

SECTION 1 – INTRODUCTION AND LITERATURE REVIEW

1.1 The Lens

Lens Function

The lens is a transparent tissue found in the eye. The primary function of the vertebrate lens is to collect and concentrate electromagnetic radiation between 400-700 nm (visible light) on the retina of the eye, providing increased sensitivity and allows for information contained by that light to be spatially resolved (Fernald, 2006; Lou, 2003; Roberts, 2001).

Lens Structure

The vertebrate lens is comprised principally of epithelial cells at varying stages of proliferation and differentiation surrounded by a thick basement membrane, or capsule, of extracellular matrix material (Rao & Maddala, 2006; Straub *et al.*, 2003). The outer zone is made up of metabolically active, structurally unspecialized cuboidal epithelial cells that cover the anterior periphery of the lens, and is present in both the embryonic and mature lens. These epithelial cells secrete the material that makes up the capsule, which is formed by the apposition of multiple layers of basal lamina composed mainly of collagen type IV (Barraquer *et al.*, 2006). The lens epithelial cells migrate from the anterior proliferative zone toward the equatorial region of the lens, known as the bow region, where they form a second discernable zone, exiting the cell cycle and commencing differentiation into elongated fibre cells. This transitive zone is the site where organelles are lost from the fibre cells. Finally, aged quiescent fibre cells reside in the core or nucleus of the lens, which is discernable at a distance 10–20% of the radius into the lens (see *fig. 1.1*; Fernald, 2006; Weber & Menko, 2006; Kyselova *et al.*, 2004; Gao *et al.*, 2004; Mathias & Rae, 2004; Shestopalov & Bassnett, 2003; Menko, 2002; Beebe *et al.*, 2001; Quinlan *et al.*, 1999; Clark *et al.*, 1999).

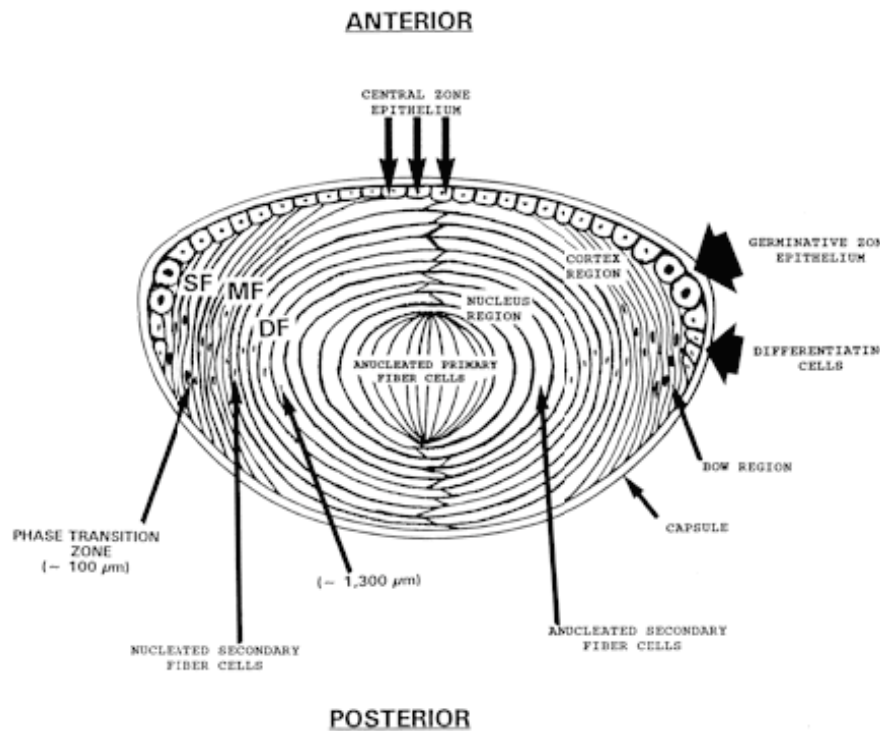


Figure 1.1 Schematic of a mammalian lens, illustrating salient points of lens structure.

Beneath the surrounding collagenous capsule, nucleated epithelial cells are seen to cover the anterior face of the lens extending back to the equatorial bow region, where they begin differentiation and elongation into fibre cells (**SF**; superficial fibres). As new fibre cells are laid over pre-existing cells (**MF**; middle fibres) the lens becomes a compacted mass of fibre cells, and the MF contributes 80-90% of the lens diameter (Gao *et al.*, 2004). The gradual loss of nuclei from the fibre cells as they track inward toward the nucleus of the lens (**DF**; deep fibres) indicates the loss of organelles from the lens fibre cell as they differentiate. Diagram from Gagna *et al.*, (1997).

The fibre cells found in the cortex of the lens elongate 50- to 100-fold, potentially reaching up to 1 cm in length when fully differentiated in an adult human lens, and are capable of elongation rates of 150 $\mu\text{m}/\text{day}$ in some species (Bassnett, 2005). Differentiation of fibre cells from lens epithelial cells involves major structural changes and partial fusion of adjacent fibre cells, as well as changes in gene expression and the expression of differentiation-specific proteins such as the crystallins, water channel and gap junction proteins, and beaded filament proteins (Rao & Maddala, 2006; Beebe *et al.*, 2001; Blankenship *et al.*, 2001). This process involves a subset of apoptotic-related proteases including the caspases (reviewed by Bassnett, 2002). Fully differentiated fibre cells lose the capability to synthesize new proteins and maintain metabolic processes, owing to the progressive loss of organelles during the differentiation process. The loss of organelles is thought to facilitate a high degree of transparency in the lens by removal of potential light-scattering mitochondria and nuclei from fibre cells that make up the bulk of the lens (Donaldson *et al.*, 2004; Frederikse *et al.*, 2004;

reviewed by Basnett, 2002; True & Carroll 2002; Moffat *et al.*, 1999; Sandilands *et al.*, 1995; Sandilands *et al.*, 1995a; Spector, 1995).

The process of epithelial cell differentiation occurs throughout life, with fibres being added the periphery of the lens fibre mass over the pre-existing fibre cells, resulting in the lens constantly increasing in size and early fibre cells progressively becoming internalised toward the lens nucleus (True & Carroll, 2002; Ireland *et al.*, 2000). Importantly, there is no turnover of lens cells, such that the fibre cells residing in the nucleus of the lens have been there the entire life of a particular organism, and thus the lens will contain cells that were present in the embryonic lens right through to adulthood (Shestopalov & Bassnett, 2003; Moffat *et al.*, 1999; Spector, 1995). As essentially all stages of lens fibre cell differentiation are present in a lens at any time, the spatial layout of fibre cells from the lens periphery to the centre thus represents a temporal profile of fibre cell differentiation (Perng & Quinlan, 2005; Donaldson *et al.*, 2004). Radial cell columns can be seen in equatorial lens sections that emanate from deep in the lens to the surface of the fibre mass. An elongating fibre cell in one of these columns becomes flattened between the cells that differentiated just before and after it. This results in the appearance of lens fibre cell in cross section having two longer sides, where it adheres to the cells in its radial column, and four shorter sides (“short sides/edges”) where the fibre cell makes contact with those in adjacent columns. These cell-cell associations cause the cross sections of fibres to approximate to flattened hexagons, and this precise arrangement may be important for lens transparency, as disruption is symptomatic in cataract patients (Beebe *et al.*, 2001).

The lens is an avascular tissue, and as such, the inner lens tissue is reliant upon the metabolically active epithelial cells for delivery of nutrients and other molecules, as well as removal of waste. The transport of molecules through the lens is carried out via a poorly understood gap junction-based internal microcirculation system that creates a functional syncytium, linking the fibre cells such that cytoplasmic continuity is maintained across the mass of cells that make up the lens (Donaldson *et al.*, 2004; Mathias & Rae, 2004; Baruch *et al.*, 2001; Moffat *et al.*, 1999).

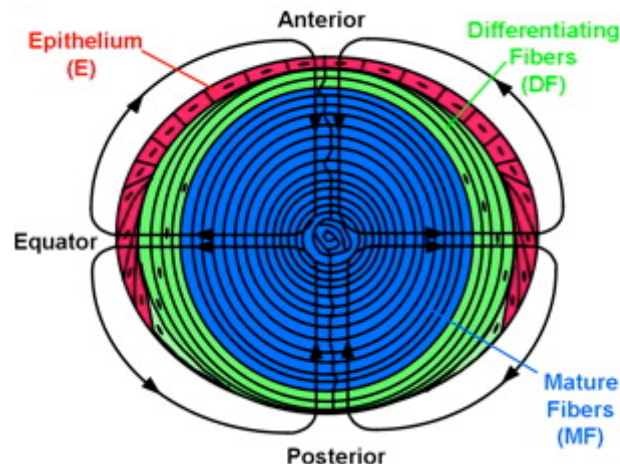


Figure 1.2 *Internal Circulation System in the Lens.*

Axial presentation of a lens indicating the direction that fluid and ions in the internal microcirculation of the lens follows (flow direction indicated by the arrows), emerging from the lens nucleus at the equator, out to the periphery. Fluid and ions then traverse the outer regions of the lens around to the anterior and posterior regions of the lens, where they then proceed to flow through the cortex back toward the nucleus. Diagram from Gao *et al.* (2004).

The lens is particularly vulnerable to insult due to its lack of organelles in mature fibre cells, as there is no mechanism for replacing or repairing damaged proteins and membranes in the interior of the lens. Consequently, any damage that is incurred by the lens will tend to be cumulative due to the lack of turnover of tissue in the lens (Bloemendal *et al.*, 2004). Additionally, the absence of turnover in the fibre cells allows a great opportunity for posttranslational modification of lens proteins to occur that may interfere with the passage of light through the lens (Ponce *et al.*, 2006; Kyselova *et al.*, 2004).

1.2 Components of the Lens

The Lenticular Cytoskeleton

The cytoskeleton is essential for the normal growth, maturation, differentiation, integrity, and function of cells in the human eye. In common with other cells, lens fibre cells possess a cytoskeletal system that is comprised of microfilaments, microtubules and intermediate filaments (Bozanic *et al.*, 2006). Lens cytoskeletal proteins are present at much lower levels than the crystallins that make up the bulk in the lens. Cytoskeletal proteins make up around 2-4% of lens proteins, though the level of cytoskeletal proteins found in a lens cell is much higher than that found in other cell types (Clark *et al.*, 1999). An assortment of cytoskeletal

proteins is found in the lens, with actin and vimentin being among the most abundant. Other cytoskeletal proteins involved in modulating the assembly, function and stability of the actin membrane skeleton such as spectrin, band 3 protein, band 4.1 protein, band 4.9 protein, myosin, tropomyosin, tropomodulin, caldesmon, α -actinin, ankyrin, ezrin and talin have also been identified in the lens. Alongside these actin associated proteins, filamentous proteins, filensin, CP49 (phakinin), beaded filaments, glial fibrillary acidic protein, and microtubule proteins including tubulin have been isolated in the lens (Guest *et al.*, 2006; Reed *et al.*, 2003; Beebe *et al.*, 2001; Blankenship *et al.*, 2001; Padgaonkar *et al.*, 1999; Matsushima *et al.*, 1997).

Cytoskeletal proteins in the lens account for only a small proportion of total protein, but they are thought to play a significant role in the maintenance of lens transparency, particularly in the stabilisation of the fibre cell during and after differentiation when the fibre cell undergoes massive changes in its dimensions (Perng & Quinlan, 2005; Menko, 2002). Additionally, cytoskeletal proteins may play an important role in the facilitation of the chaperone function of α -crystallin in the lens, which is thought to be crucial in maintaining optical clarity in the lens (Guest *et al.*, 2006; Graw, 2004; Menko, 2002). Finally, cytoskeletal proteins have been found to be associated with the formation of interlocking junction domains within the lens (Zhou & Lo, 2003).

Spectrin

The spectrin-actin membrane skeleton is expressed in all cells throughout the body, and is important for cellular shape, membrane stability, deformability, polarity, adhesion, as well as the formation of discrete membrane sub-domains and in the Golgi where it may be involved in sorting events (Beck, 2005; Thomas, 2001; Woo *et al.*, 2000). Spectrin has been extensively characterised in the erythrocyte, where it is found in a filamentous network with actin, protein 4.1 and ankyrin that forms direct and indirect connections to the membrane and is crucial for maintaining red blood cell shape and elasticity (Czogalla & Sikorski, 2005).

Structurally, spectrin is a tetramer comprised of antiparallel heteromers (α and β) of 280 and 247-460 kDa that are cross-linked by actin (reviewed by Czogalla & Sikorski, 2005; Rotter *et al.*, 2004; Thomas, 2001). Spectrin in the lens is known variously as non-erythroid

spectrin, α -spectrin, α II-spectrin or fodrin. It is similar to erythroid spectrin in terms of immunochemical cross reactivity, tetramer formation, and binding of actin, protein 4.1 and ankyrin, although it differs in some other qualitative characteristics (Winkelmann & Forget, 1993). Isolation of spectrin from lens homogenates via electrophoresis has revealed it is found primarily as 235-280 kDa bands (Robertson *et al.*, 2005; Tamada *et al.*, 2000; Fukiage *et al.*, 1997; Matsushima *et al.*, 1997). During the course of normal lens maturation, spectrin is proteolytically processed by a range of proteases to fragments ranging in mass from 60 to 160 kDa, with a highly sensitive cleavage site producing a 150 kDa band that is important in changes of cell shape (Lee *et al.*, 2001; Lee *et al.*, 2000; Fukiage *et al.*, 1997). The proteolytic activity of calpain on spectrin in the lens is known to produce fragments 145 and 150 kDa, while the action of caspase 3 is known to produce fragments of 120, 145 and 150 kDa during apoptosis, a process in which spectrin is a major substrate (Czogalla & Sikorski 2005; Robertson *et al.*, 2005; Rotter *et al.*, 2004; Lee *et al.*, 2001). Interestingly, Sjögren's syndrome, which is an organ specific autoimmune disease that affects around 4 million Americans and occurs mainly in middle-aged women, is the result of the production of autoantibodies to cleaved fragments of non-erythroid spectrin (Williams *et al.*, 2003).

Actin

As noted above, the spectrin-actin membrane cytoskeleton is an important component nearly all higher eukaryotic cell types, and actin has been purified and characterised in the lens fibres of several species (Fischer & Fowler, 2003; Lee *et al.*, 2000; Lo *et al.*, 1997). As well as being one of the major cytoskeletal proteins in the lens, actin participates in and regulates a host of events associated with fibre cell differentiation including the withdrawal of the lens epithelial cell from the cell cycle, and possibly plays a role in lens accommodation via the stabilisation of epithelium under tension (Rao & Maddala, 2006; Kivela & Uusitalo, 1998).

Actin exists in the lens both as filamentous (F-actin) and monomeric, globular actin (G-actin), a polypeptide of 47 kDa. F-actin assembles into two kinds of structural forms known as bundles and networks. The ratio between the amount of F-actin and G-actin in the lens is dependent on the stage of differentiation, with F-actin increasing as the fibre cells elongate (Rao & Maddala, 2006; Lee *et al.*, 2000). The polymerisation of G-actin into F-actin, and vice-versa, is controlled by actin modulating proteins such as gelsolin and tropomodulin, which can bind the ends of the actin molecules, or in the case of gelsolin, sever actin

filaments, thus controlling the dynamics and organisation of the filaments (Archer *et al.*, 2005; Fischer & Fowler, 2003).

The spectrin-actin membrane cytoskeleton is an important system found in cells throughout the body, and again actin, like spectrin, is found throughout the lens with its distribution altering with cell differentiation and morphological changes. F-actin in the lens epithelial cells is organised in unique, multilateral collections of stress fibres that lie beneath the apical membrane, as well as in adherens belts that are associated with cell-cell contacts and are typical of epithelial cell types (Tepass, 2002). Contrastingly, lens fibre cells contain prominent bundles of F-actin that are aligned along the vertices of the hexagonal fibre cells, and there is additionally a continuous F-actin network that underlies the entire plasma membrane of these cells (Fischer *et al.*, 2000; Lo *et al.*, 1997). Actin has been detected in the lens nucleus in some species (Lee *et al.*, 2000), where it has been found to remain associated with the nucleus fibre cell membrane. The persistence of the spectrin-actin system following organelle removal is one significant difference between the highly similar processes of lens fibre cell differentiation and apoptosis, and it has been suggested that cortical actin fibres play a central role in protecting differentiating lens fibre cells from apoptosis during and following organelle removal (Weber & Menko, 2006; Bassnett, 2002).

Tubulin

Tubulin is present in the lens as the protofilaments that form the microtubule system. Microtubules are long tubes (up to 50 nm) with a diameter 25 nm that provide mechanical stability and tracks for molecular transport by motor proteins from the kinesin and dynein families, as well as being involved in cellular events such as chromosome sorting, controlling cell polarity, and organelle localization in a large variety of cell types (reviewed by Karsenti *et al.*, 2006; Murray & Wolkoff, 2003). Tubulin itself is formed from dimers of α - and β -tubulin, each of which are approximately 55 kDa (Padgaonkar *et al.*, 1999).

Very little work appears to have been completed regarding the localisation of tubulin within the lens. Padgaonkar and co-workers (1999) followed the distribution of a selection of cytoskeletal proteins in Guinea Pig lenses that had been treated with hyperbaric oxygen, and reported the presence of tubulin in the lens nucleus. Matsushima and co-workers (1997), as well as Clark and co-workers (1999) followed the loss of cytoskeletal proteins that is seen in

the rat lens selenite cataract model, and reported tubulin was present in the lens cortex and the nucleus at reduced levels by SDS-PAGE and western blots.

Intermediate Filaments

Intermediate filament proteins are referred to as such on the basis of their average diameter (10 nm) is intermediate to that of microfilaments (characteristically 5-8 nm in diameter) and the microtubules (typically 20-25 nm in diameter; Strelkov *et al.*, 2003; Pitz & Moll, 2002). Intermediate filament proteins range in molecular weight from 45 to 150 kDa, and share a common structure based on the presence of non- α -helical N- and C-terminal domain ends and a central α -helical rod domain that is well conserved in size and predicted secondary structure (Alizadeh *et al.*, 2002). The intermediate filament system is a large and varied protein group with up to 70 known genes (DePianto & Coulombe, 2004). The intermediate filaments are thought to be involved in numerous processes, such as cell division, motility and plasticity, as well as being responsible in large part for the mechanical integrity of the cell with the other cytoskeletal systems (reviewed by Strelkov *et al.*, 2003). Six classes of intermediate filaments have been defined: class I, the acidic keratins; class II, the basic keratins; class III, desmin, glial fibrillary acidic protein, peripherin, and vimentin; class IV, the neurofilament proteins; class V, the nuclear lamins; class VI, nestin; and the lens-specific orphan intermediate filaments CP49 and filensin (Bozniac *et al.*, 2006; DePianto & Coulombe, 2004). A distinctive attribute of intermediate filament proteins is their insolubility in conditions that readily solubilise microtubules and microfilaments, which is useful property when it comes to the isolation of intermediate filaments including vimentin (Helfand *et al.*, 2003).

The lens is remarkable in its use of multiple iterations of intermediate filaments proteins. Throughout its development, the lens is known to express type I and type II cytokeratins early in lens development, followed more or less by the emergence of type III intermediate filaments (vimentin), which are present mainly in the outer epithelial cells of the lens. Lens specific beaded filaments comprised of filensin and CP49 are the last of the intermediate filaments to appear in the lens, but are also the longest surviving of this group of cytoskeletal elements (Blankenship *et al.*, 2001; Lee *et al.*, 2000; Sandilands *et al.*, 1995; Quinlan *et al.*, 1996).

Vimentin

The first intermediate filament to be identified in the lens was vimentin, which is a type III intermediate filament protein of 56 kDa predominantly expressed in cells of mesenchymal origin (Perng & Quinlan, 2005; Colucci-Gyon *et al.*, 1994).

Vimentin is known to exist in cells in a network that extends out from the cell centre in a radial organisation (Clarke & Allan, 2002). The specific role of vimentin in the lens is not well understood, and knockout studies have found that vimentin deletion does not have an obvious phenotype (Blankenship *et al.*, 2001; Colucci-Gyon *et al.*, 1994). Vimentin is found in the lens in epithelial cells and early differentiating fibre cells, but is absent from mature lens fibre cells, with its disappearance defining a specific stage of differentiation (Quinlan *et al.*, 1996). Mature lens fibres and mammalian erythrocytes share a common feature that they are devoid of vimentin filaments as well as nuclei, while lens epithelial cells and avian erythrocytes are nucleated and contain vimentin. This has led some to suggest vimentin actively contributes to the retention of the nucleus in the cell; however there is evidence that vimentin is lost from the lens fibre cell after the completion of nuclear loss (Sandilands *et al.*, 1995).

Filensin, CP49 and Beaded Filaments

Filensin (115 kDa), also known as CP95/CP115, and CP49 (~49 kDa), also known as phakosin/phakinin, are lens specific intermediate filaments that are found at all stages of lens fibre cell differentiation (Lee *et al.*, 2000; Sandilands *et al.*, 1995; Quinlan *et al.*, 1996). Filensin and CP49 combine with α -crystallins to form the unique lens filament known as the beaded filament, whose function in the lens is unknown, but which is thought to be essential for normal lens function as deduced from knock-out studies and intermediate filament function in other physiological systems (Sandilands *et al.*, 2004; Alizadeh *et al.*, 2002; Blankenship *et al.*, 2001; Ireland *et al.*, 2000; Quinlan *et al.*, 1996). The beaded filament structures of the lens fibre cell were discovered by Maisel and Perry in 1972, and have since been described in all vertebrate lenses, as well as in non-vertebrates such as the squid (Perng & Quinlan, 2005; Quinlan *et al.*, 1996).

Structure and Assembly Characteristics of the Beaded Filament

The organization of CP49 and filensin into the beaded filament in fibre cells is the only known example of intermediate filament proteins existing in something other than the usual 10nm filaments (Hess *et al.*, 1998). *In vivo*, beaded filaments are found as 5-6nm filaments regularly decorated with 15-20 nm beads that have a regularly spaced pattern and a 19-21nm axial repeat (Georgatos *et al.*, 1997). CP49 is thought to provide the filament core of the beaded filament, with filensin contributing to the peripheral regions (Sandilands *et al.*, 2004). *In vitro*, filensin and CP49 co-assemble into intermediate filaments of 10 nm when mixed in a 1:3 molar ratio that appear similar to classical smooth-surface intermediate filaments (Perng & Quinlan, 2005; Georgatos *et al.*, 1997; Goulielmos *et al.*, 1996). Also, transfection of filensin and CP49 into non-lens cells results in co-assembly of non-beaded filamentous structures (Goulielmos *et al.*, 1996).

Usually in the intermediate filament protein family there is a high degree of sequence identity between species (generally >85%), however between bovine and chicken filensin there is only a 62% level of overall primary sequence identity (Hess *et al.*, 1998). This may be in part due to the fact that different species differ in their ability and requirement to accommodate the lens for focusing, thus requiring different properties in the lens fibre cell intermediate filaments to achieve deformation as the lens is focused in a particular species (Perng & Quinlan 2005; Georgatos *et al.*, 1997). For example, the mouse lens is completely spherical and hardly accommodating, whereas the lens of a bird or human is biconvex and highly accommodating (Georgatos *et al.*, 1997).

Functions and Roles in the Lens of the beaded filament

The function of the beaded filament in the lens is poorly understood, but may be involved in stabilization and/or organization of the fibre cell membranes, and be an important structural element in the maintenance of long-term lens transparency (Ireland *et al.*, 2000; Quinlan *et al.*, 1999). A deletion mutation in the human CP49 gene (*Bfsp2*) that causes disruption in coiled-coil formation has been found in a family exhibiting an autosomal-dominant hereditary cataract (Jakobs *et al.*, 2000). This was the first discovery of a mutation in a cytoskeletal protein, and importantly non-crystallin protein, that results in cataract formation. An amino acid substitution mutation *Bfsp2* gene has also been identified in a juvenile-onset cataract, distinguished by initial lens clarity at birth followed by gradual development of opacity in the second and third decades of life (Conley *et al.*, 2000). Characterization of a naturally

occurring mutation in the *Bfsp2* gene in mice (mouse 129 strains) found that a truncated protein product is formed that destabilizes filensin and changes the morphology of the lens fibre cytoskeleton (Sandilands *et al.*, 2004).

Membrane Associated Proteins

A network of gap junctions links the fibre cells in the lens, and allows for the direct coupling between the cytoplasm of neighbouring cells (Zampighi *et al.*, 2005). The resting voltage of the lens is essentially the same wherever it is recorded, and this fact lead early lens researchers to conclude the lens was akin to a large cell, when actually the homogenous voltage witnessed was due to the intercellular connections afforded by the gap junction network (Mathias & Rae, 2004; Shestopalov & Bassnett, 2003). The gap junction-based internal microcirculation system that exists in the lens is responsible for circulating nutrients into and removal of waste from the lens nucleus. In addition to gap junctions comprised of members of the connexin family, in the lens fibre cell membrane also contains various ion channels, Na/K pump proteins, as well as the aquaporin family of water channels.

Hexameric structures of connexin molecules (connexons) interact with connexons in neighbouring cells to form the channel structure of gap junctions (Baruch *et al.*, 2001). Lens fibre cells express two connexin isoforms, Cx46 and Cx50, while the outer epithelial cells express Cx43 and Cx50 (Zhang & Qi, 2005; reviewed by Mathias & Rae, 2004). (Two nomenclatures for connexin types are commonly used, one using lowercase Greek letters that is based on closer similarity to archetypes, e.g., $\alpha 1$ and $\beta 1$, and the other using the molecular weight deduced from cDNA clones e.g. Cx46 and Cx50; Hopperstand *et al.*, 2000). Cx43 is removed from the lens epithelial cells via internalisation as well as proteosome degradation, while Cx46 and Cx50 are modified post-translation and remain in the lens nucleus (Mathias & Rae, 2004). Cx46 and Cx50 are found in high concentrations near the lens equator where they are thought to direct the outward component of the circulating current found in the lens. Interestingly, Cx50 is proteolytically processed by calpain deep within the lens, whereby its cytoplasmic tail region is cleaved in order to maintain cell coupling in acidic conditions that would normally result in the channel closing (Donaldson *et al.*, 2004).

Aquaporins in mammals comprise a family of more than 10 integral membrane proteins found in a variety of tissues where they facilitate bidirectional osmotic water transport, as well as having the ability to transport glycerol and other small solutes (Ruiz-Ederra & Verkman,

2006). The most common aquaporin in the lens fibre cell membrane is AQP0, previously known as major intrinsic protein (MIP), which is found expressly in differentiated fibre cells (Lindsey-Rose *et al.*, 2006; Ma *et al.*, 2005; Mathias & Rae, 2004). AQP0 acts as an adhesion molecule as well as a membrane water channel, and cleavage of AQP0 in the lens nucleus is thought to trigger this change of function (Gonen *et al.*, 2005; Ma *et al.*, 2005; Mathias & Rae, 2004; Gonen *et al.*, 2004). Mutations and deficiency of AQP0 cause wide ranging disorganisation in the lens fibres and result in cataracts, suggesting a structural role for these proteins also (Lindsey-Rose *et al.*, 2006). The other common aquaporin in the lens is AQP1, which is found in lens epithelial cells only. Currently, the role of AQP1 in the lens epithelium is unknown (Ruiz-Ederra & Verkman, 2006).

Crystallins

During differentiation there is a marked increase in the protein concentration of the fibre cells, which can reach 450 mg/ml (Reed *et al.*, 2003). The protein concentration in the lens is primarily due to the presence of water-soluble crystallins. The crystallins contribute between 30 and 40% of the total lens mass, and around 90% of the water-soluble protein in the lens (Ponce *et al.*, 2006; Kyselova *et al.*, 2004; Bhat, 2004; Horwitz, 2003; True & Carroll, 2002; Ueda *et al.*, 2002; Piatigorsky, 1998). Crystallins are particularly long lived owing to the nature of lens growth, and are relied upon to provide transparency, maintain viscosity and preserve the optimal refractive index in the lens. These qualities are primarily achieved by their tight, highly ordered packing within the fibre cells, and interactions between themselves, other lens proteins such as cytoskeletal proteins, and membranes (Sun & MacRae, 2005; Fernald, 2006; True & Carroll, 2002; Moffat *et al.*, 1999; Spector, 1995).

Crystallins were first described as the structural proteins of the vertebrate lens in the 1890s by Mörner, and were so named owing to their presence in the crystalline lens (Augusteyn, 2004; Graw, 1997). The main groups of crystallins found in the lens are the α and β/γ crystallins (Bhat, 2004; Horwitz, 2003). These crystallins are sometimes referred to as the ubiquitous crystallins, as they are the most common crystallins in the lens, particularly in mammals, and α and β are found in all vertebrate lenses. It was originally thought that the crystallins had evolved uniquely to function in the lens, but crystallins have since been found to be expressed in the heart, brain, and other tissues of the eye (Fernald, 2006).

α Crystallin

The α crystallins represent the major class of water-soluble proteins in the lens, and are found in all vertebrate lenses. α crystallin-type proteins comprise a large protein family that is distributed widely across species ranging from plants and bacteria to human (Franck *et al.*, 2004; Narberhaus, 2002). In the lens α crystallin is found in limited amounts in the lens epithelial cells, but its synthesis is strongly up-regulated upon differentiation into fibre cells, where they are the major protein constituent (Bloemendal *et al.*, 2004). In the fibre cells it is found as a large polydisperse multimeric assembly with a molecular weight ranging anywhere from 300 to 1200 kDa, averaging 800 kDa, and can be found in lens extracts in aggregates with molecular masses in excess of 1 MDa (Biswas & Das, 2004; Derham & Harding, 1999).

The α crystallin complex is composed of two related subunit proteins, α A- and α B crystallin of approximately 19.9 and 20.1 kDa, respectively. The α A subunit is essentially a lens specific crystallin, while α B crystallin is considered to be a ubiquitous protein particularly abundant in the heart, brain and muscle (Bloemendal *et al.*, 2004; Horwitz, 2003; Piatigorsky, 1998; Graw, 1997). The α A and B subunits exist in the mammalian lens at an approximate ratio of 3:1 respectively, although this ratio is variable depending on the age of the lens (Bloemendal *et al.*, 2004). Interestingly there appears to be no specific requirement for either subunit in the formation of a stable α crystallin multimer protein (Bloemendal *et al.*, 2004; Horwitz, 2003; Graw, 1997). The basis of the observed variation in the mass of α crystallin aggregates is not fully understood, but appears to depend on the source of the α crystallin, the purification protocols employed, and the presence of age-related post-translational modifications (Kumar *et al.*, 2005; Augusteyn, 2004).

In recent times, α crystallin has been subject of intense investigation of its structure and function, particularly in light of the fact that it is a key member of the small heat shock protein family, a large family of proteins that display chaperone-like activity. These proteins can bind denatured proteins to maintain their solubility and prevent binding and aggregation with other proteins, as well as conferring enhanced stress-resistance on cells (Sun & MacRae, 2005; Augusteyn, 2004). The structurally divergent small heat shock proteins are defined by the presence of the “ α crystallin” domain, a sequence of 80 to 100 amino acid residues located toward the C-terminal that is moderately-to-highly conserved between species (Sun &

MacRae, 2005). In the lens, α crystallin is thought to assist in the folding and stability of lens proteins, ensuring the solubility and prevention of aggregation of other proteins in the lens through an ATP-mediated process that is currently not well understood and may involve hydrophobic surface interactions between the chaperone and its target (Biswas & Das, 2004; Franck *et al.*, 2004). α crystallin has been shown to inhibit the precipitation of β and γ crystallin *in vitro*, in addition to various other proteins (Augusteyn, 2004). In the lens it is thought that the other crystallins are the major target of α crystallin chaperone function, based on extracts of 50-65 year old lenses in which the “water insoluble” fraction contained mostly α crystallin, along with some γ and β crystallins (Hanson *et al.*, 2000). Other targets in the lens that may require assistance in maintaining solubility over the long lifespan of the lens includes cytoskeletal proteins, and “housekeeping” enzymes such as glyceraldehyde-3-phosphate dehydrogenase and enolase (Horwitz, 2003).

Aggregation of proteins in the lens occurs as a result of aging when proteins unfold and denature. As there is no protein turnover or diffusion in the lens (Bloemendal *et al.*, 2004), the chaperone activity of the α crystallins may be responsible for the long-term maintenance of lens clarity through the prevention of protein aggregation that can cause light scattering and eventually cataract, a role that is in addition to its structural function. At around 40 years of age, it is thought that all the α crystallin in the nucleus of the lens are bound to denatured proteins to maintain transparency, and it is around this time that physical changes begin to noticeably affect the lens (Bhat, 2004; Truscott, 2004; Horwitz, 2003).

Further support the hypothesis that α crystallins are largely responsible for the maintenance of lens clarity through chaperone function is that owing to its abundance in the lens α crystallin could bind all of the other proteins in the lens, assuming a 1:1 stoichiometry of α -crystallin to other protein. This would prevent aggregation and insolubilization of these proteins that would otherwise to light scatter and cataract (Augusteyn, 2004). Findings that the α crystallins can also inhibit caspase function, are anti-apoptotic regulators and have auto-kinase activity indicate other general physiological functions (Zhang *et al.*, 2005; Xi *et al.*, 2003).

β and γ Crystallin

The β and γ crystallin polypeptides are recognized as members of a related β/γ super family of proteins based on amino acid sequence homology and crystallographic studies. Perhaps the most prominent feature of the β/γ crystallin family is the presence of a distinctive “Greek key” structural motif (Bhat, 2004; Branden & Tooze, 1999; Piatigorsky, 1998; Graw, 1997). The β/γ crystallins differ from the α crystallins in that they are a relatively large, multi-gene family, and no non-refractive functions have yet been found for either β or γ crystallin (Piatigorsky, 1998). In addition, both β and γ crystallin are lens fibre cell specific, although there have been reports of low-level expression in the retina, brain and testis of mice, chicken and other species (Bloemendal *et al.*, 2004; Xi *et al.*, 2003).

β crystallins are found in the lens as oligomers of up to 200 kDa, with the constitutive monomers having molecular weights of between 22 and 28 kDa. The β crystallin family is composed of acidic ($\beta A1$ - $\beta A4$) and basic ($\beta B1$ - $\beta B3$) polypeptide members (Bloemendal *et al.*, 2004). γ crystallin has a molecular weight of 20 kDa, but in contrast to oligomeric β crystallin, γ crystallin is monomeric (Giancola *et al.*, 2004). There are seven members in the γ crystallin family, γA , γB , γC , γD , γE , γF , and γS , all of which are around 20 kDa (Bloemendal *et al.*, 2004). There also is evidence for another γ crystallin in some mammals known as γN (to denote that it is a *New* γ crystallin; Wistow *et al.*, 2005). γN crystallin is a recently discovered crystallin with genes found in rats, mice, chicken, humans and chimps. γN crystallin has features similar to both γ and β crystallin and could be described as an evolutionary bridge or “missing link” between the two related γ and β crystallin proteins (Wistow *et al.*, 2005).

1.3 Calpain and the Lens

The calpain enzymes are a family of calcium-activated cysteine proteases of considerable interest because of their implication in numerous physiological and pathological events (Cuerrier *et al.*, 2007; Sanders & Donkor, 2006). Their association with the development of opacity in the lens is of particular significance (reviewed by Zatz & Starling, 2005; Goll *et al.*, 2003; Reed *et al.*, 2003). The principle calpain isoform in the lens is calpain II/m-calpain, but other calpain isoforms are also present, including calpain I/ μ -calpain, Lp82, Lp85 and calpain

10 (Reed *et al.*, 2003). Calpain I and II are ubiquitous, and Lp82, a lens specific splice variant of calpain 3, is present in several animals including sheep (Robertson *et al.*, 2005) and rodents, but not in human lens (reviewed by Goll *et al.*, 2003). Calpain II has been immunologically detected in rat lens during early development and persists to maturity. Lp82, while being detected earlier in development than calpain II, has been found to be present at a markedly decreased level in mature lenses (Reed *et al.*, 2003).

Many proteins are partially hydrolysed by the action of calpain, however no natural specific substrates have been identified (Chicharro *et al.*, 2006). Calpain may be involved in basic cellular functions, such as differentiation, cell cycle, signal transduction and apoptosis, along with its possible role in pathological conditions (Reed *et al.*, 2003). There is accumulating evidence that calpain is implicated in cataractogenesis (Biswas *et al.*, 2005; Robertson *et al.*, 2005; Sanderson *et al.*, 1996; Azuma *et al.*, 1995; David & Shearer, 1986, 1984). It is thought that calpain, particularly calpain II, is responsible for the formation of opacities in the lens via the calcium-activated degradation of α and β crystallins that leads to their insolubilization, resulting in the scattering of light in the lens (Zatz & Starling, 2005; Matsushima *et al.*, 1997). This is backed up by assays of calpain where it has been found to proteolyze crystallins *in vitro*, and through the use of calpain inhibitors that can prevent the progression of cataract in rodent lenses (Biswas *et al.*, 2005).

Cataract is the leading cause of blindness worldwide, and it is thought between 17 and 19 million people are affected by cataract, accounting for 43% of global blindness (WHO estimate), while an estimated 28,000 new cases are reported each day (Kelly *et al.*, 2005; Kyselova *et al.*, 2004; Matsui *et al.*, 2003). The associated economic burden of cataracts amounts to around \$USD5-6 billion annually, with surgery costs over \$3 billion in the US alone, where 1.3 million cataract operations are performed annually (Ruiz-Ederra & Verkman, 2006; Kyselova *et al.*, 2004; Taylor & Hobbs 2001). A wide variety of insults to the lens can result in a rise in calcium levels, with intracellular concentrations reaching up to 1 mM (reviewed by Goll *et al.*, 2003; and Duncan *et al.*, 1994), sufficient to activate calpain II (calpain II is capable of being activated by 400 μ M calcium; Zatz & Starling, 2005; Goll *et al.*, 2003). Aging and diabetes are the primary factors associated with the majority of cataract cases, with smoking, low antioxidant intake, gout, drugs, metabolic disturbances, family history and congenital disorders as additional causative factors (Biswas *et al.*, 2005; Graw, 2004). The aging process naturally results in the accumulation of light-scattering opacities, at

first slowly, but accelerating around middle age and especially in older age, such that at around 80 years old, more than 50% of people in the USA are affected with cataracts (Mares, 2004). Aging is generally associated with compromised lens function and a decrease in reserves of antioxidants such as GSH (reduced glutathione) as well antioxidant enzyme activity (Kyselova *et al.*, 2004). These cataracts are often referred to as age-related cataracts, which distinguishes them from cataracts that can develop due to other complications, such as congenital, metabolic disorders (e.g. diabetes-induced cataract) and trauma (Taylor & Hobbs, 2001).

Calpain Activation & Regulation

The temporal and spatial regulation of calpain activity is essential because calpain is an abundant cytoplasmic protease, capable of cleaving many signalling and structural proteins (Perrin & Huttenlocher, 2002). For instance, it has been estimated that if all of the calpain residing in muscle were proteolytically activated simultaneously, the entire complement of Z-disks in skeletal muscle would be destroyed in less than five minutes (Goll *et al.*, 2003).

In inactive calpain the two subdomains of domain II (see *fig. 1.3*), in which the active site is located, are arranged with a gap of approximately 10 Å separating cysteine 105L from histidine 262L and asparagine 286L (the catalytic triad residues). This gap renders calpain unable to bind peptide substrates and therefore catalytically inactive (Strobl *et al.*, 2000). In this state, it resides in the cytosol, however upon increased intracellular calcium concentration and binding, and translocation to the membrane, the gap is reduced to approximately 3.7 Å, thereby allowing the catalytic triad to be completed and the calpain molecule activated.

This mechanism was elucidated from observations of numerous cysteine proteases (particularly the catalytic cysteine and histidine residues), of which calpain is a family member, as well as studies on calpain I and calpain II (Suzuki *et al.*, 2004; Jia *et al.*, 2002; Goll *et al.*, 2003). While the properties of calpain have been extensively characterised, the role of calcium in the activation of the proteolytic activity of calpain is less well understood. The structural changes and effects of calcium binding has been a much debated topic over the years (Jia *et al.*, 2002; Dutt *et al.*, 2000).

Difficulty in studying the conformational changes and sites of calcium occupation occurring upon calcium binding arises due to the inability to obtain crystals of this form of calpain, as activation of calpain triggers aggregation, precipitation, autolysis and degradation (Dutt *et al.*, 2000). Thus structural information and the effects of calcium activation have been made from inactive, truncated and mutated forms of calpain, and the details of the required conformational changes are speculative (Jia *et al.*, 2002; Reverter *et al.*, 2001a). It is thought that binding of calcium to the EF-hands in the IV-VI domains should not change its overall structure (Reverter *et al.*, 2001). This is because both the calcium-bound and unbound form of the EF-hand structure is known (there is a large amount of literature about it due to their presence in other proteins; Dutt *et al.*, 2002), and binding produces only a small change in their conformation indicating that it may not provide the required shifting of the domain II subdomains to bring the active site residues together (Strobl *et al.*, 2000; Goll *et al.*, 2003). This is backed up by the fact that calpain in which all EF-hands have been mutated (and therefore, assumingly inactive/incapable of binding calcium) is still activated upon calcium binding at high enough concentrations (Dutt *et al.*, 2000). For these reasons, it is believed that the calmodulin-like domains are possibly more structural than explicitly regulatory with regards to calcium binding (Reverter *et al.*, 2001). Also, it suggests calcium binding sites must exist on calpain aside from the EF-structures (Goll *et al.*, 2003).

While the function of domain III has been difficult to determine, owing to its lack of sequence homology to any known protein (Hosfield *et al.*, 1999), its central location and proximity to the catalytic domain suggests it may be important in the activation of calpain. Notably the acidic loop, with its many negatively charged side chains seems to be of particular interest in calpain activation (Reverter *et al.*, 2001). The acidic loop is in direct contact with domain IIb, which possess a number of basic (lysine) side chains that are attracted to the negative charges of the acid loop, which is highly conserved in the calpain family (Goll *et al.*, 2003). Binding of calcium to the acidic loop could reduce or alleviate the negative potential of the acidic loop, thus decreasing the electrostatic interaction between it and domain IIb (Reverter *et al.*, 2001a). To this end, it has been found that the acidic loop of calpain B (*Drosophila*) does indeed bind calcium (Alexa *et al.*, 2004). This would then allow fusion of the catalytic domain, as domain IIb would be free to move toward IIa, which is clamped down via many polar contacts to domain III, as well as being held by the N-terminal α -helix of domain I (see *fig. 1.3*; Alexa *et al.*, 2004).

This mechanism, termed the “electrostatic switch mechanism” could also account for the differences in calcium between calpain I and calpain II, as calpain II has less acidic residues in its acidic loop, and may consequently require less calcium reduce the electrostatic attraction of domain IIb to III (Strobl *et al.*, 2000). Other investigations where lysine residues of domain IIb were removed via point mutations significantly reduced the calcium requirement of activation, and replacement of two acidic residues in the acidic loop caused a large decrease in the half-maximal calcium requirement of rat calpain II (250 μM down to 7 μM ; Alexa *et al.*, 2004). However, studies that utilized expressed domain IIa/IIb from calpain I have shown that they can bind one calcium atom, which induces a conformational change that brings together the catalytic triad, indicating that at least some of the activation switch of calpain resides in the catalytic domain itself (Goll *et al.*, 2003; Moldoveanu *et al.*, 2002).

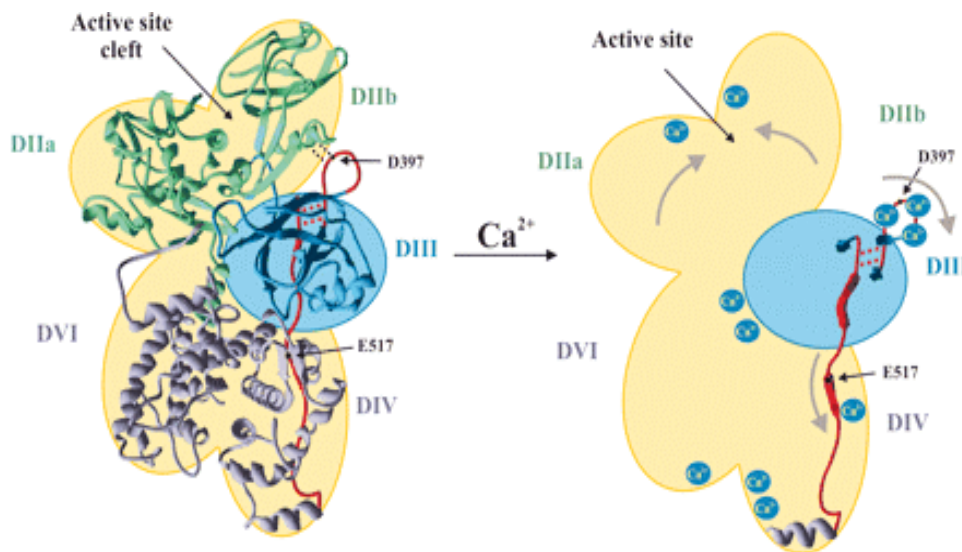


Figure 1.3 Proposed activation mechanism of calpain by calcium

Depicted are the crucial local conformational changes and sub domain movements (the latter indicated by grey arrows in the structure to the right), which occur upon calcium binding leading to calpain activation. Domain III (in blue), binds sub domain IIb via electrostatic interaction between acidic and basic residues in each of the respective regions (shown as two dotted lines near D397). Binding of calcium to the acidic residues disrupts this attraction, and binding in domain IV also results in conformational changes that are transduced to domain III resulting to its pulling down (i.e. as depicted), allowing domain IIb to move toward IIa and complete activation of calpain. The domain structures that comprise calpain are labelled as in the text (Adapted from Alexa *et al.*, 2004).

Calpain Inhibitors

Calpastatin is an endogenous calpain regulatory protein found in the body, and is the only specific calpain inhibitor that has been identified (Biswas *et al.*, 2005; Goll *et al.*, 2003; Donkor, 2000). Calpastatin has a large mass (120 kDa) and consequent low membrane permeability, and thus is thought to be of little therapeutic use (Biswas *et al.*, 2005; Moldoveanu *et al.*, 2004; Todd *et al.*, 2003). The absolute selectivity of calpastatin has raised interest in developing synthetic calpain inhibitors, and more than 50 endogenous and exogenous/synthetic calpain inhibitors have been described (Zatz & Starling, 2005). These inhibitors may be subdivided into 4 classes, the largest and perhaps best understood of which are the peptidyl aldehydes, such as leupeptin, which was originally isolated from *Streptomyces* species (Carragher, 2006; Donkor, 2000). Leupeptin and other peptidyl aldehyde inhibitors inhibit calpain by way of the aldehyde functional group reversibly binding the active site cysteine, which it does so in a calcium dependent manner (Biswas *et al.*, 2004; Fukiage *et al.*, 1997).

The second distinct sub-group of calpain inhibitors are the peptidyl epoxides, which includes the irreversible inhibitors E64 (trans-epoxysuccinyl-L-leucylamido-4-guanidino-butane) and E64c. E64 and its peptidyl epoxide derivatives covalently bind to the thiol group of the active site cysteine residue, thereby blocking calpain proteolytic activity (Biswas *et al.*, 2005; Moldoveanu *et al.*, 2004).

The third sub-class of calpain inhibitors is the peptidyl α ketoamides, which are third generation reversible inhibitors that exhibit improved cellular permeability and solubility than peptidyl epoxides and aldehydes, in addition to circumventing the metabolic instability of the aldehydes *in vivo* (Carragher, 2006).

It is important to note is that several of the inhibitors mentioned so far are not specific for calpain. Other cysteine proteases such as plasmin, trypsin and papain, as well as the proteasome complex, may be affected by these calpain inhibitors, which potentially could cause side effects in physiological systems were they to be used in a therapeutic setting (Carragher, 2006; Moldoveanu *et al.*, 2004; Donkor, 2000).

The final sub-class of calpain inhibitors are the non-peptide inhibitors, such as ATA and quinolinecarboxamide. These inhibitors are reversible and non-competitive fourth generation

inhibitors that have been developed and display greater specificity for calpain over other cysteine proteases (Carragher, 2006).

Calpain Inhibitors as Anticataract Agents in Animal Models

Several calpain inhibitors have been tested as anticataract agents in various animal models, including rats, sheep and pigs, both in organ culture as well as *in vivo* (e.g. Robertson *et al.*, 2005; Biswas *et al.*, 2004; Sanderson *et al.*, 1996; Lampi *et al.*, 1992; Shearer *et al.*, 1991; Azuma *et al.*, 1991). Success in these trials has been limited, particularly due to low water solubility of the compounds coupled with low membrane permeability for first generation inhibitors including E64 (Biswas *et al.*, 2005). E64 has also been found to offer poor protection from proteolysis for lens cytoskeletal proteins such as vimentin and spectrin that are important proteins for lens function as well as prime substrates for calpain degradation (Guest *et al.*, 2006; Biswas *et al.*, 2005; Sanderson *et al.*, 1996). Additionally, it has been found for some inhibitors such as E64 that calpain inhibition *in vivo* is less potent than *in vitro* lens culture system, and some peptidyl aldehyde inhibitors have been found to exhibit lens toxicity (Biswas *et al.*, 2005; Azuma *et al.*, 1992).

Fukiage *et al.* (1997) synthesized a peptidyl aldehyde calpain inhibitor, *SJA6017* (*N*-[4-fluorophenylsulfonyl]-L-valyl-L-leucinal), in an attempt to ameliorate the shortcomings of these other calpain inhibitors, particularly in terms of cell penetration and calpain inhibition. The efficacy of *SJA6017* to inhibit calpain and its affect on calcium ionophore-induced cataracts in cultured rat lenses was investigated, and it was found that *SJA6017* reversibly bound to and strongly inhibited calpain, while reducing nuclear opacity and reducing proteolysis of lens proteins. Comparative studies of a range of calpain inhibitors in rodent lenses also found that *SJA6017* displayed superior efficacy as an anticataract agent (Biswas *et al.*, 2005).

With regards to non-rodent lenses, similar results have been found in an *in vitro* pig lens culture system, where *SJA6017* considerably reduced the progress of induced cataract, (Biswas *et al.*, 2004), while Robertson *et al.* (2005) found that in sheep that developed hereditary cataracts opacification was temporarily delayed following treatment with *SJA6017*.

1.4 The Lens Cytoskeleton and Cataract

While the majority of research into cataracts tends to focus on calpain-induced degradation of α and β crystallins, no typical calpain cleavage sites have been detected from crystallins in aged human lenses (Nakajima *et al.*, 2006; Ma *et al.*, 2005). This suggests that for calpain to play a role in human cataract, proteolysis of proteins other than crystallin must be important, either additionally or principally. The lens cytoskeleton comprises a small proportion of the total lens protein; however it is thought that it plays an important role in the development and maintenance of transparency in the lens (Guest *et al.*, 2006; Perng & Quinlan, 2005; Menko, 2002; Quinlan *et al.*, 1999). Actin, vimentin, spectrin and filensin have all been found to be substrates or potential substrates for calpain proteolysis, which may fit in with the currently accepted model for some types of cataract formation where calpain has been implicated (Guest *et al.*, 2006; Perng & Quinlan, 2005; Sanderson *et al.*, 2004; Reed *et al.*, 2003; Lee *et al.*, 2001; Quinlan *et al.*, 1999; Matsushima *et al.*, 1997; Marcantonio 1992; Marcantonio & Duncan, 1991). A number of diseases have been linked to mutations and disruptions of cytoskeletal proteins, and breakdown of lens cytoskeletal proteins may be a major factor in cataract formation (Sandilands *et al.*, 2004; Lee *et al.*, 2001; Quinlan *et al.*, 1999; Matsushima *et al.*, 1997).

Sanderson *et al.* (2000) reported that loss of the cytoskeletal proteins, spectrin, vimentin, and filensin, seen during incubation of rat and bovine lenses with a calcium ionophore was prevented on incubation in a calcium-free medium and reduced by calpain inhibitors. Matsushima *et al.* (1997) reported similar results in rats with selenite-induced cataract, and that cytoskeletal proteins were among the earliest proteins degraded in this model indicates that their breakdown may play a significant role during the early stages of cataract formation. Inhibition of cytoskeletal breakdown with calpain inhibitors has been associated with a reduction in ionomycin-induced lens damage (Shearer *et al.*, 1997). As mentioned above, mutations in the beaded filament gene *Bfsp2*, the gene for the lens specific intermediate filament protein CP49, have been associated with inherited and juvenile-onset cataracts in humans (Hejtmancik & Kantorow, 2004; Conley *et al.*, 2000; Jakobs *et al.*, 2000). Additionally, changes in cytoskeletal proteins have been detected in congenital and childhood cataracts in humans, suggestive of a role in cataract etiology (Guest *et al.*, 2006; Matsushima *et al.*, 2000).

At Lincoln University, a flock of sheep (Coopworth-Romney cross) are maintained that naturally develop a hereditary cataract. The ovine cataract is cortical in nature and emerges at 1-2 months of age, developing bilaterally over approximately 10 months (Lee, 2006; Robertson *et al.*, 2005; Brooks *et al.*, 1982; Brooks *et al.*, 1982-1983). A sheep based model for human cataracts may present a better alternative to currently available models such as rodent lenses (Robertson, 2003). The ovine lens is large, and the bi-convex lens shape more closely resembles that of a human. Additionally, the ovine lens is an accommodating lens, as is the human lens, and as such may share more commonality than the spherical and poorly accommodating rodent lens (Lee, 2006; Lo, 1988). The absence of alternative natural large animal cataract models makes the ovine cataract a convenient model for such studies and reproducibility of the cataract enhances the claim for an ovine model (Robertson *et al.*, 2005).

Among the aims of researching the ovine cataract is to further the understanding cataract progression in other species, including humans. Additionally, inhibition of the ovine cataract has been used as a platform in the development of potential cataract treatments that are based on the inhibition of calpain proteolysis. Further investigation into the nature of the ovine cataract will clarify its usefulness as a model for human cataracts

1.5 The Use of Immunohistochemistry to Study the Lens

Principles of Immunohistochemistry

Immunohistochemistry, the process of localizing and identifying proteins in tissue sections by exploiting the principle of specific antibody binding to antigens in biological tissues (Mao *et al.*, 1999) coupled with fluorescent or enzymatic markers, is a sensitive technique that offers a high degree of resolution. Immunohistochemistry is sometimes referred to interchangeably with immunocytochemistry (Bratthauer, 1999). The distinction between the two that is that the latter term is more suited to describe the general technique of visualising protein using labelled antibodies when it is applied to biological samples collected in some way other than via histology, such as for example from cell culture.

The first step in immunohistochemistry is fixation of tissue, as it is essentially impossible to perform immunohistochemical microscopic studies with living specimens (Melan, 1999). Fixation is required to preserve the cellular features, prevent tissue autolysis, and prevent or reduce the movement of labile proteins and carbohydrates (Taylor & Levenson, 2006). Tissue

may be fixed by chemical or physical (freezing) processes. The most common chemical fixative is formaldehyde, usually made from its solid hydrated polymeric form paraformaldehyde (PFA) dissolved in phosphate-buffered saline (PBS) solution (Taylor & Levenson, 2006; Montero, 2003; Melan, 1999).

Formaldehyde is favoured because of its high degree of tissue penetration, and its preservation of morphological detail and maintenance of immunoreactivity, especially when compared to other fixatives such as glutaraldehyde or solvent-based fixatives (e.g. acetone/methanol; Bratthauer, 1999; Melan, 1999). For example rapid fixation (1-3 hours) of rodent lenses in concentrated PFA solutions has been found to offer excellent tissue preservation, but at the expense of antigen preservation (Jacobs *et al.*, 2003). Qualities associated with the use of formaldehyde fixatives are ease of storage, low cost, availability, and its long history as a primary fixative in histology and the resultant familiarity which that brings (Taylor & Levenson, 2006; Berod *et al.*, 1981).

Tissue is sectioned after fixation. In order to cut the thin (<20 µm) sections required for immunohistochemistry, tissue must be embedded in a support medium, and this is achieved by either freezing or embedding it in paraffin wax. The latter technique requires dehydration as paraffin is not miscible with water present in cells. This is achieved by exposing the tissue to a graded alcohol series containing increasing proportions of alcohol and decreasing amounts of water. Paraffin is not miscible with ethanol however, and xylene likewise not with water; therefore the dehydrated tissue must next be infiltrated with xylene prior to embedding in molten paraffin. The paraffin is then cooled and solidified prior to precision microtome sectioning of the tissue. Removal of paraffin is required before the sections may be stained, and this is achieved by clearing in xylene, followed by rehydration (Bratthauer, 1999).

Sectioning frozen tissue, also known as cryosectioning, involves freezing the tissue in liquid nitrogen, or less commonly on dry ice or in super-chilled alcohol, embedded in a frozen medium sectioning medium such as *Tissue-Tek*[®] O.C.T. (for *Optimal Cutting Temperature*) compound (Bratthauer, 1999a). Preparing tissue for cryosectioning often requires a cryopreservation step, which involves exposing the tissue to PBS containing gradually increasing amounts of cryoprotectant, such as sucrose. Cryopreservation assists in the prevention of cellular damage induced by the stresses of freezing, possibly by affecting the

formation of ice crystals, although this process is poorly understood (Karlsson & Toner, 1996).

Paraffin processing is the favoured processing technique in routine pathological diagnostic laboratories, where immunohistochemistry is used for diagnosis of diseases with more specificity such as cancer (Niki *et al.*, 2004). Paraffin processing is favoured as it provides superior preservation of tissue morphology, and is conducive to retrospective studies for potentially several decades owing to the apparent stability of proteins in wax block (Walker, 2006; Krenacs *et al.*, 1999). A degree of antigenicity may be compromised during fixation and processing of tissue, as the action of the chemical fixative causes cross-linking of proteins, thereby altering their properties. Additionally, the other chemicals and physical extremes that the tissue is exposed to during the embedding process, including heat from molten paraffin, may alter the immunological properties of an antigen (Taylor & Levenson, 2006; Bratthauer, 1999; Krenacs *et al.*, 1999; Melan, 1999). Sections obtained from tissue that has been frozen are considered to have superior antigen preservation, by virtue of the absence of chemical modification of the target tissue that fixatives introduce. Although frozen sections can suffer from inferior morphological preservation that may be attributable to the stresses of freezing and thawing, this method of immunohistochemical analysis is considered to be the closest to *in vivo* conditions (Bratthauer, 1999a). Additionally, it offers the benefit of being relatively quick and comparatively simple process.

Immunohistochemistry in the Lens

Myriad techniques have been used in the processing of lenses from other species for immunohistochemistry. Rodent lenses have been processed following two general strategies; *a*) fixation followed by sectioning either from paraffin blocks (e.g. Blankenship *et al.*, 2001) or from frozen blocks (e.g. Jacobs *et al.*, 2003; Grey *et al.*, 2003), or *b*) sectioning from fresh frozen tissue blocks (i.e. no prior fixation), followed by fixation after sections were obtained (e.g. Alizadeh *et al.*, 2003; Reed *et al.*, 2003; Reed *et al.*, 1999; Kistler *et al.*, 1985). There is an interesting lack of concurrence among the research on rodent lenses regarding the strength of fixatives made using PFA as the primary agent of fixation. Values can be found in the literature ranging from 0.75% PFA in Jacobs *et al.* (2003) and Grey *et al.* (2003), to 4% for Blankenship. Chicken lenses may be fixed prior to sectioning in 4% PFA (Beebe *et al.*, 2001), while embryonic chicken lenses require a more gentle fixation solution containing 1% PFA

prior to cryosectioning (Lee *et al.*, 2000). Lee *et al.* (2000) also sectioned adult lenses, but these were sectioned unfixed, and were fixed post sectioning in 1% PFA.

Immunohistochemical studies on bovine lenses (e.g. Girao *et al.*, 2005), especially the type of fixation method applied to these larger lenses, are particularly useful for formulating an immunohistochemical protocol for the ovine lens, as they share more features with the ovine lens than rodent lenses. In addition to similar proportions, bovine lenses are soft and are thought to be accommodating lenses (Augusteyn & Stevens, 1998), whereas rodent lenses are hard and are known to be hardly accommodating (Georgatos *et al.*, 1997). Finally, bovine lenses offer the advantage of having been reasonably well studied with regards to fixation and sectioning compared to ovine lenses.

SECTION 2 – EXPERIMENTAL RATIONAL

2.1 Aim of Current Investigation

The aim of this research project was to investigate the distribution of cytoskeletal proteins in the lens, and to examine the effects of calpain proteolysis and cataract on these proteins, with the goal of establishing the role of the cytoskeleton in the ovine cataract model.

An immunohistochemical approach will be used to achieve these objectives that requires the development and optimisation of a protocol that was specific for the ovine lens.

Proteolysis of cytoskeletal proteins with calpain and in the presence of inhibitors also will be investigated to evaluate the roll of calpain in the ovine cataract model.

2.2 Hypothesis

It is proposed that proteolysis the of cytoskeleton in the ovine lens by calpain will result in breakdown of proteins similar to that seen in cataract, indicative of a role for this protease in cataractogenesis through the proteolysis of the lens cytoskeleton. (i) Inhibition of calpain with novel inhibitors will result in the absence of proteolytic products associated with calpain.

Additionally, (ii) immunohistochemical localisation of cytoskeletal proteins in the ovine lens will reveal a characteristic distribution for that selected protein and will be distributed in the lens following a pattern similar to that seen in other species.

2.3 Outline of Sections

A variety of techniques have been utilised in order to investigate the ovine lens cytoskeleton and test the hypothesis that calpain drives proteolysis of the cytoskeleton in the lens, including immunohisotochemistry and a lens based cell free assay of exogenous calpain and lens cytoskeletal extracts. The efficacy of novel calpain inhibitors in the lens based cell free

assay also was investigated. There are 3 major experimental sections that comprise this investigation, in addition to a concluding section.

Section 3 Optimisation of a Method for the Immunohistochemical Processing of the Ovine Lens

In this section, a method for processing the ovine lens for immunohistochemistry was investigated, as no method could be identified in the literature that was specific for the ovine lens. This required evaluation of common methods used for rodents and other large mammalian lenses, and the adoption and optimisation of useful elements therein for use in an immunohistochemical investigation of the ovine lens (see Section 5).

Section 4 The Effect of Calpain on Lens Cytoskeletal Proteins

A method for investigating the effect of exogenous calpain on lens cytoskeletal proteins was adapted, with the aim of establishing a role for the proteolysis of these proteins by calpain in the ovine lens. Lens cytoskeletal extracts were incubated in the presence of calpain, calcium, and one of a selection of calpain inhibitors, including two that have been developed as part of an ongoing research project investigating calpain inhibitors as a treatment for cataracts. Novel calpain inhibitors were included to examine their potential to inhibit cytoskeletal proteolysis, while observing the ability of exogenous calpain to proteolyse lens cytoskeletal proteins and comparing to what has been observed in cataract.

Section 5 Immunohistochemical Localisation of Cytoskeletal Proteins in the Ovine Lens

An immunohistochemical survey of the ovine lens cytoskeleton was completed in order to examine its structure and compare with what is observed in other species.

Section 6 Conclusions and Future Directions

This final section emphasises the experimental evidence garnered from the preceding experimental sections in relation to the original hypothesis, as well as covering possible future research opportunities that may follow from the work completed here.