999) has suggested that diabetic cataract is due to a failure of the lens to regulate its volume. A
working hypothesis advanced to explain the damage phenotype observed in diabetic cataract is shown in Figure 5.1. In this model osmotic and/or oxidative stress induced by hyperglycaemia is thought to affect the signalling pathways that modulate the phosphorylation status of the NKCC1 and KCCs in the lens. In this project, I have attempted to test this hypothesis by monitoring the phosphorylation status of NKCC1 and KCC3 in bovine lenses organ cultured in high glucose. As already discussed this approach is dependent on the specificity of the antibodies used and the utility of the model of diabetic cataract adopted.

In order to assess NKCC1 phosphorylation changes in response to high glucose, I chose to develop an acute experimental model of diabetes in the bovine lens. To achieve this I investigated whether organ culturing bovine lenses in high glucose could mimic the effects of hyperglycaemia on the lens observed in diabetes. Interestingly, while I discovered that the damage phenotype of cell swelling leading to major cell damage was similar to that seen in diabetic rat lenses (Bond et al., 1996) and human diabetic lenses, the location of this damage was not consistent with previous reports, in which cell swelling was restricted to the deep outer cortex region. The damage phenotype I observed in peripheral fibre cells may be linked to the location of aldose reductase in the bovine lens. It would be interesting to determine whether aldose reductase expression in the bovine lens is more restricted to peripheral cells which might account for the major changes in fibre cell volume that I see in this region.

Interestingly, nuclear magnetic resonance spectroscopy of glucose and sorbitol in bovine lenses cultured in a high glucose (50mM) medium demonstrated that their accumulation corresponded to the lens periphery and cortex after days 1, 2 and 4 (Sawada et al., 2003). The other interesting feature I noticed in my hyperglycaemic model was that no cataract was detected. Longer culturing times (post 72 hours) will be useful in determining when cataract development occurs and if this is associated with cell swelling and damage in the deeper lens
Chapter 5 – Conclusions and Significance

cortex. In a recent study, bovine lenses were incubated in a high glucose (50mM) medium for 5 days to determine hyperglycaemia induced refractive changes (Mehta et al., 2015). A trend towards myopia was observed with increasing hyperglycaemia; however this was not statistically significant. When lenses were stepped-down from hyperglycaemia to normal physiological levels of glucose, a hyperopic shift was observed in line with published clinical studies; however this was also not statistically significant. The authors suggest that bovine lenses are able to offset the raised osmotic pressure from high glucose levels in the short-term by a process of osmoregulation and that repeated osmotic stress or longer term exposure may be required to induce the changes in refraction that are seen clinically. Finally, it was observed that no significant decrease in NKCC1 phosphorylation was detected in my hyperglycaemic model at 2 hours. This may be linked to the time point selected. Given that a gain in lens weight and the time taken for cell swelling and damage occurs at a much slower time course for lenses cultured in high glucose relative to hypotonic conditions, selecting a slightly longer time point (e.g. 4 hours) may result in a significant decrease in the phosphorylation status of NKCC1-P.

Overall, the hyperglycemic model I have developed may be an interesting model to characterise further. The lack of any detectable cataract may suggest that this is a good model for studying the events that are triggered in response to high glucose that precede cataract development. It is therefore possible that this model could potentially be used for examining intervention strategies aimed at controlling fiber cell volume via the manipulation of CCCs or their regulators to delay diabetic cataract.
Figure 5.1 Proposed model for dysregulation of cell volume regulators in diabetic cortical cataract. (A) In the normal lens, the highly ordered rows of hexagonal fibre cells are a result of the tightly regulated balance between the net ion efflux and influx maintained by appropriate KCC and NKCC upstream signalling. (B) Osmotic and/or oxidative stress generated by hyperglycaemia in diabetic cortical cataracts lead to a decrease in the upstream kinase function and/or increase in phosphatase activity resulting in a decrease in the phosphorylation status of NKCC1 and KCC activity. The subsequent decrease in NKCC1 and increase in KCC activity together produces an increase in ion efflux in the lens periphery and an increase in ion influx in the deeper lens, that manifests as peripheral cell shrinkage and deeper fibre cell swelling observed in diabetic cataract.
References


Gusev, G., Agalakova, N., 2010. Regulation of K–Cl cotransport in erythrocytes of frog Rana temporaria by commonly used protein kinase and protein phosphatase inhibitors. Journal of
References

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References

References


References


References

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References

## Appendix & Supplementary Data

### Table A1: Intensity Ratios of NKCC1-P/NKCC1-T signals in the bovine lens outer cortex.

<table>
<thead>
<tr>
<th>Osmotic Challenge/Treatment</th>
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### Table A2: Percentage change in wet weights of lenses incubated in different osmotic conditions and treatments at 2, 24, 48 and 72 hours.

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bKCC3 61  SEMSGATTSLATVALDQSADRTSNDPQDVITEDPSQNSITGEHSQLLDDGHKKARNAYLNN 120
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bKCC3 241  IATNGVVFAGSYFMSRLAGPEFGGAVGCLFYLGTITFAAAMYILGAIEIFLYIVPRAA 300
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Appendix & Supplementary Data

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bKCC3  661  CALQTLLRTPNWPFRFRYYHWALSFMGMSICLAMFISSWYYAIVAVMIQIYKIEYQ  720

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Appendix & Supplementary Data

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GGSEVITIYS

bKCC3 1141  GGSEVITIYS 1150
## Table A4: Homo sapiens and Bos taurus NKCC1 sequence Alignment

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hNKCC1 900  VDMYLNFHADFIQDYGVVVIIRLKEGLIHDLSLQGEQELLSSQEKSEPDKVDVVSVSVEYKK 959
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hNKCC1 960  SDLTSKPSESEPKTHVEVEDEGKATQPLLKESKPGP1VPLMVADQKLLEASTQFKQK 1019
bNKCC1 952  SDLTSKPSESEPKTHVEVEDEGKATQPLLKESKPGP1VPLMVADQKLLEASTQFKQK 1008
hNKCC1 1020  GKTIDVWLFDGGGLLLLIPYLLTTTKKKDKCIRVFGKGIKNIRHHDIBMATELLSKF 1079
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| bNKCC1  | 1009 | GKTIDVWWLFDDGGGLTLPLLTTKKKDCKIRVFIGGKIRHDHRAM TLL+KF |
| hNKCC1  | 1080 | RIDFSDIMVLGDINTKPKKENIAFEIIEPYRLHEDDKEQDIADKMEDEPWRITDNEL |
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| hNKCC1  | 1140 | ELYKTKTYRQIRNLLEHSSSTANIIVMSLPVARKGAVSSALYMAWLEALSKDLPPILL |
| bNKCC1  | 1129 | ELYKTKTYRQIRNLLEHSSSTANIIVMSLPVARKGAVSSALYMAWLEALSKDLPPILL |
| hNKCC1  | 1200 | VRGNHQSVLFYS 1212 |
| bNKCC1  | 1189 | VRGNHQSVLFYS 1201 |
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