The Influence of extracellular matrix on lens epithelial cell viability

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The Influence of Extracellular Matrix on Lens Epithelial Cell Viability

By

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Declaration:

I declare that the experiments described in this thesis were carried out by myself in the Department of Biological and Biomedical Sciences, University of Durham, under the supervision of Prof. Roy A. Quinlan. This thesis has been composed by myself and is a record of work that has not been submitted previously for a higher degree. All references have been consulted by myself unless stated otherwise.

Frederique M.D. Tholozan

I certify that the work reported in this thesis has been performed by Frederique M.D. Tholozan, and during the period of study, who has fulfilled the conditions of the Ordinance and Regulations governing the Degree of Doctor of Philosophy.

Roy A. Quinlan

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Dedication:

'The presence of those seeking the truth is infinitely to be preferred to those who think they've found it' *Terry Pratchett.*

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List of Abbreviations:

a.a: amino acid

ADAM: a disintegrin and metalloprotease

AIDS: acquired immuno-deficiency syndrome

AIF: apoptosis inducing factor

ALPS: autoimmune lymphoproliferative syndrome

ANT: adenine nucleotide translocator

AOP: anti-oxidant protein

Apaf: apoptotic protease-activating factor

AQP0: aquaporin 0

ARC: age-related cataract

ASK: mitogen-activated protein kinase kinase kinase

ATP: adenine tri-phosphate

BCA: bicinchoninic acid

BIR: baculovirine inhibitor of apoptosis repeat

BLAST: basic local alignment search tool

BMI: body mass index

BRAC: breast cancer associated

BSA: bovine serum albumin

BSS: basic saline solution

CAD: caspase activate DNase

CARD: caspase- activating recruitment domain

CCC: continuous curvilinear capsulorhexis

cDNA: cyclic deoxyribo nucleic acid

Dab2: disabled-2

DAXX: death associated protein

DD: death domain

DED: death effector domain

dd H_2O: distilled deionised H_2O

DFF: DNA fragmentation factor

DISC: death-inducing signalling complex

DNA: deoxyribo nucleic acid

DM: myotonic dystrophy

DMEM: Dulbecco's minimun eagle's medium

DMPK: myotonic dystrophy protein kinase

dNTP: dinucleotide tri-phosphate

DTT: dithiothreitol

ECCE: extra-capsular cataract extraction

ECL: enhanced chemiluminescence

ECM: extra-cellular matrix

EDTA: ethylene diamine tetra acetate

EGF: epidermal growth factor

ELISA: enzyme-linked immunofluorescence substrate assay

EMT: epithelial to mesenchymal transition

ER: endoplasmic reticulum

ERK: extracellular-signal-regulated kinase

FADD: Fas-associated death domain

FAK: focal adhesion kinase

FANC: Fanconi anaemia

FCS: foetal calf serum

FGF: fibroblast growth factor

FLIP: c-Fas associated death domain-like IL-1-converting enzyme-like inhibitory protein

$FLIP_L$: FLIP long

GAF: GAGA factor

GAG: glucosaminoglycan

GFAP: glial fibrillary associated protein

GSH: reduced glutathione

HBS: HEPES buffered saline

HDGF: hepatoma-derived growth factor

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFC: high-frequency capsulotomy

HGF: hepatoma growth factor

HIPK: homeodomain interacting protein kinase

HSE: heat shock element

HSF: heat shock factor

HSP: heat shock protein

HSPG: heparan sulfate proteoglycan

HUVEC: human umbilical vein endothelial cell

IEMA: immunoenzymometric assay

IAP: inhibitor of apoptosis protein

ICAD: inhibitor of caspase activated DNase

ICCE: intra-capsular cataract extraction

IFN: interferon

IGF: insulin-like growth factor

IGFBP: IGF binding protein

IGF-IR: IGF-1 receptor

IKK: inhibitor of I-κB kinase

ILK: integrin linked kinase

IOL: intra-ocular lens

IOP: intra-ocular pressure

IRS: insulin receptor substrate

JNK/SAPK: c-jun amino-terminal kinase/ stress-activated protein kinase

LEDGF: lens epithelium derived growth factor

LSR1: Lens specific regulatory element 1

LSR2: Lens specific regulatory element 2

MAPK: mitogen activated protein kinase

MAPKAP: mitogen activated protein kinase activated protein

MCP: multicatalytic proteinase

MEK: MAPK/ERK kinase

MIP: major intrinsic protein

MMP:matrix metallo-proteinase

MTS:3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetratzolium

mRNA: messenger ribonucleic acid

NAD: nicotine amide deoxyribonucleotide

Nd:YAG: Neodynium:Yttrium-Aluminium-Garnet

NF-κB: necrosis factor-κB

NGF: nerve growth factor

NIK: NF-κB inducing kinase

NK: natural killer

NOD: nucleotide-binding oligomerization domain

OFZ: organelle-free zone

OVD: ophthalmic viscosurgical device

PAGE: poly-acrylamide gel electrophoresis

PARP: polyADP-ribose polymerase

PBS: phosphate buffered saline

PBR: peripheral benzodiazepin receptor

PCO: posterior capsule opacification

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PDK-1: phosphoinositide-dependent kinase 1

PDTC: pyrrolidine dithiocarbamate

PEX: pseudo-exfoliation syndrome

PF-HB1: progressive familial heart block 1

PI3K: phospho-inositide 3 kinase

PIP3: phospho-inositide phosphate 3

PK-A/-B/-C: protein kinase A/B/C

PMMA: polymethyl methacrylate

PTP: permeability transition pore

PTPC: permeability transition pore complex

r: Pearson product moment correlation coefficient

RAIDD: RIP- associated protein with death domain

RIP: receptor interactive protein kinase

RNA: ribonucleic acid

RNAse: ribonuclease

ROCKI: Rho kinase I

ROS: reactive oxygen species

ROSE: rat ovarian surface epithelium

RP: retinitis pigmentosa

RPE: retinal pigment epithelial

RT: reverse transcriptase

RT-PCR: reverse-transcriptase polymerase chain reaction

SARA: Smad anchor for receptor activation

SDS: sodium dodecyl sulphate

sHSP: small heat shock protein

SIP-1: Smad interacting protein-1

SMA: smooth muscle actin

SOD: super-oxide dismutase

SODD: silencer of death domain

SPARC: secreted protein, acidic and rich in cysteine

SRM: serious risk material

sRNAi: small RNA interference

STAT: signal transducer and activator of transcription

STRE: stress related regulatory element

TβR: TGFβ receptor

TBS: Tris-buffered saline

TCA: tri-chloro acetic acid

TGF: transforming growth factor

TIMP: tissue inhibitor of MMP

TMB: tetramethylbenzydine

TNF: tumour necrosis factor

TNFR: tumour necrosis factor receptor

t-PA: tissue plasminogen activator

TRADD: TNFR-associated death domain

TRAF: TNFR-associated factor

TRAIL: TNF-related apoptosis-inducing ligand

TRAIL-R: TNF-related apoptosis-inducing ligand receptor

TRAP1: TβRI associated protein

TTBS: Tween 20-Tris-buffered saline

TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

UV: ultra violet

VDAC: voltage-dependent anion channel

VEGF: vascular endothelial growth factor

Abstract:

Posterior capsular opacification is the main complication of cataract surgery and results from the proliferation, migration and differentiation of lens epithelial cells remaining in the capsular bag. To better understand this pathological cell behaviour, I investigated the interactions between lens epithelial cells and the bovine lens capsule *in vitro* and their effect on cell viability. As determined by a colorimetric cell proliferation assay, *in vitro* culture of cells directly on the bovine lens capsule resulted in maintained cell viability in the presence of staurosporine in both lens epithelial cell lines tested, but in neither of the two non-lens cell lines tested. As determined by immunoblotting and reverse-transcriptase polymerase chain reaction (RT-PCR), cell viability on the bovine lens capsule could further be correlated to the presence of both αA-crystallin and αB-crystallin expression. A positive correlation of cell viability on the lens capsule with vimentin and HSP27 expression was also found in a smaller set of cell lines. As determined by gelatin zymography and immunoblotting, MMP-2 was expressed by lens epithelial cells, led to the release of FGF-2 and IGF-1 from the lens capsule and correlated with lens epithelial cell viability.

Taken together, these results suggest that the lens capsule can act as a store of releasable growth factors available to the lens epithelial cells, with effects on their protein expression and cell viability.

1. Introduction:

1.1. Lens Biology:

The lens, by allowing the focusing of images onto the retina, is an essential part of the visual system and is a conserved component of the eye throughout the animal kingdom, with evidence of a lens structure being found even in some dinoflagellates species (Gehring, 2002). In vertebrates, differential transcription factor expression during embryogenesis leads to the establishment of two continuous, but morphologically very distinct, cell populations within the lens, the proliferative anterior epithelial cells and the differentiated posterior fibre cells, with the latter the bulk of the lens mass. Transparency and a constituting refractive index gradient throughout the fibre mass are crucial to the lens function and both are made possible by the chaperone and osmotic properties of the crystallin proteins.

1.1.1. From the ectoderm to the lens vesicle:

During Embryogenesis, the future lens originates from the lens placode on the head ectoderm and progresses through to the closed lens vesicle stage under the direction of a transcription factor cascade. The initial induction of the ectoderm occurs in two phases. The early phase establishes the surface ectoderm lens-forming competence during gastrulation and the neural plate stage, when signals from neighbouring neural folds lead to Sox3, Otx2 and Pax6 expression. During the later

phase of ectoderm induction, the optic vesicle, a lateral protrusion of the diencephalon that will ultimately become the retina, comes in contact with the lens-competent ectoderm. This induces a decrease in ectoderm Otx2 expression, while maintaining Pax6 expression (Ashery-Padan and Gruss, 2001). From then on, Pax6 orchestrates lens development.

Pax6 controls lens placode formation via an early expression of sFRP2 and Sox2, followed by a later expression of CMaf and LMaf, Prox1 and FoxE3 (Ashery-Padan and Gruss, 2001). During those later stages of the lens placode formation, Pax6 expression is maintained by BMP7 and through the FGFRs (Wawersik *et al.*, 1999; Faber *et al.*, 2001). Pax6 also maintains Six3 expression, which was initiated by Otx2 at the presumptive ectoderm stage and contributes to further Pax6 expression in a positive loop (Kawakami *et al.*, 2000). At the cellular level, these new transcription factors have conflicting effects on protein expression. This can be interpreted as an early presence of all the transcription factors able to promote fibre differentiation which are being antagonised by another set of transcription factors, eventually enforcing an epithelial phenotype. At the level of crystallin expression, this competition is manifested in the limitation of αA-crystallin transcription by FoxE3 (Blixt *et al.*, 2000) in the presence of the pro-α-crystallins transcription factors Pax6 (Cvekl and Piatogorski, 1996) and Mafs (Kondoh, 1999). Pax6 also represses β-crystallin expression (Kondoh, 1999), while the antagonistic effects of the pro-transcription Prox-1 and the anti-transcription Six3 result in repression of γ-crystallins expression (Lengler *et al.*, 2001). Non-crystallin examples include FoxE3

predominance over Prox1 to repress Cdkn1c expression (Blixt *et al.*, 2000), thus preventing early cell cycle exit, which would result in reduced proliferation and, hence, a smaller adult lens.

Progressing to the next developmental stage, the cells of the lens placode thicken, probably followed by apical contraction of zonulae adherens and their associated actin bundles (Lo *et al.*, 2000) to produce an invagination of the placode into what is termed the lens pit. Further invagination forms the lens vesicle which maintains close proximity with the optic vesicle (Francis *et al.*, 1999) and is temporarily in contact with the surface ectoderm by the lens stalk, which is later degraded under FoxE3 supervision (Blixt *et al.*, 2000). Lens vesicle formation coincides with a decrease in Sox2 expression, which, being linked to lens placode formation, is no longer needed and an increase in Sox1 expression, which will be later employed during primary fibre differentiation (Kondoh, 1999). At that stage, the cells of the lens vesicle form a unicellular layer, with their basement membrane, the lens capsule (detailed in section 3-1-2), facing outwards. For the first time, the anterior cells and the posterior cells are exposed to different micro-environmental conditions, i.e. the developing anterior aqueous humour and posterior vitreous humour. These different conditions are associated with the first signs of lens cell polarisation, such as the expression of connexin 46 and connexin 50 (Jiang *et al.*, 1995) and of p57, a cyclin dependent protein kinase inhibitor, in the posterior cells (Lovicu *et al.*, 1999).

1.1.2. Lens polarisation and primary fibres differentiation:

Outside the lens, tissue polarisation is controlled by different growth factors in the anterior (Van Setten *et al.*, 2002) and posterior (Koyama *et al.*, 2003) chambers of the eye. Experiments on the embryonic chick eye lens have shown that aqueous humour factors derived from the embryonic plasma induce proliferation in epithelial cells (Hyatt and Beebe, 1993), while vitreous humour factors induce induces elongation and crystallin production (Beebe *et al.*, 1980). Both factors antagonise each other, as adding aqueous humour to the vitreous humour abolishes cell elongation (Hyatt and Beebe, 1993) and their effects on the determination of lens cell phenotype are so strong that when the exposure to those factors is inversed, such as in the early experiments using a 180^0 rotation of the already differentiated lens (Coulombre and Coulombre, 1963), the original epithelium is induced to form fibres and an epithelial monolayer appears on the originally-posterior side. Insulin-like growth factor (IGF) -2, in combination with fibroblast growth factor (FGF) -2 or platelet-derived growth factor (PDGF), has been proposed as the pro-epithelium aqueous factor (Hyatt and Beebe, 1993), while IGF-1 has been identified as the pro-fibre vitreous factor (Beebe *et al.*, 1986).

Inside the lens, the exposure to different growth factors translates into a decrease in the levels of pro-epithelial transcription factors, such as Pax6 and FoxE3, in the posterior cells only, allowing the remaining transcription factors to induce fibre differentiation. In the anterior cells, however, transcription factor levels are unchanged and the epithelial phenotype is retained. The posterior cells' differentiation into the primary fibres is under the control of Prox1, which mediates p57-induced exit from the cell cycle and, together with Sox1 (Nishigushi *et al.*, 1998), maintains, but

does not initiate, cell elongation into primary fibres (Kondoh, 1999). Extensive expression of α-, β- and γ-crystallins in the fibre cells is essential to the correct differentiation of the fibres (reviewed in Graw, 1999) and to the properties of the future lens (detailed in sections 1.1.4.2 and 1.1.5.1). Accordingly, α-crystallin expression is up-regulated by cMaf, β-crystallin expression is de-repressed by the absence of Pax6, and γ-crystallins' expression is de-repressed and induced by the absence of Six3 and the retention of Prox1, respectively (Kondoh, 1999).

Primary fibre elongation also correlates with the establishment of a cell-to-cell communication network, which will later allow nuclear fibres' survival in the avascular lens. This is manifested by the initiation of basal major intrinsic protein (MIP) expression (Yancey *et al.*, 1988), connexin 46 and connexin 50 expression at the basal and apical ends of the fibres (Jiang *et al.*, 1995) and the transition from E-cadherin to N-cadherin expression (Kondoh, 1999). When the fibres reach the anterior epithelial cells, apical ends of both epithelial and fibre cells exhibit zonulae adherens with associated actin bundles and fascia adherens bridge epithelial cells and primary fibres (Lo *et al.*, 2000).

Primary fibre cell denucleation, which is necessary for the transparency of the future adult lens nucleus, follows (detailed in section 1.1.4.1).

1.1.3. Secondary lens fibres:

1.1.3.1. Secondary lens fibres differentiation:

Once the primary fibres are established as the lens nucleus, secondary fibre differentiation starts from the bow region of the lens, which is the only site of Prox1 and p57 expression from this stage onwards (Wigle *et al.*, 1999; Lovicu and McAvoy, 1999). Sox1 and cMaf, however, continue to be expressed throughout the fibre mass and promote continuous crystallin expression (Kondoh, 1999).

Induction of secondary lens fibre differentiation is predominantly driven by FGF-1 and FGF-2 (reviewed in Chamberlain and Mc Avoy, 1997). Both FGF forms are more concentrated in the vitreous humour than in the aqueous humour and, as the lens epithelial cells reach the posterior bow region, the increased FGF exposure triggers an initial migration response, followed by differentiation. Amplification of the response also occurs, as stimulation of the FGF pathway leads to up-regulation of FGFRs.

Differentiation itself is first characterised by cell elongation, the result of K+ intracellular retention, leading to water uptake and increase in cell volume (Parmelee and Beebe, 1988). Elongating secondary fibre cells face the epithelial cells via their apical ends and the posterior capsule via their basal end (Lo *et al.*, 2000). They are pushed along the outer and inner cortex by the younger fibres leaving the equatorial

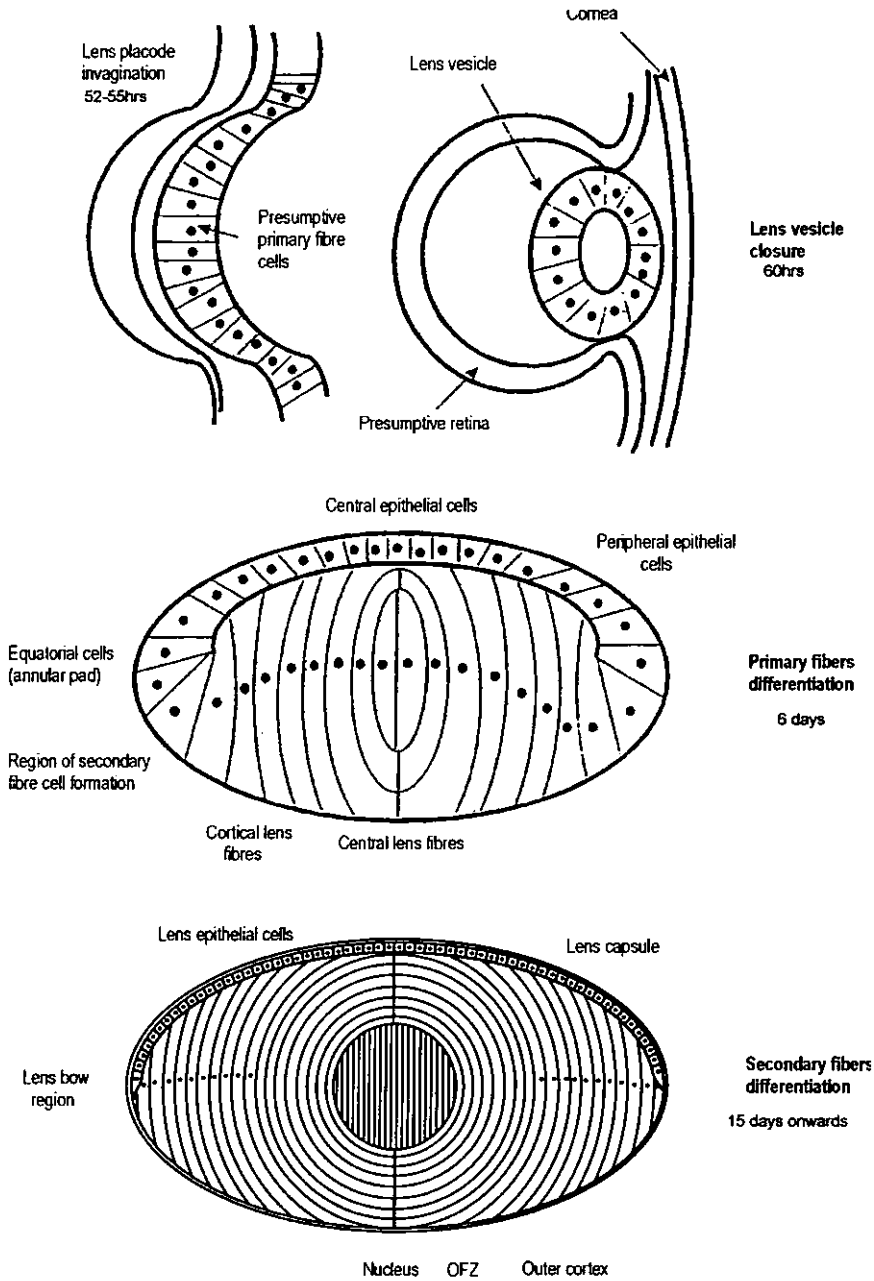


Figure 1.1: The stages of lens development in the chick. The figure was modified from Wride, 2000 using CorelDRAW 8.

region and encircle the older fibres at the centre of the lens. Hence, the fibre mass is made of concentric layers of progressively older fibres.

The high elongation rate varies little between fibres from the outer to the inner cortex, at around 145μm per day in the chicken lens, (Bassnett and Winzenburger, 2003) and requires constant addition of membrane material to the plasma membrane. To this end, single and bundled microtubules run parallel to the plasma membrane and mediate membranous vesicle transport. The microtubule centrioles are found exclusively at the fibres' apical ends and, as a result, all the minus ends of the microtubules are at the apical side, i.e. the anterior side of the lens, and all plus ends are at the basal side, i.e. the posterior side of the lens. The microtubule role in plasma membrane addition is limited to the outer cortex of the lens, as they are not found in fibres after organelle loss (Lo *et al.*, 2003; Dahm *et al.*, 1997). Elongation stops when facing fibres contact, first at the posterior suture, then at the anterior one (Bassnett *et al.*, 2003). Contact between fibres at the sutures is associated with a disassembly of both basal and apical zonulae adherens, correlating with detachment from the posterior capsule and the lens epithelium, respectively, and a decrease in N-cadherin expression, correlated with an increased vinculin expression and the extensive formation of membrane interdigitations along the long sides of the fibres (Beebe *et al.*, 2001). Hence, the mature fibres are in contact only with the immediate younger and older fibre layers and cannot directly access the ions, metabolites and growth factors present in the aqueous and vitreous humours. Loss of organelles, which is essential for transparency, follows (detailed in section 1.1.4.1).

1.1.3.2. Secondary lens fibres maintenance:

As the fibre mass is not vascularised, the differentiated fibres' survival depends on the diffusion of metabolites through the lens, which is known to involve both intracellular and extracellular pathways (Goodenough *et al.*, 1980).

Although the epithelial cells and the fibre cells are juxtaposed at their apical ends, before the fibre cells reach the sutures, dye loading studies have shown that metabolite transport directly from the epithelium to the fibres is very restricted (Rae *et al.*, 1996). It also appears not to involve Gap junctions, as these are absent from the epithelium-fibre interface (Dahm *et al.*, 1999; Bassnett *et al.*, 1994). Rather, metabolite transport to the mature fibres relies on diffusion from the peripheral lens fibres, which are still transcriptionnaly active (Faulkner-Jones *et al.*, 2003). In the outer cortex, intracellular diffusion is restricted to Gap junctions (Shestopalov *et al.*, 2000), which limits transport to small water-soluble molecules (Alberts *et al.*, 2002), i.e. prevents protein diffusion. However, in the inner cortex and nucleus, where denucleation of cells prevents protein turn-over, diffusion of macromolecules is made possible by the formation of a syncitium, developmentally established from the lens nucleus outwards and mediated by discrete, but abundant, cytoplasmic fusions (Shestopalov *et al.*, 2000). This syncitium includes a small number of nucleated cells, allowing the diffusion towards the innermost layers of the fibre mass of both membranous and cytoplasmic proteins newly produced by younger peripheral cells (Shestopalov *et al.*, 2003). Gap junctions are also retained and rendered pH-

insensitive by connexin50 cleavage (Grey *et al.*, 2003), allowing them to stay active in the acidic lens nucleus (Mathias *et al.*, 1991).

Extracellular diffusion, allowing transport such as GLUT3-mediated glucose uptake, also occurs in the lens cortex, but abruptly stops at the transitional zone. This correlates with MP20 re-localisation from the cytoplasm to the plasma membrane, resulting in increased cell-to-cell adhesion and the formation of a physical barrier to extracellular diffusion (Grey *et al.*, 2003). Preventing metabolites from accumulating in the inter-membranous space, this barrier eliminates refractive index discontinuity between adjacent fibre cells.

1.1.4. Lens transparency:

1.1.4.1. Establishment of lens transparency:

Lens transparency is achieved by the programmed degradation of all major organelles during fibre differentiation, giving rise to a central organelle-free zone (OFZ) which coincides with the visual axis of the lens.

The steps of organelle loss have been extensively studied in the chick lens. In this system, the Golgi apparatus is the first organelle to be lost, with degradation initiated at the equator and completed within a few cell layers into the outer cortex (Bassnett, 1995). Mitochondrial loss is initiated at E8, after re-localisation of the

mitochondria, which in peripheral fibre cells are elongated and evenly distributed throughout the cytoplasm, to perinuclear clusters, and is characterised by fragmentation, followed at E10 by swelling and cristae disruption. By E12, mitochondria are absent from the fibre cells (Bassnett and Beebe, 1992).

Nuclear loss, first described by Kuwabara and Imaizumi in 1974, is initiated in the OFZ, after endoplasmic reticulum (ER) loss (Bassnett and Mataic, 1997), but simultaneous with mitochondria loss (Basnett, 1992), and has been suggested to share common pathways with apoptosis, as Bcl2 and Bad, caspase-1 and capsase-3 are all expressed in chick fibre cells during development (Wride *et al.*, 1999). Bcl-2 over-expression (Fromm and Overbeek, 1997) and caspase-1 inhibition (Wride *et al.*, 1999) have also been shown to result in decreased nuclear degeneration. In contrast to apoptosis, however, nuclear loss takes several days to complete and is not associated with nuclear membrane blebbing (Bassnett and Mataic, 1997), but rather with CyclinB/p34^{cdc2} complex activity and nuclear location, which is reminiscent of mitosis-related nuclear membrane loss (Gao *et al.*, 1995). Nuclear loss may be initiated by intracellular pH, 0_2 or Ca^{2+} changes directly resulting from the loss of the ER and/or the mitochondria (Bassnett, 2002), or by increased tumour necrosis factor (TNF) -α signalling (Wride and Sanders, 1998). It is executed by early transcription shutdown (Dahm *et al.*, 1998) followed by nuclear lamina breakdown, involving nuclear pore aggregation (Dahm and Prescott, 2002), phosphorylation by the CyclinB/p34^{cdc2} complex and nuclear membrane degradation (Bassnett and Mataic, 1997). Nuclear membrane breakdown extends to the contiguous ER membrane system, which is degraded at the same time (Bassnett, 1995). These nuclear

membrane changes are accompanied by partial uncoiling of the heterochromatin to form, with the euchromatin, a homogenous pool of 30nm-thick fibres, followed by segregation and export or degradation of all nuclear proteins, except histones which stay associated to the DNA fraction. The strong nuclear localisation of γ-crystallin, CP49 and filensin at this stage is an indication of their possible role in DNA uncoiling and protein segregation. Further DNA uncoiling follows to form 10nm fibrils, with eventual degradation of both DNA and histones (Vrensen *et al.*, 2004). DNase I, DNase II and DNase II-like acid Dnase (DLAD) have been proposed as candidates for DNA digestion (Bassnett *et al.*, 1997; De Maria and Arruti, 2004; Nishimoto *et al.*, 2003) and the ubiquitin-proteasome pathway, members of which are present and active in the lens cortex (Pereira *et al.*, 2003), as candidate for histones degradation (Vrensen *et al.*, 2004).

A number of mice models have been established which present with persitent cell nulei in the fibre cell compartment (*a*8 connexin knock-out mouse (Rong *et al.*, 2002), ectopic AP-2*a* mouse (West-Mays *et al.*, 2002) and E2F-1 knock-out mouse (McCaffrey *et al.*, 1999)). All three models produce cataractous mice. However, the presence of other important lens disruptions, such as increased lens cell proliferation, vacuole formation and decreased gap junction size, make it difficult to determine the precise effects that persistent nucleation alone has on the development of these lenses.

1.1.4.2. Maintenance of lens transparency:

Although the ubiquitin-proteasome is present and efficiently degrades damaged proteins in the lens epithelium and outer fibre layers, its activity in the lens nucleus is reduced due to a loss of ubiquitin-conjugating enzymes (Pereira *et al.*, 2003). Therefore, maintenance of transparency in the lens relies heavily on the chaperone activity of the lens crystallins to prevent protein aggregation (Bloemendal *et al.*, 2004).

α-crystallin is found as a high molecular complex of 800 kDa composed of an arrangement of two polypeptides of 20kDa each, αA- and αB-crystallin , in a three to one ratio (Wistow and Piatigorsky, 1988). The chaperone activity of α-crystallin relies on ATP binding of αB-crystallin, which induces conformational changes and the exposure of hydrophobic residues which bind amino acids (a.as) on the chaperone' substrate, i.e a misfolded or unfolding protein. The crystallins' hydrophobic residues are spaced with polar a.as, allowing the bound substrate to maintain a refoldable state and preventing it from aggregating with other unfolfed proteins (Haslbeck, 2002). Site-directed mutagenesis studies have shown the importance of Asp69, situated in a conserved charged cluster (Smulders *et al.*, 1995), and Arg116, in the C-terminal domain (Kumar *et al.*, 1999), for αA-crystallin chaperone properties and the importance of Arg120 (Kumar *et al.*, 1999), Thr144 and Gly147 (Derham *et al.*, 2001), all situated in the C-terminal region for αB-crystallin chaperone properties. This C-terminal region allows αB-crystallin to bind its fellow sHSPs αA-crystallin and HSP27 (Liu *et al.*, 1999).

α-crystallin is the main protector of lens transparency in the lens, as shown when lens total soluble protein fractions, which remain transparent after prolonged heating, lose this ability when α-crystallin is selectively removed (Rao *et al.*, 1995). Targets of α-crystallin include the β-crystallins and γ-crystallins, which it binds to form a soluble high molecular weight aggregate (Rao *et al.*, 1995). Another α-crystallin target is the cytoskeleton, as α-crystallin participates, with CP49 and filensin, in the lens-specific beaded filament (Prescott *et al.*, 1996) and is known to increase solubilisation of the intermediate filaments vimentin and CP49 (Nicholl et Quinlan, 1994). Proteins of the plasma membrane also possibly associate with α-crystallin, as a fraction of α-crystallin is palmitoylated, indicating plasma membrane anchorage (Manenti *et al.*, 1990).

The relative importance of the α-crystallin constitutive polypeptides, αA-crystallin and αB-crystallin, in lens transparency differs, as αA-crystallin (Brady *et al.*, 1997), but not αB-crystallin (Andley *et al.*, 2001), knockout mice develop cataract.

The transparency of α-crystallin itself is mediated by the repulsive short range interactions between individual α-crystallin molecules, which insure that, instead of aggregating at high concentration, such as the 450mg/ml found in the human lens nucleus (Horwitz, 2003; Fagerholm *et al.*, 1981), the individual molecules form a regular spatial arrangement, resulting in elimination of light scatter by destructive interference (Tardieu, 1998).

The efficacy of α-crystallin in maintaining lens nucleus transparency is limited by the original α-crystallin content of the differentiated lens fibre. Protein synthesis is thought not to occur in inner fibres as ribosomal ribonucleic acid (rRNA) is extensively degraded, thus preventing the transcription of otherwise stable mRNA (mitochondrial RNA) (Faulkner-Jones *et al.*, 2003). With age, the soluble α-crystallin fraction progressively decreases, then disappears from the nucleus (Rao *et al.*, 1995). Concomitantly, the high molecular weight soluble fraction increase, correlating with a decrease in α-crystallin activity (Fujii *et al.*, 2003) and eventually reaching a size allowing light scatter (Rao *et al.*, 1995). This decrease in activity might be the effect of a loss of α-crystallin structure with age, probably due to oxidation (detailed in section 1.2.2). With time it would eventually result in an overall failure of chaperone efficiency and the formation of opacity-causing aggregates.

α-crystallin activity can also be indirectly affected by disrupting molecular transport within the lens mass. As an example, the induction of nuclear opacities in mouse models of galactose cataract is thought to involve hydration and subsequent swelling of the hyperosmotic sugar-rich lens, leading to fibre membrane damage allowing $Ca^{2+}$and Na^{+} intracellular influx (Hightower and Misiak, 1998). Calcium is present in the healthy lens as membrane bound aggregates throughout the fibre mass extracellular space, with the exception of GAP junction regions, and, after organelle loss, can also occasionally be found as small precipitates in the cytoplasm, possibly bound to β-crystallin (Van Marle *et al.*, 1997). The increase in calcium documented

in galactose cataract (Hightower and Misiak, 1998) is compatible with the *in vitro* Ca^{2+}-induced decrease in α-crystallin chaperone activity, with higher Ca^{2+} concentrations also inducing decreased thermal stability, loss of tertiary structure and partial unfolding (del Valle *et al.*, 2002). Calcium also affects α-crystallin chaperone function indirectly by activating calpain-mediated membrane protein degradation, thus increasing the pool of crystallin-binding proteins.

1.1.5. Lens refractive index:

1.1.5.1. Establishment of lens refractive index:

The exact direction of the incoming light path through the lens depends on the lens' refractive properties. The plasma membranes of the fibre cells constitute a major discontinuity along the light path through the fibre mass. Changes to the plasma membranes' structure, as in the CP49 knockout mouse, which exhibit loss of interdigitation and variation in diameter along the fibre, result in increased light scattering and disrupted focal length (Sandilands *et al.*, 2003). In the outer cortex, fibres are tightly packed following a regular hexagonal arrangement maintained by actin bundles, which run parallel to the long axis at each corner of the fibre (Lo *et al.*, 1997). This regularity allows concentration of the light path along a single axis with minimal scatter in all other directions and compensate for the difference in refractive index between membrane and cytoplasm (Michael *et al.*, 2003). Plasma membrane morphology, however, changes with lens fibre differentiation and membranes in the

lens nucleus present extensive interdigitation, mediated by a coordinated push-pull action of actin branching and clathrin-AP2 complex, respectively, between neighbouring cells (Zhou and Lo, 2003). This should result in increased light scattering. However, the composition of the membrane itself also changes with fibre differentiation and both sphingomyelin content and cholesterol to phospholipids ratio are greatly increased in membranes in the nucleus, which reduces the refractive index of the membranes. Meanwhile, as the cytoplasmic refractive index is increased in the nucleus, both cytoplasm and plasma membrane reach comparable refractive indexes and constitute an homogenous medium in which light scattering cannot be produced (Michael *et al.*, 2003).

The increase in cytoplasmic refractive index is due to the massive up-regulation of crystallin expression, which in the nuclear fibre cells accounts for over 90% of the total protein fraction (Horwitz, 2003). In the lens epithelium and as explained in section 1.1.1, Pax-6 up-regulates αA-crystallin and αB-crystallin expression (Cvekl and Piatogorski, 1996; Gopal-Srivastava *et al.*, 1996), but represses β-crystallin expression (Duncan *et al.*, 1998). Meanwhile, γ-crystallin expression is suppressed via the methylation of its promoter region (Peek *et al.*, 1991), and the conflicting effects of Prox1 and Six3 (see section 1.1.1). In the fibre cells, and as explained in section 1.1.2, crystallin expression is strongly up regulated, with cMaf up-regulating α-crystallin expression in the absence of Pax6 and FoxE3 (Kondoh, 1999) and Prox1 and cMaf inducing β-crystallin expression (Cui *et al.*, 2004). Six3 down-regulation, coupled with Sox 1 up-regulation, also allows γ-crystallin promoter

demethylation and expression (Peek *et al.*, 1991; Nishigushi *et al.*, 1998). This general up-regulation lead to an increase in crystallin concentration from the peripheral fibre cells inwards (Kenworthy *et al.*, 1994) and gives rise to an increasing refractive index. However, most fibre cells are metabolically inactive (Bassnett and Winzerburger, 2003) and the increase in crystallin concentration within the inner cortex and lens nucleus cannot rely solely on protein expression or diffusion from younger fibres. In the inner parts of the lens, establishment of the crystallin gradient also depends on progressive cell dehydration from the cortex to the nucleus, which increases protein concentration without requiring further crystallin expression and flattens the fibres (Shiels *et al.*, 2001). The importance of the integrity of this water efflux in establishing the crystallin refractive gradient is illustrated in the *Aqp0*$^{-/-}$ mouse lens. The *Aqp0*$^{-/-}$ lens is characterised by greatly reduced water diffusion out of the differentiating fibres, leading to decreased nuclear crystallin concentration and subsequent decreased refractive index and increased focal length (Shiels *et al.*, 2001).

1.1.5.2. Maintenance of lens refractive index:

Maintenance of the crystallin concentration gradient in the syncitium part of the lens, which in the adult lenses encompasses the cortex up to the innermost nucleated fibres (Shestopalov *et al.*, 2003), requires a shift in osmotic properties between cortical and nuclear crystallins. Both the osmotic properties of α-crystallins and β-crystallins are based on short range repulsive interactions, which lead to an exponential increase in osmotic pressure at high concentration (Kenworthy *et al.*,

1994) and are incompatible with the passive maintenance of a concentration gradient. Studies on bovine crystallins show that, compared to cortical α-crystallins, nuclear α-crystallins are less phosphorylated, which is associated with lower ionic charge, and have a higher tendency to aggregate. Both modifications lower the nuclear α-crystallins' osmotic pressure, allowing them to equilibrate at a higher protein concentration with the cortical α-crystallins (Kenworthy *et al.*, 1994). A similar mechanism has been suggested for maintaining the β-crystallin protein gradient. γ-crystallins, which are subject to attractive short-range interactions, do not exhibit such large increases in osmotic pressure at high concentration (Kenworthy *et al.*, 1994).

1.1.6. Lens accommodative properties:

1.1.6.1. Establishment of lens accommodation:

Accommodation, which results in the formation of a focused image on the retina, involves a sequence of events in and outside the eye. It involves recognition and translation of the unfocused nature of the image on the retina, followed by activation of a neuromotor response towards the ciliary body (Mordi and Ciuffreda, 2004). Contraction of the ciliary body is transmitted to the lens capsule by the ciliary fibres, resulting in decreased curvatures of mainly the anterior capsule and, to a lesser extent, the posterior capsule (Garner and Yap, 1997). At the same time, the equatorial lens diameter decreases, coupled to a forward displacement of the anterior part of the lens only, with the vitreous depth remaining constant (Garner and Yap, 1997). Such

deformation was originally proposed by Fisher in 1971 to result from differential capsule pressure, due to increased capsule thickness at the equator. However, the variations in capsule thickness with age do not correlate with the observed loss of accommodation and the viscoelastic properties of the capsule are more compatible with an ability to evenly distribute pressure on the lens surface (Krag and Andreassen, 2003). The biochemical basis of the lens capsule's elasticity will be detailed in section 3-1-2.

The fibre mass deformation under capsular pressure affects the lens nucleus, which is uniformly stretched, resulting in increased thickness and forward displacement of the embryonic lens nucleus, while both the anterior and posterior cortex are largely unaffected (Dubbelman *et al.*, 2003). Nuclear shape changes correlate with the deformation of fibre cells, regulated by tPA (Tripathi *et al.*, 1888) and stabilised by adherens junctions (Lo, 1988), and cytoplasmic content redistribution (Tiffany and Koretz, 2002).

Crystallins, which account for almost all of the nuclear cytoplasmic proteins (Horwitz, 2003), are redistributed via the syncitium (Shestopalov *et al.*, 2000). Their non-Newtonian viscous properties insure that their viscosity is high in the absence of capsule pressure, thus efficiently maintaining the lens shape, but the viscosity decreases quickly with increasing pressure, insuring a quick dispersal during accommodation (Tiffany and Koretz, 2002).

1.1.6.2. Maintenance of lens accommodation:

Age-related changes are the main challenge to the maintenance of accommodation. With age, the curvature of the lens increases, which increases the power of the lens and should make the eye myopic. The observation that this does not occur has been termed the 'lens paradox' (Smith and Pierscionek, 1998). This increase in lens curvature with age is naturally compensated by a decrease and equalisation of the refractive index in the nuclear and inner cortex regions, which correlate with a reduced soluble crystallin fraction and decreased power of the lens. The outer cortex refractive index gradient remains unchanged (Moffat *et al.*, 2002).

Older lenses are also characterised by a thickened and stiffened capsule and a compacted cortex region, which reduce pressure transmission from the ciliary body to the nucleus and prevent curvature changes (Krag and Andreassen, 2003). The ciliary body contractile ability, however, is retained (Pardue and Sivak, 2000) and accommodation can be restored by surgically replacing the fibre mass by a soft polymer filling (Koopmans *et al.*, 2003).

1.2. Primary cataract:

1.2.1. Cataract prevalence worldwide:

Cataract affects 110 million people worldwide, 25 million of which are registered blind (Paik and Dillon, 2000; Ornek *et al.*, 2003) and its treatment, cataract

surgery, is the most important procedure in financial terms in the health service (Apple *et al.*, 2000). As the post-surgical development of posterior capsular opacification (PCO, detailed in 1.3) indicates the pre-surgical presence of primary cataract, the cellular and molecular changes having led to cataract might still affect the physiology of the lens epithelial cells that remain after surgery, and therefore their behaviour during PCO progression and treatment.

1.2.2. Age-related cataract:

Age-related cataract (ARC) accounts for half of blindness cases worldwide (WHO Fact Sheet N143) and has been associated with a range of environmental and genetic risk factors. A large number of epidemiological studies in geographically distinct populations have been conducted and shown that the incidence of nuclear, cortical and posterior subcapsular opacities, which are the main morphological types of ARC, increases with age, female sex, dark iris colour, UV-B exposure and use of corticosteroids (Sperduto, 1994; AREDS report 5, 2000). Morphologically, ARC is associated with discrete lens fibre defects such as cytoplasmic globular body aggregation, loss or hyper-ruffling of the plasma membrane and discharge of cytoplasmic material in the extra-cellular space. These defects are mainly found in the cortex region, not the nucleus region, although the nucleus is the opacified region (Al-ghoul *et al.*, 1996), which highlights the importance of outer fibre integrity for the correct function of the inner nuclear fibres.

At the cellular level, a predominant feature of ARC is an extensive lens protein oxidation (Truscott and Augusteyn, 1977), which occurs mainly by direct photo-oxidation of tryptophan, tyrosine, phenylalanin, cysteine and cystine residues on lens proteins by UV-B irradiation (Davies and Truscott, 2001). UV-filtering compounds in the lens can also become photo-oxidised, following which they bind other lens proteins (Berry and Truscott, 2001). O_2 content has been shown to be low in the lens core regions and is therefore unlikely to produce extensive oxidative stress in the absence of a co-existing mitochondrial disorder (McNulty *et al.*, 2004). UV-B exposure is aggravated by age, as 3OHKG, a product of tryptophan metabolism which acts as a natural UV filter in the lens, decreases with age (Wood and Truscott, 1993), allowing increased UV stress within the lens. UV-B irradiation is associated with oxidation of α-, β- and γ-crystallins in the fibre cells (Andley and Clarck, 1989a; Andley and Clarck, 1989b; Andley and Clarck, 1989c) with effects on the chaperone ability of the α-crystallins and aggregation tendency of the β- and γ-crystallins. UV-B exposure also correlates in the epithelial cells with a reduction in glutathione levels, which increases the overall oxidative stress, and a disruption of Na^+/K^+ membrane pumps, disrupting the osmolar balance and eventually resulting in an increase in intracellular Ca^{2+} (Hightower and McCready, 1992). This Ca^{2+} increase further affects crystallin aggregation (detailed in section 1.1.4.2). Unsurprisingly, UV-B irradiation correlates with opacification in animal models (Hightower and McCready, 1992). Increased calcium content resulting from capsular breakdown is also a feature in the mouse model of accelerated senescence, which develops cataract (Ashida *et al.*, 1994). This model also records increased β-crystallin cross-linking and high

molecular weight aggregate formation as well as decreased levels of soluble γ-crystallin.

Genetic background is also a significant risk factor for ARC. Down-regulation or mutations of genes involved in oxidative stress resistance or chaperone activity and up-regulation of genes involved ionic transport, although initially resulting in mild phenotypes, will increase the susceptibility of the lens to environmental stresses over time and increase the likelihood of developing late onset cataract (Hejtmancik and Kantorow, 2004). An example of such a mechanism was found in the Japanese population in which decreased galactokinase activity due to an A198V substitution leads to galactitol accumulation and osmotic swelling of the lens fibres (Okano *et al.*, 2001). In mouse models of diabetic cataract, such fibre swelling has been shown to allow Ca^{2+} intracellular influx (Hightower and Misiak, 1998). Presence of the mutant allele associated with a greater risk of adult onset cataract.

1.2.3. Congenital cataract:

Inherited human cataracts are associated with mutations affecting a restricted set of protein families, which are nevertheless deeply involved in lens function.

Mutations involving the crystallin genes disrupt either the chaperone capacity of the α-crystallins or increase the aggregation tendencies of the β- and γ-crystallins. In humans, three α-crystallin mutations resulting in cataract have been reported. A

C413T substitution in the αA-crystallin gene, corresponding to a R16C substitution in the protein, is associated with nuclear cataract, together with corneal defects of various severity, indicating a role of αA-crystallin outside the lens during eye development (Reddy *et al.*, 2004). In the αB-crystallin gene, two cataract-causing mutations have been described, 450delA (Berry *et al.*, 2001) and A358G (Perng *et al.*, 1999). The 450delA mutation results in a frameshift after the 149th protein residue and the introduction of thirty-five new C-terminal residues, which is likely to affect the solubility and chaperone activity of the crystallin (Carver and Lindner, 1998). The A385G mutation introduces a R120G substitution in the protein and has been linked with cataract and desmin-related myopathy. *In vitro* studies on recombinant R120G αB-crystallin indicate that the mutation results in partial unfolding of the protein and increases its binding to intermediate filaments, leading to decreased solubility and promotion of bundling of the filaments (Perng *et al.*, 1999). Mutations affecting the β- and γ-crystallins have also been found to cause various forms of congenital cataract. Two mutations have been reported in βA-crystallin; G474A or G474C, which introduces a splice site (Reddy *et al.*, 2004) and 279delGAG, corresponding to 91delG in the protein (Ferrini *et al.*, 2004). In βB-crystallins, two non-sense mutations, G658T and C475T, have been described (Reddy *et al.*, 2004). γ-crystallins have often been found to be mutated in human cataracts, with five distinct substitutions mutations in the γD-crystallin gene, two of which, C43T, resulting in R14C, and G176A, resulting in R58H, reduce the protein's solubility and promote aggregation (Reddy *et al.*, 2004). Of the three mutations detected in the γC-crystallin gene, A13C, resulting in a T5P substitution in the protein sequence and associated

with Coppock-like cataract, decreases the *in vitro* stability and solubility of the protein, which would increase its aggregation tendency, even though its binding to αA-crystallin is unaffected (Liang, 2004). A secondary effect of this mutation would be that a greater proportion of the α-crystallin would be bound to T5P γC-crystallin, therefore less α-crystallin would then be available for other unfolding proteins in the lens, resulting in a general decrease in lens protein solubility.

Other cataract-causing mutations affect proteins involved in cell-to-cell junctions, which are important for the maintenance of the differentiated fibre cells (detailed in section 1.1.3.2) and can put indirect stress on the chaperone system. AQP0 is responsible for water transport away from the differentiating fibre cells (Shiels *et al.*, 2001) and has been linked with two distinct forms of human congenital cataracts. Resulting from either a C413G or an A401G substitution in the gene sequence, both mutations affect conserved residues at the tightest part of the water channel (Hejtmancik and Kantorow, 2004; Agre and Kozono, 2003). Disruption of water transport can induce fibre swelling, with consequences on the establishment of the refractive index (see section 1.1.5) and on chaperone-threatening Ca^{2+} intracellular influx following plasma membrane rupture (see section 1.1.4). Gap junctions can also be disrupted, as a number of distinct mutations in both connexin 46 and connexin 50 have all been shown to associate with nuclear pulverulent cataract. Some of these mutations are directly linked to the transport function of the proteins, with the C262T substitution of connexin 50 affecting the second transmembrane region and the A188G substitution of connexin 46 affecting the region involved in the coupling of hemi-channels (Francis *et al.*, 1999). As these mutations will affect the

transport of small molecules only, they are likely to disrupt the general metabolism of the fibre cells.

Lastly, mutations affecting transcription factors involved in lens development result in lens defects, although some are not restricted to the lens and disrupt other tissues within the eye. G38A, in the *PITX3* gene expressed in the lens placode and pit, has been proposed to affect DNA-binding ability (Francis *et al.*, 1999). 943InsG, in the *FoxE3* gene, a mutation separate from the well-described F98S which causes the *dyl* mouse phenotype (Blixt *et al.*, 2000), has been detected in familial human cataract (Semina *et al.*, 2001). Transcription factors directly involved in crystallin expression have also been involved in congenital cataract. G1670C in *MAF*, which induces α-crystallin expression in the fibre cells (see section 1.1.2) and four separate substitutions in the HSF4 protein sequence, which regulates both small and large heat shock proteins expression, (Reddy *et al.*, 2004) are likely to indirectly induce extensive protein aggregation, due to a general decrease in chaperone proteins.

1.2.4. Disease cataract:

Cataract can also develop as a secondary symptom of a polysystemic disease disrupting pathways or proteins non-exclusive to the lens.

The best documented example of disease cataract is diabetes-related cataract, in which water permeability of the plasma membrane is increased, leading to fibre swelling (Fisher, 1985). The lysine, proline, arginine and threonine sidechains of the

crystallin proteins are also oxidised to form carbonyl groups, affecting chaperone activity (Jain *et al.*, 2002). Diabetes-related cataract might also be exacerbated by a co-existing diabetic retinopathy. Retinopathy is characterised by apoptosis of pericytes and subsequent increased endothelial cell proliferation (Hammes *et al.*, 2002), which, coupled to an increased angiotensin II vitreous humour level, eventually results in neovascularisation of the retina (Funatsu and Yamashita, 2003). It is conceivable that this retinal proliferating cell population might affect the growth factor composition of the vitreous humour. Access to those growth factors would also be facilitated by lens capsule degradation, and hence possible greater lens capsule permeability, also observed in diabetes (Lin *et al.*, 1995), with effects on the lens cells both before and after the cataract operation. Indeed, Neodynium:Yttrium-Aluminium-Garnet (Nd:YAG) laser capsulotomy (detailed in 1.3.7) is also more common in diabetic cataract (Hayashi *et al.*, 2002). Another condition where cataract develops secondary to retinopathy is Bloch-Sulzberger-syndrome, which is primarily characterised by abnormal skin inflammation and pigmentation (Kassmann-Kellner *et al.*, 1999).

Another condition commonly associated with cataract is myotonic dystrophy (DM1), which in the great majority of cases correlates with an unstable CTG repeat at 19q13.3 (Morris, 2000). The main gene affected in DM1 is DMPK, the 3'untranslated region of which encompasses the CTG repeat. The expansion prevents DMPK mRNA from leaving the nucleus but instead it binds and sequesters RNA-binding proteins, thus disrupting the processing of other mRNAs (Morris, 2000). Six5, a member of the Six family of developmental transcription factors, is downstream of the CTG repeat

and down regulated in DM1. As Six5, but not DMPK, is expressed in the eye, it has been suggested that Six5 is responsible for the eye phenotype of DM1 (Kawakami *et al.*, 2000). In the eye, DM1 is manifested by ocular motor weakness (Versino *et al.*, 1998), retinal lesions and early adult onset cataract (Gjertsen *et al.*, 2003). DM1 is also associated with increased complications after cataract surgery, such as capsulorhexis contracture and a PCO incidence of about 50%, the upper limit of PCO incidence in the general population (Gjertsen *et al.*, 2003). PCO resilience is also increased in DM1, with several Nd:YAG laser treatments required to clear the opacification (Gjertsen *et al.*, 2003).

1.2.5. *Primary cataract surgery:*

Primary cataract surgery has been performed for centuries (Linebarger *et al.*, 1999) and is highly successful at restoring visual acuity (Powe *et al.*, 1994). The procedure follows a series of steps, namely anaesthesia, corneal incision, anterior chamber stabilisation, capsulorhexis, cataract removal, intra-ocular lens (IOL) positioning, ophthalmic visco-surgical device (OVD) removal and wound closure (reviewed in Linebarger *et al.*, 1999). The range of techniques available at each step allows customisation to the needs of the patient, which can be weighted against the specific post-operative complications of each technique. Although the corneal cells, which are highly exposed to surgical devices throughout the procedure, and the retinal cells, which can be affected by any disruption to the vitreous humour, can be damaged during surgery, this introduction will be limited to the surgical steps most relevant to the lens.

1.2.5.1. Stabilisation of the anterior chamber:

Preventing the collapse of the anterior chamber, due to aqueous humour loss through the corneal incision, prevents, amongst other things (Linebarger *et al.*, 1999), forward displacement of the lens against the cornea. Maintenance of the anterior chamber space is achieved by injection of a transparent OVD. A high molecular weight OVD, characterised by a high viscosity, is best to maintain a physical space between the cornea and the anterior lens capsule (Tetz and Holzer, 2000). It is therefore recommended for paediatric surgery, as increased capsule elasticity and sclera rigidity in children leads to anterior displacement of the lens and increases the risk of capsule tear formation during capsulorhexis (Jeng *et al.*, 2004).

1.2.5.2. Anterior capsulorhexis:

All anterior capsulorhexis methods used involve a small opening of the anterior capsule and do not affect the viability of the lens epithelial cells (Radner *et al.*, 2004). The most common method used to open the anterior capsule is continuous curvilinear capsulorhexis (CCC), which regular shape ensures equal tension around the opening and decreases the risk of capsular tears. The main complications of this technique are after cataract (detailed in section 1.3) and delayed capsule contraction (Miyake *et al.*, 1998). Others methods include high frequency capsulotomy (HFC), in which use of high temperatures increases the risk of radial capsule tears and of post-

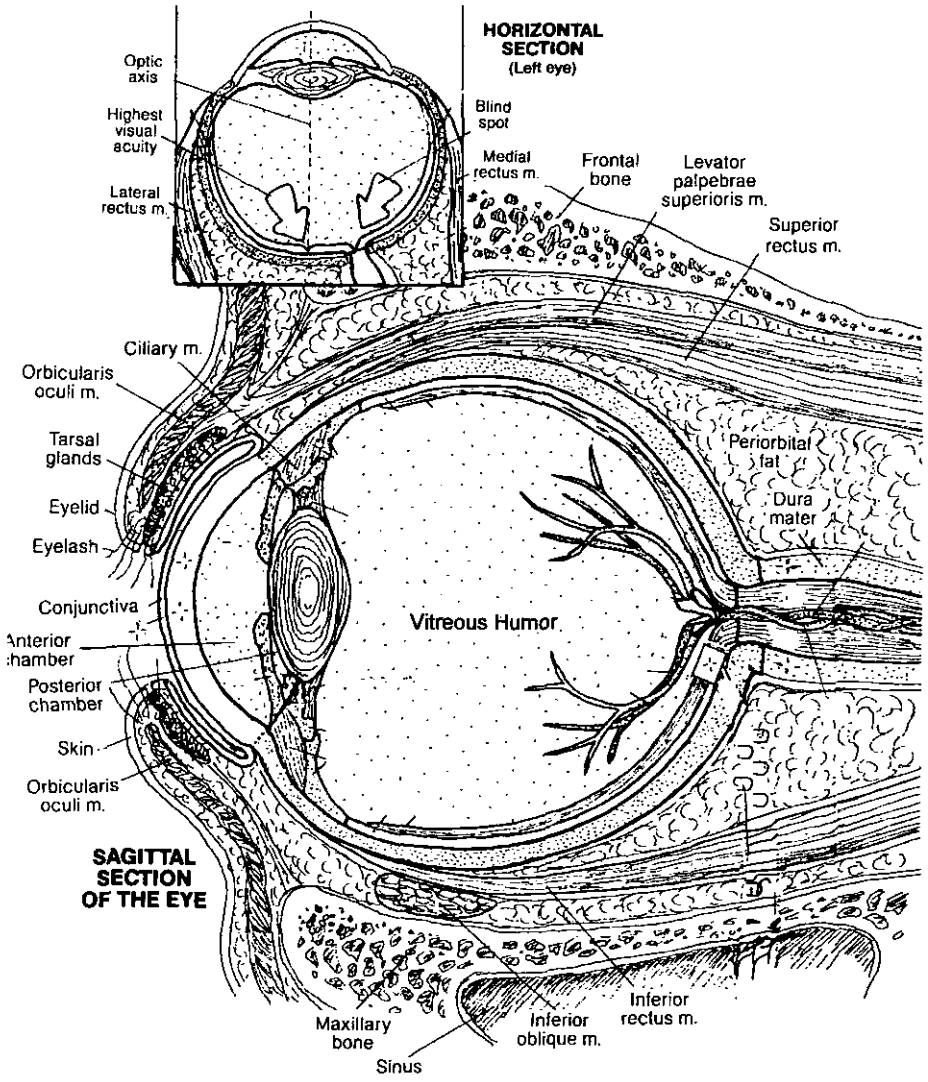


Figure 1.2: The gross anatomy of the eye. Picture taken from Kapit and Elson, 1993 using Coral DRAW 8.

operative false positioning of the IOL (Radner *et al.*, 2004) and Nd:YAG laser phacolysis, which use isn't yet approved in humans, but has been used in rabbits to open the anterior capsule up to 24h before surgery, allowing softening of the fibre mass when it contacts the aqueous (Radner *et al.*, 2004). Its obvious drawback is that it allows the lens cells direct access to aqueous growth humour factors overnight, which might promote long-term proliferation post-surgery.

1.2.5.3. Lens material removal:

Removal of the opacified fibre mass is the endpoint of cataract surgery. However, a non-exhaustive capsular bag clean up can lead to many active lens epithelial cells being left on the capsule. These remaining lens cells are the primary cause of PCO.

Hydrodissection of the fibre mass is first performed by injecting basic saline solution (BSS) between the anterior capsule and the cortex to separate the lens nucleus from the lens cortex while maintaining a layer of cortical fibres against the capsule, thus protecting the posterior capsule from tearing. Then, initial mechanical nuclear break-up creates large fractures in the nucleus, the number and position of which depend on the technique used (Linebarger *et al.*, 1999). Although removal of the nucleus is possible following minimal mechanical disruption (Akura *et al.*, 2000), extensive nuclear breakup before extraction is common. Thorough fibre separation

and removal from the capsule follows and is most commonly done by phacoemulsification. Phacoemulsification relies on ultrasonic vibrations produced by electrical stimulation of either a wire coiled around a stack of thin metal strips or a compressed ceramic crystal, coupled with an irrigation/aspiration pump for material removal (Linebarger *et al.*, 1999). It is associated with minimal IOP variation and, compared to phacoemulsification-free extra-capsular cataract extraction (ECCE), with greatly decreased post-operative inflammation (Linebarger *et al.*, 1999).

Nd:YAG laser can also be used, whereby short energy pulses are transmitted via a quartz fibre optic to a titanium probe, resulting in the emission of shock waves which break up the lens nucleus. The disrupted lens fragments are then aspirated using an irrigation/aspiration system (Werner *et al.*, 2000). The total energy, number of pulses and duration of the procedure increase with the hardness of the nucleus, which increases with the maturity of the cataract. When compared to phacoemulsification, the laser technique involves less surgical trauma, less focal heating and less total energy (Werner *et al.*, 2000). Large-scale studies also show a very low rate of complications, either during or three months after surgery (Kanellopoulos, 2001). Following nuclear material removal, further hydrodissection is used, coupled with irrigation/aspiration, to remove any remaining cortical fibres.

1.3. After cataract:

1.3.1. After cataract prevalence worldwide:

If the disappearance of intra-capsular cataract extraction (ICCE) during primary cataract surgery has eliminated the risks of haemorrhage, vitreous loss, disappearance of the aqueous-vitreous barrier and retinal detachment, the retention of the capsular bag characteristic of ECCE leads to widespread after cataract (Hersh *et al.*, 1999). After cataract, or PCO, is nowadays the most common complication of cataract surgery, occurring in 10% to 50% of patients depending on age and surgery method (Powe *et al.*, 1994; Saxby *et al.*, 1998), and is particularly common after paediatric cataract surgery (Apple *et al.*, 2000; Mullner-Eidenbock *et al.*, 2003). As a result, it is also a significant drain on the health budget and second only to primary cataract surgery, which is the most costly overall procedure in the US (Apple *et al.*, 2000).

1.3.2. *Lens cell changes with after cataract:*

Lens epithelial cells remaining in the capsular bag after surgery are the primary cause of after cataract. A rat model of cataract surgery (Lois *et al.*, 2003) demonstrated that anterior lens epithelial cells proliferate in the space between the anterior and the posterior capsule as soon as 24h after surgery. After a few days, they can fill the entire bag but do not migrate out onto the anterior side of the anterior capsule.

A closer study of cell migration in the human and bovine capsular bag (Saxby *et al.*, 1998) reveals that migration onto the posterior capsule is done first by a monolayer of epithelial-like cells, with cells at the leading edge having greater

proliferative ability and radial actin filaments. Multilayering occurs once the posterior capsule is fully covered by the initial monolayer, with α-SMA expressing-fibroblasts-like cells present in the upper layers. This behaviour is associated with the fibrotic form of PCO (see section 1.3.3).

The appearance of fibroblast-like cells results from the epithelial-to-mesenchymal transition (EMT) of the lens epithelial cells under the influence of TGFβ-2. TGFβ-2 is the predominant TGF-β isoform in the aqueous humour and is produced locally by epithelial and equatorial lens cells, as well as retinal pigment epithelial (RPE) cells (Meacock *et al.*, 2000). After cataract surgery, all three TGFβ isoforms are present on IOL matrix deposits (Saika *et al.*, 1998). Outside the lens, TGFβ induce EMT via an ERK-independent remodelling of cortical actin into actin stress fibres, tight and desmosomal junction disassembly, ERK-dependent matrix re-modelling and, via SIP-1 (Moustakas *et al.*, 2002), E-cadherin junction disassembly, eventually resulting in reduced cell-cell interactions and increased motility (Zavadil *et al.*, 2001). In *in vitro* culture of lens epithelial cells, TGFβ-induced EMT results in increased fibronectin, collagen I and αSMA expression and decreased laminin, collagen IV and crystallin expression (Lee *et al.*, 1999). This is compatible with the changes in protein expression and the cell migration across the lens capsule observed during PCO progression (see section 3-1). αSMA expression of lens epithelial cells during after cataract also induces cell contraction and is responsible for capsular wrinkling (Wormstone *et al.*, 2004). Additional evidence of the direct role of TGF-β isoforms in lens epithelial cell EMT can be found in the

mouse sub-capsular cataract model, where overexpression of both TGFβ-1 and TGFβ-2 has been used to generate multi-layered, opacified, fibroblasts-like plaques in place of the original epithelium (Sun *et al.*, 2000).

In a separate rat model, cells at the equatorial region elongated and their nuclei aligned along the anterior-posterior axis of the lens, indicating an attempt at fibre differentiation, while cells lining the anterior capsule were showing signs of apoptosis (Lois *et al.*, 2003). A rabbit model (Saika *et al.*, 2001) also showed regeneration of lens fibres, accompanied by extra-cellular matrix (ECM) deposition, along the capsule's equator. This behaviour is associated with Soemmering's ring formation (*see section* 1.3.3).

1.3.3. Morphological types of after cataracts:

Four types of morphologically different PCO have been recorded. Fibrous PCO is due to fibrosis of either the anterior or the equatorial lens epithelial cells after their migration onto the posterior capsule (Apple *et al.*, 2000) and can be associated with lens capsule wrinkling (Cheng *et al.*, 2001). If a primary cataract is allowed to develop for several years before being removed, due to long waiting time before surgery, fibrosis of the equatorial cells on the posterior capsule can also be observed at the time of cataract surgery and is termed 'acute PCO' (Apple *et al.*, 2000). Soemmering's ring is an opaque, thick ring found along the equator and originates from the attempted differentiation into fibres of the cells of the lens bow region which, in the normal lens, would leave the epithelium at the equator and elongate to

differentiate into lens fibre cells. Soemmering's ring develop after rupture of the anterior capsule and extrusion of lens cortex into the aqueous following either a traumatic shock ECCE (Apple *et al.*, 2000). Elschnig pearls, sometimes seen co-existing with a Soemmering's ring, originate from individual equatorial cells which migrate onto the posterior capsule and swell to a rounded shape (Cheng *et al.*, 2001). Lastly, liquefied PCO occurs when the edge of the remaining anterior capsule fully adheres to the IOL, recreating an enclosed space within the capsule. This space gradually fills with a white substance, which has been suggested to be formed by a mix of ECM degraded by the fibrotic lens cells and aqueous humour. Liquefied PCO can be differentiated from endophthalmitis and uveitis (see 1.3.5.1) by the absence of inflammation and from capsular block syndrome by the time of onset and the colour of the liquid (Miyake *et al.*, 1998).

1.3.4. Visual implications of after cataract:

After-cataract is associated with an increase in opacification density of the posterior capsule, which correlates with a decrease in visual acuity (Hayashi *et al.*, 1998). The impact on visual acuity, however, is only manifested when the opacification reaches the visual axis, i.e. the centre of the posterior capsule, and is more evident either in long-term follow-up (Halpern *et al.*, 2002) and/or for higher PCO grades (Abhilakh-Missier *et al.*, 2003).

1.3.5. Aggravating factors for after cataract:

1.3.5.1. Eye-related complicating factors of after cataract:

A number of ocular conditions are known to increase the prevalence and severity of PCO when co-existing with cataract. At the cellular level, some of these aggravating conditions correlate with an increase in aqueous humour growth factors traditionally associated with lens cell proliferation or epithelial-to-mesenchymal transition. The growth factors increased are hepatoma growth factor (HGF), vascular endothelial growth factor (VEGF) and TGF-β in glaucoma (Hu and Ritch, 2001; Hu *et al.*, 2002; Ochiai and Ochiai, 2002; respectively), VEGF in uveitis (Fine *et al.*, 2001) and FGF-2 in pseudoexfoliation syndrome (Gartaganis *et al.*, 2001).

Glaucoma involves an increased intra-ocular pressure due to impaired function of the trabecular meshwork. Co-existing glaucoma and cataract can be operated on simultaneously with good results (Donoso *et al.*, 2000) and the long-term PCO can be reduced by mitomycin C exposure for a few minutes during surgery (Shin *et al.*, 1998). Uveitis is a chronic, mild inflammation of the eye induced by a wide variety of agents (Foster *et al.*, 1999). Cataract is a frequent complication of uveitis, and commonly leads to post-operative anterior capsular opacification (Ram *et al.*, 2002), which can be resistant to Nd:YAG laser treatment and necessitate IOL removal (Foster *et al.*, 1999). Pseudoexfoliation syndrome (PEX) is a generalised disorder characterised by an excessive production of extracellular matrix material. In the lens, the anterior capsule becomes covered with melanin deposits, released from broken iris vessels (Naumann *et al.*, 1998), and the over-production of matrix by pre-equatorial cells at the point of zonular attachment leads to extensive detachment of

the zonular fibres (Naumann *et al.*, 1998). The rate of PCO has also been reported to be higher in PEX eyes (Tetz *et al.*, 1999).

Another condition correlating with increased PCO, as well as anterior capsule fibrosis and contraction (Hayashi *et al.*, 1998), is retinitis pigmentosa (RP), which refers to the progressive loss of rods and cones in the retina and results in night blindness and progressive loss of peripheral vision (Baumgartner, 2000). PCO is more disabling in RP patients, due to an already reduced vision field, and is thought to develop preferentially via the presence of capsular folds, which facilitate epithelial cell migration past the IOL barrier (Ando *et al.*, 2003).

1.3.5.2. Age-related factors:

Age is another influencing factor for cataract and PCO, with the changes in lens capsule breaking point with age resulting in younger capsule being harder to open and older ones being easier to break (Krag and Andreassen, 2003). After surgery, lens cells from younger patients proliferate at a faster rate (Wormstone *et al.*, 1997), resulting in PCO rates in children of over 50% after up to 5 years follow-up (Knight-Nanan *et al.*, 1996). Systematic posterior capsulotomy during cataract surgery has been recommended for children under 6 years old to prevent PCO, while still allowing IOL implantation (Jensen *et al.*, 2000).

1.3.6. Risk reduction at the time of surgery:

Current surgical procedures can be optimised to prevent the presence of lens cells and their proliferation and migration on the capsule, thus decreasing the incidence of PCO.

The original number of lens cells left in the capsular bag after surgery can be greatly decreased by the use of extensive cortical clean up via hydrodissection (see section 1.2.5). However, it can be overlooked by surgeons whose incomplete cortical clean up can leave significant numbers of actively dividing cells, leading to Soemmering's ring formation (Apple *et al.*, 2000). Extensive cortical clean up has also been shown to help decrease the duration of phacoemulsification and of the irrigation/aspiration step and the post-operative inflammation due to leftover cortex material (Peng *et al.*, 2000). Increased lens epithelial cell visualisation, which facilitates cortical clean up, can be achieved by using the OVD Viscoat (Budo *et al.*, 2003).

After surgery, the long-term extent of lens cells proliferation and migration is also affected by the type of IOL introduced during surgery. Positioning of the whole IOL inside the capsular bag, rather than out of the bag or only partly in the bag, has increased from 10% to 60% since the 1980s and allowed the use of the IOL as a physical barrier to lens cell proliferation (Apple *et al.*, 2000). Using a CCC diameter smaller than the IOL's allows the IOL to adhere to the anterior capsule beyond the capsulorhexis' limit and creates a physical barrier that should prevent lens epithelial

cells' direct exposure to aqueous growth factors, thus limiting their induction of proliferation, migration and EMT (Peng *et al.*, 2000). However, a complete IOL to capsule adherence around the CCC can lead to liquefied cataract (Miyake *et al.*, 1998). IOL design itself can also significantly affect the progression of PCO. It is now accepted that a square-edged IOL reduces PCO compared to a round-edged IOL (Nagata and Watanabe, 1996). However, there have been conflicting reports as to whether this reduction results from the contact inhibition of cell proliferation at the sharp edge of the IOL (Nishi and Nishi, 1999; Bhermi *et al.*, 2002). Mathematical modelling, however, has shown that the pressure of the IOL onto the capsule is increased by both the size of the IOL and a sharp edge, which provides a better physical barrier to cell migration (Boyce *et al.*, 2002). A decreased compressibility of the IOL haptics results in an increased number of folds forming at the haptic's point of contact with the lens capsule, which scatter light and facilitate lens epithelial cells proliferation past the IOL and onto the posterior capsule (Meacock and Spalton, 2001). The material composition of the IOL is also an important factor, with hydrophobicity of the IOL leading to a rapid adhesion to the posterior capsule, preventing cell proliferation (Saika, 2004; Schauersberger *et al.*, 2001). The IOL material can also regulate the extent of EMT on the capsule. Silicone, although incompatible with lens cell proliferation on the IOL itself, is associated with more fibrosis on the capsule (Saika, 2004), eventually resulting in more overall opacification than acrylic or PMMA IOLs (Werner *et al.*, 2000).

Other devices can be used to control cell migration. A sharp-edged, open, bending ring, introduced in the capsular bag at the time of the surgery, has been

shown to reduce the number of capsular folds, the amount of anterior capsule shrinkage and the lens cell proliferation onto both IOL and posterior capsule. The ring, which stretches the capsular bag, works as a physical barrier to anterior lens cell proliferation and prevents contact between the anterior capsulorhexis edge and the IOL (Nishi *et al.*, 2001).

Experimental research into the control of PCO at the time of surgery has also been conducted using rabbit models with promising results. Bipolar diathermy involves a pencil with an electrode area exposed at the tip, which is placed against the capsule during cataract surgery and transmits electrical energy, leading to the thermal destruction of the lens epithelial cells. Short-scale studies in rabbits have proven this method to be effective at preventing PCO, although the tip of the pencil should be inserted between the iris and the external side of the capsule, with the electrode being exposed only on the side in contact with the capsule, to minimise damage to the iris (Bretton *et al.*, 2002). Radioactive stress, delivered by the low rate decomposition of P^{32} atoms embedded in a PMMA capsular tension ring, has also been shown to effectively reduce lens epithelial cell proliferation in rabbits in a dose-dependent manner, without damage to the iris (Joussen *et al.*, 2001).

1.3.7. Treatment of after cataract post-surgery:

Due to the high prevalence of PCO after cataract surgery, PCO treatment by posterior capsulotomy is a significant surgical procedure, both in terms of frequency and cost (Apple *et al.*, 2001). Posterior capsulotomy is most commonly done by

Nd:YAG laser, which has replaced the earlier and more invasive surgical discission. The procedure, which can be done without prior pupil dilation to limit the working area to the active visual field, involves focusing the laser on the side of the capsule in contact with the vitreous to create a pressure wave which breaks and eventually creates a central opening in the posterior capsule. A small opening of 2 to 3 mm is enough to restore visual acuity and contrast sensitivity, but a bigger one is needed to reduce glare (Aslam *et al.*, 2003). Complications of Nd:YAG laser capsulotomy include vitreous opacification, due to lens cell material diffusing into the vitreous (Kumagai *et al.*, 1999) and lining of the posterior opening with Elschnig pearls, which is common after capsulotomy and can sometimes lead to the complete occlusion of the opening (Kurosaka *et al.*, 2002). In a significant number of eyes, however, these Elschnig pearls spontaneously disappear with time, possibly due to collapse into the vitreous or cell death (Kurosaka *et al.*, 2002). IOLs are also affected during the capsulotomy. The IOL surface can be damaged by the laser and the creation of an opening in the posterior capsule also affects the pressure distribution of the IOL against the capsular bag and induces a slight change in IOL position towards the back of the eye. Neither changes has significant effect on visual function (Aslam *et al.*, 2003). Posterior vitreous detachment and consequential retinal breaks have been shown not to be significantly increased after Nd:YAG laser treatment (Sheard *et al.*, 2003 and Ranta *et al.*, 2000; respectively). The extent of visual acuity recovery after Nd:YAG treatment also depends on the type of the original after-cataract, with fibrotic PCO being associated with a better outcome than the pearl form of PCO (Cheng *et al.*, 2001).

1.4. Apoptosis induction as a mean to prevent PCO:

While the surgical methods of PCO prevention at the time of surgery are mainly based on the restriction of lens epithelial cell migration, the frequent necessity of posterior capsulotomy shows that they are not fully effective. As a result, research into PCO prevention is thriving and has been focused on the complete elimination of the lens epithelial cells. Apart from a small number of studies using thermal stress or hydrolysis (Bretton *et al.*, 2002; Maloof *et al.*, 2003), most current research relies on apoptosis induction.

1.4.1. Mechanisms of apoptosis:

In the lens, as for all cell types, cell death occurs by two mechanisms, necrosis (Proskuryakov *et al.*, 2003) and apoptosis (Kerr, 2002; McConkey, 1998). Necrosis is a passive process associated with severe stress and results in cell swelling, membrane rupture and diffusion of intracellular contents in the cell medium, potentially affecting neighbouring cells and inducing an inflammatory response (Chandra *et al.*, 2000). In contrast, apoptosis is an active process (Wadewitz and Lockshin, 1988), induced by two main pathways (detailed in section I-4-1) and characterised by cytoplasm contraction, due to water loss following K+ and Cl- efflux (Bortner and Cidlowski, 1998), membrane blebbing, chromatin condensation and DNA degradation, resulting in the formation of apoptotic bodies eventually engulfed by phagocytes or neighbouring cells (Bortner and Cidlowski, 1998). Apoptosis is involved in development (Yamashita, 2003), wound healing (Greenhalgh, 1998), ageing (Zhang

and Herman, 2002), and, when disrupted, in tumour progression and autoimmune disease (Mullauer *et al.*, 2001).

1.4.1.1. Apoptosis induction:

1.4.1.1.1. the extrinsic pathway:

1.4.1.1.1.1. steps of the extrinsic pathway:

The extrinsic pathway of apoptosis relies on the binding of soluble or adjacent-cell-bound ligands to transmembrane death receptors.

Six death receptors have been identified, all belonging to the TNF receptor (TNFR) superfamily. Their extracellular domain contain two to four cyteine-rich regions, involved in ligand binding, while their intracellular region contains a conserved 80 amino acids death domain, which binds to adapter proteins (Curtin and Cotter, 2003).

The Fas/CD95 death receptor is involved in the apoptosis of antigen-presenting cells by cytotoxic T lymphocytes and in the apoptosis of activated T cells at the end of an immune response (Curtin and Cotter, 2003). Fas mutations are associated with autoimmune lymphoproliferative syndrome (ALPS) type I and bladder, gastric and colon cancers in humans (Mullauer *et al.*, 2001). CD95 binding

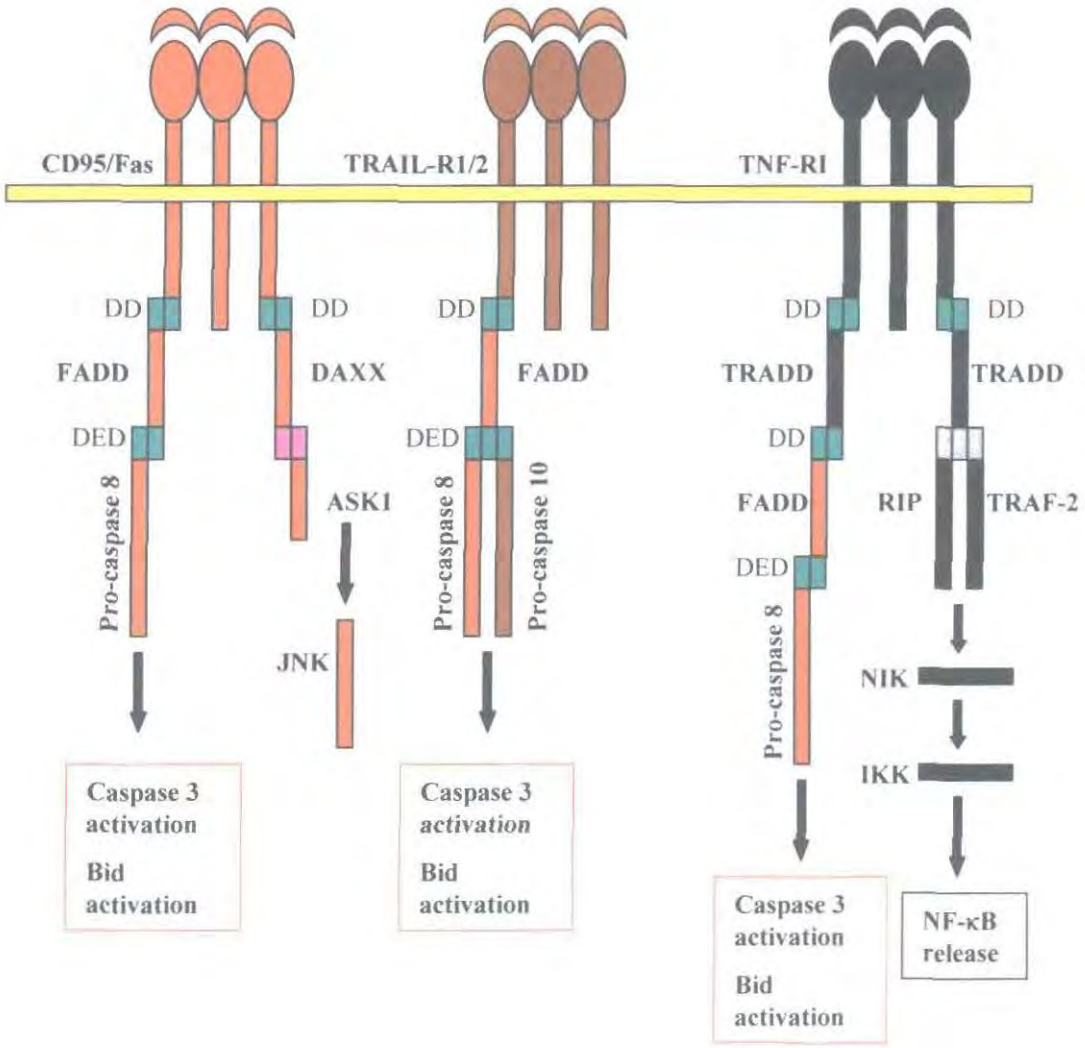


Figure 1.3: Steps of the extrinsic apoptotic pathway. Extracellular ligand binding induces transmembrane receptor trimerisation and binding of adaptor proteins, which themselves promote binding and activation of initiators of intracellular apoptotic pathways. Diagram made by myself based on the pathways described in 1.4.1.1.1. For a definition of the abbreviations used, see the abbreviation list.

of its ligand, the expression of which is restricted to activated T cells, induces receptor trimerisation and aggregation of the intracellular death domains (DDs), which leads to Fas-associated death domain (FADD) recruitment. FADD is an adapter protein which possess one DD for binding to CD95 and one death effector domain (DED) for binding pro-caspase 8. Pro-caspase 8 biding results in autocleavage and activation and allows caspase 8 cleavage of caspase 3. The CD95-FADD-pro-caspase8 complex is termed death-inducing signalling domain (DISC) (Gupta, 2001). The adapter death-associated protein (DAXX) can also bind to the CD95's DD, followed by ASK1 recruitment and JNK activation (Curtin and Cotter, 2003).

The TNF-related apoptosis-inducing ligand (TRAIL) death receptors, TRAIL-R1 and TRAIL-R2, are widely expressed and induce apoptosis following TRAIL binding in a FADD-dependent manner, with DISC formation, followed by caspase 8 and caspase 10 activation (Almasan and Ashkenazi, 2003).

The TNFR-I death receptor is induced to trimerise following TNF binding. TNFR-I's DD aggregation leads to TNFR-associated death domain (TRADD) binding. TRADD is capable of recruiting several adapter proteins, namely FADD, resulting in an induction similar to CD95-mediated apoptosis, or TNFR-associated factor (TRAF-2) and receptor interactive protein kinase (RIP), which activate NF-κB inducing kinase (NIK). NIK activates the inhibitor of I-κB kinase (IKK), which phosphorylates and inactivates IκB, allowing NFκB release, nuclear translocation and activation of gene transcription (Gupta, 2001).

1.4.1.1.1.2. regulation of the extrinsic pathway:

An increase in the resistance to the extrinsic pathway of apoptosis can be achieved by regulation of protein activity and expression at each step of the pathway.

The activity of membrane-bound Fas ligand can be decreased by matrix metallo-proteinase (MMP) -7 cleavage, and MMP-7 expression correlates with Fas-mediated apoptosis resistance in tumour cells (Curtin and Cotter, 2003).

Decoy death receptors, which bind ligands but lack the intracellular death domain can also be either expressed on the cell membrane, like TRAILR3 and TRAILR4, or secreted into the extracellular space, like DcR3, thus reducing the amount of ligand binding to functional death receptors (Curtin and Cotter, 2002).

Adapter proteins function can be disrupted by the competitive binding of silencer of death domain (SODD) to TNFR-I, which prevents trimerisation, and of c-Fas associated death domain-like IL-1-converting enzyme-like inhibitory protein (FLIP) to FADD, which prevents pro-caspase 8 binding and activation (Gupta, 2001). Adapter proteins can also be inactivated by phosphorylation, such as FADD phosphorylation by protein kinase (PK)-Cζ and homeodomain-interacting protein kinase (HPIK) -3 and TRADD phosphorylation by HIPK2 (Curtin and Cotter, 2002).

Lastly, caspase 8 function can be prevented by p35 binding of its active site (Shi, 2002).

An increase in the sensitivity to the extrinsic pathway of apoptosis can also be induced. Both NK cells and cytotoxic T lymphocytes can up-regulate Fas receptor expression, through an IFN-γ and STAT-1-dependent pathway.

1.4.1.1.2. the intrinsic pathway:

1.4.1.1.2.1. steps of the intrinsic pathway:

The intrinsic pathway of apoptosis can be triggered by a range of insults, such as cytokine withdrawal, anoikis, which refers to the triggering of apoptosis upon destruction of the cell-matrix adhesion (Sprick and Walczak, 2004; Frisch and Screaton, 2001), and oxidative stress (Chandra *et al.*, 2000). These apoptotic stimuli activate a subgroup of proteins from the Bcl-2 family, the BH3-only proteins, including Bad and Bim, which promote the activation of the pro-apoptotic subgroup of the Bcl-2 family, Bax and Bak (Sprick and Walczak, 2004). All four proteins are translocated to the outer membrane of the mitochondria (Szabadkai and Rizzuto, 2004). Bad then inactivates the inner membrane-bound Bcl-2 and Bcl-x, allowing permeability transition pore complex (PTPC) formation (Gupta, 2001). The PTPC consists of a peripheral benzodiazepin receptor (PBR) and a voltage-dependent anion channel (VDAC), which bind cytosolic hexokinase on the outer membrane, creatine kinase bridging VDAC to the adenine nucleotide translocator (ANT) in the intermembranous space and ANT itself, bound to matrix Cyclophilin D, in the inner membrane. The PTPC allows diffusion of ions and water into the mitochondrial

matrix with subsequent swelling, membrane rupture and intermembrane cytochrome c release (Belzacq *et al.*, 2002). When first released, cytochrome c translocates to the ER, where it induces Ca^{2+} release, which enters the mitochondria, resulting in further swelling and membrane rupture, eventually releasing more cytochrome c (Szabadkai and Rizzuto, 2004). Released cytochrome c binds to Apaf-1, an adapter protein homologue to the product of the *C.elegans* CED-4 gene (Saraste and Pulkki, 2000) and member of the nucleotide-binding oligomerisation domain (NOD) family, which contains an N-terminal caspase activating recruitment domain (CARD), a central nucleotide-binding oligomerisation domain and a C-terminal intracellular ligand-binding domain. The resulting Apaf-1/cytochrome c complex is termed the apoptosome. The heptameric symmetry of Apaf-1 allows CARD-mediated binding of seven pro-caspase 9 monomers, leading to caspase dimerisation and active site formation (Shi, 2002).

1.4.1.1.2.2. regulation of the intrinsic pathway:

Resistance to the intrinsic pathway of apoptosis can be mediated by resistance to oxidative stress, with Bcl-2 over-expression resulting in increased reduced glutathione (GSH) nucleus content. Once induced, the intrinsic pathway can be regulated by modulation of caspase 9 activity. XIAP, a member of the inhibitor of apoptosis protein (IAP) family, possesses two baculovirine inhibitor of apoptosis repeat (BIR) domains, with BIR3 biding to the N-terminus of caspase 9 small sub-unit resulting in physical obstruction of the caspase 9 substrate binding site (Shi, 2002).

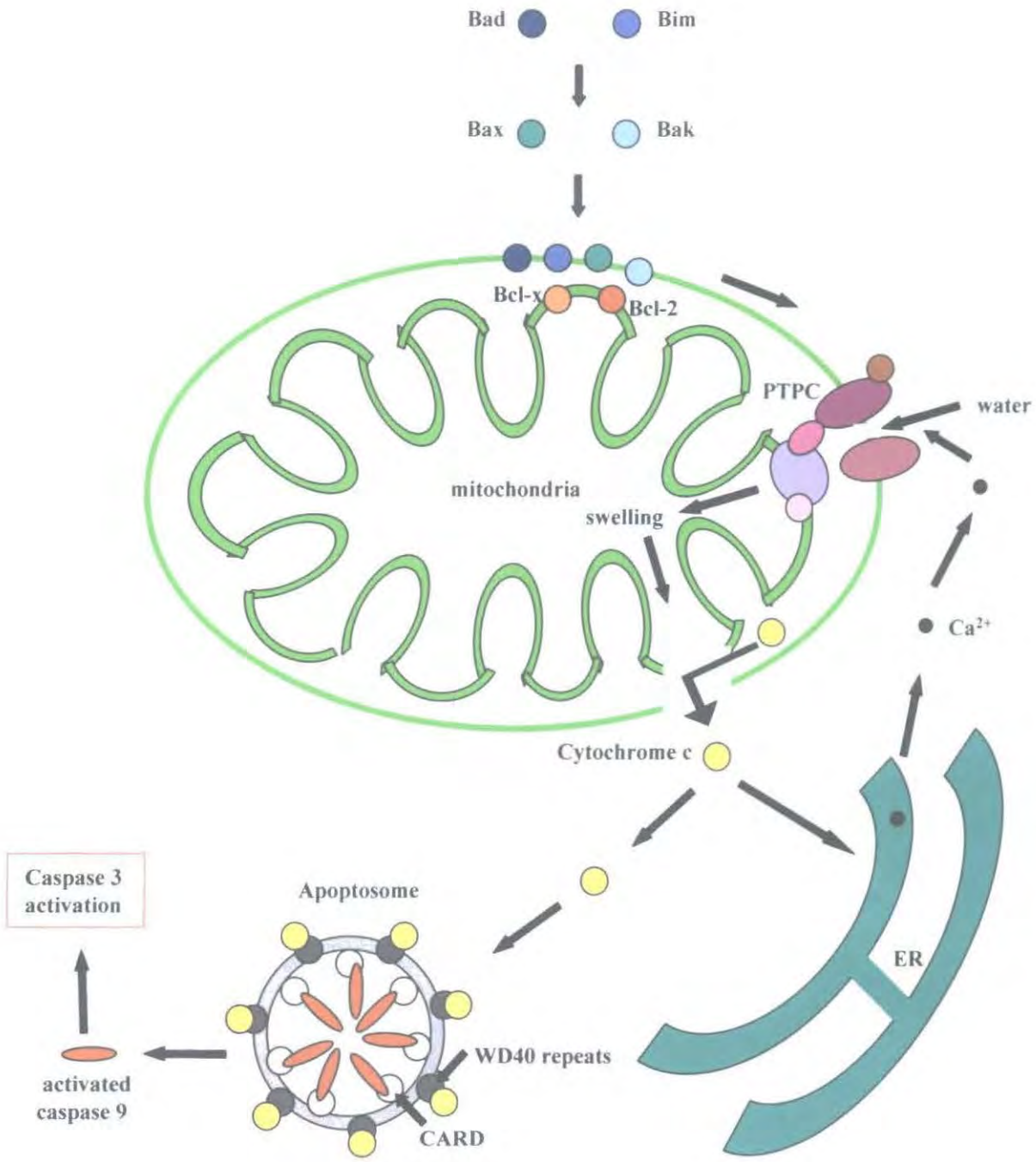


Figure 1.4: Steps of the intrinsic apoptotic pathway. Oxidative damage induces pro-apoptotic Bcl-2 family members translocation to the mitochondrial membrane with subsequent PTPC formation, release of cytochrome c, positive feedback loop from the ER Ca^{2+} stock, and apoptosome formation. Diagram made by myself based on the pathways described in 1.4.1.1.2. For a definition of the abbreviations used, see the abbreviation list.

XIAP can be antagonised by two mitochondrial intermembrane proteins, Smac/DIABLO and HtrA2/Omi, which are released upon mitochondrial degradation and sequester XIAP, allowing caspase 9 activity and subsequent caspase 3 activation (Sprick and Walczak, 2004). Caspase 9 activity can also be reduced by direct phosphorylation by Akt/protein kinase B (Cross *et al.*, 2000).

1.4.1.1.3. crosstalk between intrinsic and extrinsic pathways:

Both intrinsic and extrinsic pathways are linked by Bid, a pro-apoptotic Bcl-2 family member which, following Fas-induced caspase 8 activation, is cleaved by caspase 8 and translocates to the outer mitochondrial membrane, to participate in PTP induction and cytochrome c release (Gupta, 2001). This has been suggested as a way to overcome XIAP inhibition of caspase 8-activated caspase 3, by, on the one hand, increasing the pool of caspase 9-activated caspase 3 and, on the other hand, allowing Smac/DIABLO and HtrA2/Omi release and XIAP sequestration (Sprick and Walczak, 2004). By decreasing the levels of free XIAP while increasing the levels of its substrate, this eventually results in an excess of activated caspase 3 and the progression of apoptosis.

1.4.1.2. Apoptosis execution:

1.4.1.2.1. Executioner caspases activation:

Caspases define a family of cysteine proteases first described in *C.elegans* and activated during apoptosis. Caspases are separated into two functional groups. The initiator caspases contain a long N-terminal domain encoding one CARD, as in caspase 9, or two DEDs, as in caspase 8, which mediate binding to large complexes during the induction phase of apoptosis and facilitate initiator caspase autoactivation. Executioner caspases, caspase 3, caspase 6 and caspase 7, are devoid of this N-terminal domain and are activated directly by an upstream caspase (Sprick and Walczak, 2004).

Caspase activity depends on four amino acid loops, two contributed by the large N-terminal subunit and two contributed by the small C-terminal subunit, which form a substrate-binding groove, with loops 1, 3 and 4 allowing binding to a four amino acid residue and loop 2 containing the catalytic cysteine. Substrate binding is specific for an X-Glu-X-Asp residue sequence, with cleavage occurring after the Asp (Shi, 2002). In the inactive effector caspase, loop 2 is rotated, preventing access to the catalytic cysteine and loops 3 and 4 are turned away from each other. Asp cleavage between the two subunits by an initiator caspase results in conformational change and formation of a functional active site (Shi, 2002). Caspase activity also requires homodimerisation, with the stability of the active site of one monomer being increased upon substrate binding by the loop 2 of the other monomer, which doesn't form its own active site (Shi, 2002).

Caspases cleave a wide range of substrates during apoptosis (Saraste and Pulkki, 2000), eventually resulting in DNA degradation and apoptotic body formation.

1.4.1.2.2. Nuclear degradation:

Morphologically, nucleus degradation is characterised by nucleus shrinkage and chromatin condensation, followed by DNA fragmentation and nuclear breakup, or karryorhexis (Saraste and Pulkki, 2000).

Chromatin condensation is mediated by caspase 3 activation of the protein acinus and caspase 6 cleavage of the nuclear membrane proteins NuMa and lamins and results in collapse of the DNA-histone network (Robertson *et al.*, 2000). Caspase-independent chromatin condensation can also be achieved by the Ca^{2+}-dependent nuclear scaffold protease, which mediates lamin cleavage (McConkey and Orrenius, 1997) and possibly by the apoptosis inducing factor (AIF), translocated from the mitochondria to the nucleus (Robertson *et al.*, 2000). DNA degradation then occurs, giving rise to large fragments of 300kbp and 50kbp, and smaller multiples of 180 bp to 220 bp in length, which corresponds to the spacing of topoisomerase II, lamin B and histone H1 along the DNA, respectively (Robertson *et al.*, 2000). Topoisomerase II and lamin B are direct caspase substrates, while histone H1 can be bound by DNA fragmentation factor (DFF) -40, itself released from cytosolic DFF45 binding by cleavage of DFF45 by caspase 3 or caspase 7 (Robertson *et al.*, 2000). Degradation of

these DNA-associated proteins allows DNA access to DNases, namely the caspase-released DFF40 (Robertson *et al.*, 2000), but also the Ca^{2+}-dependent endonuclease DNase I, which is released from the ER during the intrinsic pathway and benefits from the elevated Ca^{2+} concentration in apoptotic cells (McConkey and Orrenius, 1997) and from caspase-induced cleavage of its inhibitor gelsolin (Robertson *et al.*, 2000). DNA damage repair is prevented by caspase 3 cleaving of DNA-PK, which is involved in DNA double strand break repair and polyADP-ribose polymerase (PARP), wich participates in complexes involved in DNA repair and replication (Robertson *et al.*, 2000).

1.4.1.2.3. Cytoskeletal degradation:

Cytoskeletal protein cleavage is an important part of the apoptotic process. Apart from the straightforward degradation of the cytoskeletal network, such as caspase 6 cleavage of the muscle-specific desmin, yielding an N-terminal fragment which induces both desmin and vimentin filament aggregation (Chen *et al.*, 2003), and eventual loss of actin bundles, microtubules and keratin filaments (Dommina *et al.*, 2002), cytoskeletal modifications are also linked to cell shape and adherence changes. Cell detachment and rounding are associated with the disappearance of focal adhesions induced by caspase cleavage of focal adhesion kinase (FAK), a member of the focal adhesion complexes which bridge the ECM-binding integrins (reviewed in Qin *et al.*, 2004) to the actin cytoskeleton (Carragher *et al.*, 2001). Membrane blebbing is mediated by caspase cleavage and activation of Rho kinase (ROCK) I,

which promotes actomyosin network contractility (Dommina *et al.*, 2002). Targeted actin microfilament or microtubule disruption has also been shown to induce a caspase-independent dephosphorylation of Akt/protein kinase B (Flusberg *et al.*, 2001), resulting in decreased Bcl-2 expression and Bad phosphorylation (Cross *et al.*, 2000), therefore increasing general pro-apoptotic signalling in the cell.

The cytoskeleton is also involved in stabilisation of the apoptotic bodies formed at the end of the apoptotic process via increased cross-linking to integral plasma membrane proteins, resulting from Ca^{2+}-dependent transglutaminase activity (McConkey and Orrenius, 1997). This stabilisation prevents rupture of the apoptotic bodies and spillage of intracellular material, which would lead to an inflammatory response. Recognition and phagocytosis of the apoptotic bodies is due to Ca^{2+}-dependent membrane phospholipid redistribution, with inner surface phosphatidylserine becoming transported to the outer surface of the plasma membrane (McConkey and Orrenius, 1997).

1.4.2. Apoptosis in the lens:

Although the various apoptotic pathways seem to offer a wealth of potential targets to induce cell death in lens epithelial cells, it is important to realise that apoptosis is an integral part of both the healthy and cataractous lens. As a result, the lens epithelial cells, which are still active after cataract surgery have already been exposed to a range of apoptotic stresses and possess a range of anti-apoptotic factors, which need to be taken in account when devising an apoptosis-inducing strategy.

1.4.2.1. Apoptosis exposure in the healthy lens:

Apoptosis is an integral feature of lens development and differentiation.

Apoptosis is first detectable at the stage of the lens placode, when apoptotic cells are present between the lens placode and the optic vesicle and at the junction between the lens placode and the surface ectoderm (Mohamed and Amemiya, 2003). After invagination, apoptosis is again essential for the regression of the lens stalk, which bridges the lens vesicle to the surface ectoderm. Failure of the cells of the lens stalk to undergo apoptosis after vesicle closure leads to a permanent connection between the lens and the cornea, resulting in microphthalmia in mouse models and Peters anomaly in humans (Ozeki *et al.*, 2001).

The stage of lens development which has been most compared to apoptosis, however, is the later-occurring secondary fibre differentiation. Organelle loss during secondary lens fibre differentiation involves the expression and activity of proteins commonly associated with apoptosis, such as the early expression of the *c-fos*, *c-jun* and *c-myc* genes and the requirement of p53 expression for denucleation (Zelenka *et al.*, 1997). Nuclear degradation is also associated with decreased Bcl-2 expression and increased Bcl-x cleavage, which suggests a promotion of apoptosis, and can be greatly reduced in lens cells populations by caspase inhibition, with cathepsin D activity being responsible for the remaining degenerating nuclei (Wride, 2000).

Caspase activity is also involved in α-spectrin and β-spectrin cleavage during lens fibre maturation from the cortex to the nucleus. Spectrin cleavage by caspase 3 results in the localised degradation of the plasma membrane-spectrin-actin network complex, allowing membrane blebbing and, eventually, facilitating the formation of the extensive membrane protrusions and fusions present in the lens nucleus (Lee *et al.*, 2001).

Caspase 3 is also involved in the connexin 50 cleavage observed in the nucleus, which allows Gap junctions to function independently of pH (Yin *et al.*, 2001).

Lastly, apoptosis might also be a feature in all ageing lenses, as xanthurenic acid, a product of tryptophan degradation which accumulates in the lens with age, correlates with intracellular Ca^{2+} increase, cytochrome c release, caspase 3 activation and nuclear and plasma membrane degradation (Malina *et al.*, 2002).

1.4.2.2. Apoptosis exposure during cataract:

Induction of cataract has often been associated with lens epithelial cell apoptosis in both humans and animal models (Li *et al.*, 1995). TGF-β-induced cataract, which models sub-capsular human cataract, presents a high number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) - positive lens epithelial cells, especially in the region of the multilayered plaque

adjacent to the lens fibres (Maruno *et al.*, 2002). Rat models of diabetic cataract also show an increase in TUNEL-positive lens epithelial cells with galactose overfeeding, which is correlated with aldose reductase and subsequent p53 expression (Takamura *et al.*, 2003). UV-B exposure correlates with increased and sustained c-fos expression in both epithelial and fibre cells and DNA degradation comparable to an apoptotic DNA ladder (Li and Spector, 1996). The direct use of H_2O_2 to induce cataract is also associated with c-fos expression and DNA fragmentation (Li *et al.*, 1995) and with PARP cleavage, chromatin condensation and nuclear membrane disappearance in selenite-induced cataract (Tamada *et al.*, 2000).

Apoptosis of lens epithelial cells is also a feature of some forms of human cataract. While no or very few apoptotic cells can be detected in the epithelium of normal adult lenses, they are present in capsulorhexes from cortical and sub-capsular cataracts (Li *et al.*, 1995). Lens epithelial cells from anterior polar cataract have decreased Bcl-2 expression (Lee *et al.*, 2002), but detectable levels of Fas and caspase 3 (Majima *et al.*, 2003). Lens epithelial cells from cataract associated with diabetic retinopathy also exhibit increased Fas-induced apoptosis (Okamura *et al.*, 2002). This tendency, however, may not be a predominant feature of human cataract as the lens epithelium in senile cataracts (Okamura *et al.*, 2002) and posterior subcapsular cataracts (Andersson *et al.*, 2003) shows only reduced levels of apoptosis and conflicting results have been presented about the existence of apoptosis in nuclear cataracts (Li *et al.*, 1995; Lee *et al.*, 2002).

1.4.2.3. Anti-apoptotic factors in the lens:

Exposure of the lens epithelial cells to apoptotic mechanisms frequently occurs during the normal life of the organism, in the form of organelle degradation during fibre differentiation (detailed in section 1.1.3), oxidation following decrease in UV filter efficiency (detailed in section 1.2.2), and during cataract (detailed in section 1.4.2.2). As a result, the lens epithelial cells exhibit a large number of anti-apoptotic pathways.

1.4.2.3.1. Anti-oxidants:

Exposure of lens epithelial cells to oxidative stress induces the expression of a wide number of anti-apoptotic genes, such as Fanconi Anaemia (FANC)-A, FANCG and breast cancer-associated (BRAC) 2, which are involved in DNA repair, Oxysterol binding protein (OSB) - related protein (ORP) 150, which is a caspase 3 inhibitor, and α–1-anti-trypsin, an anti-apoptotic proteinase inhibitor (Goswami *et al.*, 2003).

But the lens epithelial cells also express a number of proteins directly involved in the regulation of the oxidative pathways. Superoxide dismutase (SOD), which neutralises the O_2^- anion into H_2O_2, is present under two forms in the lens, the cytosolic SOD1 and the mitochondrial SOD2. Independently down-regulating the expression of both forms *in vitro* had shown that both promote survival in lens

epithelial cells, with SOD2 down-regulation allowing increased cytochrome c leakage into the cytoplasm and subsequent caspase 3 activation (Reddy *et al.*, 2004). In the young lens epithelium, H_2O_2 is degraded by the glutathione redox cycle and catalase, with GSH also involved in reducing lens proteins damage from interaction with sugar compounds. With age, however, GSH production decreases, due to impaired cysteine transport into the lens epithelial cells, and the lens oxidative stress increases (Reddan *et al.*, 1999). Other proteins responsible for lens protection are the endogenous anti-oxidant protein (AOP) -2, which has been suggested to bind and sequester both intracellular and extracellular reactive oxygen species (ROS) and H_2O_2, thus preventing oxidative damage, while exogenous AOP2 promotes cell survival in diabetic cataract models, which involve both osmotic and oxidative stresses (Kubo *et al.*, 2004).

1.4.2.3.2. Growth factors:

Lens epithelial cells express a large number of cell-surface receptors for growth factors such as epidermal growth factor (EGF), FGF, IGF-I, PDGF and, in the chicken lens, HGF and VEGF (Zelenka *et al.*, 1997). Although most of these growth factors are involved in proliferation and differentiation of lens cells, some of them are also involved in survival. Insulin presence is necessary for early lens development *in vitro*, where it prevents apoptosis by down-regulating c-fos and c-jun expression (Rampalli and Zelenka, 1995). Apoptosis prevention can also be mediated via activation of the IGF-1 receptor (IGF-IR) pathway. Ligand binding and

phosphorylation of IGF-IR allows binding and phosphorylation of insulin receptor substrate (IRS) -1, which activates phospho-inositide 3 kinase (PI3K), resulting in increased phospho-inositide phosphate 3 (PIP3) levels. PIP3 then binds and activates Akt/PKB, which directly inactivates the pro-apoptotic Bad and caspase 9 and indirectly down-regulates Fas ligand expression, while increasing Bcl-2 and Bcl-x expression. PIP3 also activates phospho-inositide-dependent kinase (PDK) -1, which contributes to Akt/PKB, as well as PKA and PKC, activation (Vincent and Feldman, 2002). Type I PKA has been suggested to inactivate Bad, while activation of the classical PKC-α and PKC-β and the atypical PKC-ι and PKC-ζ are correlated with increased cell survival (Cross *et al.*, 2000).

Another important anti-apoptotic growth factor is lens epithelium-derived growth factor (LEDGF), which is produced, secreted and re-internalised by the lens epithelial cells (Ayaki *et al.*, 1999). LEDGF is required for the survival of lens epithelial cells *in vitro* in the absence of serum (Singh *et al.*, 2000), and the presence of anti-LEDGF antibodies is correlated with lens epithelial cell death *in vitro* (Singh *et al.*, 2000) and with Vogt-Koyanagi-Harada disease and sympathetic ophthalmia *in vivo* (Yamada *et al.*, 2001). LEDGF expression is activated in the presence of both thermal and oxidative stress (Sharma *et al.*, 2000) and correlates with increased resistance to both stresses *in vitro* (Singh *et al.*, 1999). LEDGF mediates its effects by binding to the heat shock element (HSE) and stress related regulatory element (STRE) within the AOP2, HSP27 and αB-crystallin promoters, thus inducing their expression (Fatma *et al.*, 2001; Singh *et al.*, 2001).

1.4.2.3.3. Heat shock proteins:

Heat shock proteins are heavily involved in lens epithelial cell survival.

HSP70, the expression of which is up-regulated in lens epithelial cells with H_2O_2-induced (Goswami *et al.*, 2003) and selenite-induced (Belusko *et al.*, 2003) oxidative stress, directly binds and inactivates Apaf-1, pro-caspase 3 and JNK1 in an ATP-dependent manner (Garrido *et al.*, 2001). Together with its co-chaperone BAG, HSP70 can also activate Bcl-2 (Garrido *et al.*, 2001) and sequester FADD, thus blocking both Fas-mediated apoptosis and anoikis (Sreedhar and Csermely, 2004).

HSP27, the activation of which is mediated by LEDGF after oxidative or thermal stress (see 1.4.2.3.2), prevents Bid translocation to the mitochondrial membrane and its subsequent involvement in cytochrome c release, as well as directly binding cytosolic cytochrome c and pro-caspase 3 (Paul *et al.*, 2002; Samali *et al.*, 2001). MAPKAP kinase 2 phosphorylation of HSP27 dimers also allows HSP27 binding to Daxx, which blocks the Fas-mediated JNK kinase apoptotic pathway (Sreedhar and Csermely, 2004). HSP27 also inhibits Smac/DIABLO release (Sreedhar and Csermely, 2004), thus increasing the anti-apoptotic effects of IAPs.

αA-crystallin and αB-crystallin, which are highly expressed in the lens, confer resistance to Fas ligand and TNF-α induced apoptosis (Andley *et al.*, 2000), as well as UVA-induced apoptosis (Liu *et al.*, 2004). Both α-crystallins can bind and

sequester Bax and the short, pro-apoptotic form of Bcl-x, which interferes with mitochondrial PTP formation (Mao *et al.*, 2004). Protection against UVA, however, involves distinct mechanisms for each crystallin, with αA-crystallin-induced protection being correlated with Akt/PKB activation and αB-crystallin-induced protection involving decreased Raf-1 activity and subsequent MEK1/2 and ERK1/2 inhibition (Liu *et al.*, 2004). αB-crystallin has also been shown to bind pro-caspase 3, preventing its maturation (Kamradt *et al.*, 2002; Kamradt *et al.*, 2001).

1.4.3. Apoptosis induction in after-cataract research:

Against such an impressive number of anti-apoptotic pathways available to the lens epithelial cells, an equally wide range of apoptotic agents has been tested. Most of them, however, are generic and could present a risk to other cell populations in the eye, especially the endothelial corneal cells and the RPE cells.

1.4.3.1. Oxidative stress:

Experimental use of oxidative stress can be mediated by application of UV-B, which induces H_2O_2 production via the degradation of aqueous ascorbate (Goswani *et al.*, 2003), and results in p53-dependent apoptosis in rat lens epithelial cells *in vivo* (Michael *et al.*, 1998; Sun *et al.*, 2001). Direct exposure to H_2O_2 *in vitro* leads to apoptosis via DNA degradation and activation of PARP, which leads to NAD

consumption, eventual exhaustion of ATP and failure of DNA repair (Spector *et al.*, 2002). General caspase activation is also detectable in H_2O_2-induced apoptosis, with the notable exception of caspase 9 (Choudhary *et al.*,2002), suggesting that the mitochondrial pathway of apoptosis is not involved. Lipid oxidation, a consequence of H_2O_2 exposure, is sufficient to induce apoptosis and caspase 3 activation *in vitro* (Choudhary *et al.*, 2002), and synthetic lipid peroxides such as tertiary butyl hydroperoxide induce apoptosis levels comparable to H_2O_2 *in vitro* (Spector *et al.*, 2002).

The use of oxidative stress, though efficient at promoting apoptosis in lens epithelial cells, is nevertheless non-specific and the potential induction of apoptosis in non-lens cell populations of the eye do not make it a suitable tool for treating PCO in humans.

1.4.3.2. Direct activation of the apoptotic pathways:

The direct activation of members of the apoptotic pathways has also been used to induce lens epithelial cell death.

Direct exposure to Fas ligand or Fas-activating antibody *in vitro* successfully induces apoptosis (Nishi *et al.*, 2001; Hueber *et al.*, 2003), while IFN-γ treatment fails to activate caspase-3 and results in only a limited decrease in viability, even though capsase 8 is activated and Bcl-2 expression decreased (Awasthi and Wagner, 2004).

Anoikis can significantly reduce cell proliferation when mediated by ethylene diamine tetra acetate (EDTA) in *in vivo* rabbit models for up to three months and with only short–term postoperative complications (Inan *et al.*, 2001). The disintegrin salmosin, which is much more effective at inhibiting cell attachment than soluble RGD peptide, also selectively inhibits lens epithelial cells proliferation on both fibronectin and laminin-based matrices *in vitro* (Kim *et al.*, 2002), which is particularly relevant as the expression of both ECM components is known to be up-regulated in lens epithelial cells in after-cataract (Zhang *et al.*, 2001).

Again, as effective as these methods may be, they are still too non-specific for safe use in humans.

1.4.3.3. Lens-specific apoptogens:

In order to reduce the risk of apoptotic induction to other cell populations in the eye, a small number of lens-specific apoptogens have been developed.

Ricin is a potent non-specific inhibitor of protein synthesis consisting of two chains, the active chain A and the cell-binding chain B (Meacock *et al.*, 2000). The association of the chain A to a lens epithelial cell monoclonal antibody allows specific selection and, after proving its efficiency at inhibiting human lens epithelial cell proliferation *in vitro* (Tarsio *et al.*, 1997), the immunotoxin conjugate has been tested in human patients *in vivo*, where it was associated with a significant decrease in

cell proliferation onto the IOL up to a year after surgery (Meacock *et al.*, 2000) and in the need for secondary posterior capsulotomy for up to three years after surgery (Clark *et al.*, 1998). Both studies, however, found a tendency for increased post-operative inflammation. Also, despite a low dispersion rate in the eye, the immunotoxin still displays low-affinity binding to corneal endothelial cells and RPE cells (Meacock *et al.*, 2000).

Another example of such a conjugate is the polylysine-saporin complex, with the polylysine binding specifically to heparan sulphate chains on the lens capsule, allowing cellular take-up of the toxic saporin moiety (Bretton *et al.*, 1999; David *et al.*, 1992).

1.4.3.4. Alternatives to apoptosis induction:

Specific apoptosis induction in lens epithelial cells *in vivo* has proved difficult to achieve and a new school of thought has emerged that the complete elimination of the lens epithelial cells might not be necessary, or even desirable, to prevent PCO. Indeed, as long as the proliferating cells do not invade the visual axis and its surrounding area, no significant sight loss occurs. Also, in the absence of lens epithelial cells, the lens capsule wouldn't be maintained and might weaken, facilitating capsule rupture and IOL displacement. Therefore, some studies have focused on the inhibition of proliferation and/or migration of lens epithelial cells on the lens capsule, without inducing their apoptosis.

Disrupting collagen secretion, with the lysyl hydrolase inhibitor minoxidil (Saika and Ohnishi, 2001), or FGF-binding heparin secretion, with suramin (Rieck *et al.*, 2004) result in decreased proliferation and migration, without any apoptotic effects. The MMP inhibitor Ilomastat also prevents lens epithelial migration (Wong *et al.*, 2004), which, as will be shown in Chapter 4 of this study, can be related to growth factors levels. The efficiency of these drugs reflects the importance of growth factors and cell-matrix interactions in the invasive behaviour of lens epithelial cells on the lens capsule.

1.5. Summary:

In this introduction, I have shown how current research into after-cataract prevention is hindered by the high resistance of lens epithelial cells to apoptosis (see sections 1.4.2) and the low specificity of the apoptogens used (see section 1.4.3). In this study, I have aimed to identify lens-specific agents of lens epithelial cell viability. In the long-term, such agents could be disabled to decrease lens-specific proliferation, migration or apoptosis resistance, thus slowing down the progression of PCO and delaying the need for posterior capsulotomy. To this end, I set up an *in vitro* model whereby lens epithelial cells were cultured onto their natural basement membrane *in vivo* (detailed in section 3.1), the lens capsule.

In Chapter 2, I examined the influence of the presence of the lens capsule on the viability of lens epithelial cells *in vitro*, both in the absence and the presence of

apoptogen. To determine whether any influence of the capsule was lens-specific I also examined whether the presence of the lens capsule affected the viability of non-lens cell lines.

I have also stressed the importance of crystallins (see sections 1.2.3, 1.1.4, 1.1.5 and 1.4.2) and growth factors (see sections 1.1.2, 1.1.3, 1.3.2 and 1.4.2) in the development, functions and pathologies of the lens. Therefore, I then focused my attention on the potential involvement of crystallins and growth factors in the mediation of the capsule's effect on lens epithelial cell viability. In Chapter 3, I examined the expression of the intra-cellular anti-apoptotic factors αA-crystallin, αB-crystallin and HSP27, to test whether it correlated with cell viability on the lens capsule. In Chapter 4, I examined the medium levels of FGF-2 and IGF-1, also to test whether they correlated with increased cell viability on the lens capsule. I also identified MMP-2 as a soluble factor able to lead to the release of FGF-2 and IGF-1 from the lens capsule, with consequences on lens epithelial cell viability.

2. The lens capsule maintains lens cell viability:

To investigate the role of the lens capsule in lens cell viability, it is first necessary to be familiar with the nature of the lens capsule itself.

2.1. Introduction: Structure and function of the lens capsule:

The lens originates from the invagination of the ectoderm at the lens placode (detailed in 1.1.1), and it is the basal membrane of the ectoderm cells that constitutes the original lens capsule.

The thickening of the lens capsule is ensured by the continuous deposition of ECM by the lens epithelial cells, and as a result the anterior lens capsule, which retains lens epithelial cells throughout life, increases in thickness and strength with age (Krag and Andreassen, 2003). In contrast, the posterior capsule, whose lens epithelial cells elongate into primary fibre cells to form the lens nucleus (Faber *et al.*, 2002), doesn't significantly increase in thickness with age (Krag and Andreassen, 2003) and is, essentially, unmodified from its prenatal form.

The main structural components of the lens capsule are characteristic of those found in other basement membranes and each performs specific functions.

2.1.1. Proteoglycans and lens development:

Proteoglycans are composed of a protein core to which are attached a variable number of one or several types of glycosaminoglycans (GAG). A GAG is a long, linear chain of sulphated and anionic disaccharide repeats that make the GAG hydrophilic and therefore voluminous. The main types of GAGs are hyaluronan, chondroitin sulphate and dermatan sulphate, heparan sulphate and keratan sulphate. They attach to the serines of the protein core via a link tetrasaccharide. Some proteoglycans are secreted into the ECM, while others are retained at the cell's surface (Alberts *et al.*, 2002).

In the lens, chondroitin sulphate, dermatan sulphate and several heparan sulphate proteoglycans are synthesised by the lens epithelial cells and deposited in the lens capsule (Winkler *et al.*, 2001; Goes *et al.*, 1999; Mohan and Spiro, 1991). The size and distribution of these proteoglycans vary between anterior and posterior capsules, with the aqueous side of the anterior lens capsule being characterised by larger proteoglycan complexes (Landemore *et al.*, 1999). As the hydrophilic character of GAGs favours gel formation and increased resistance to pressure (Alberts *et al.*, 2002), these larger outer anterior structures might be involved in dealing with the stress endured by the anterior capsule during accommodation.

However, chondroitin sulphate and heparan sulphate have mainly been linked with developmental processes. Chondroitin sulphate expression, in both basal and apical laminae in the chick lens placode, is necessary for lens vesicle formation and

closure and has been suggested to act via Ca^{2+}-mediated microfilament contraction at the cells' apical end and via H_2O influx-mediated intercellular pressure at the cells' basal end (Gato *et al.*, 2001). Heparan sulphate proteoglycans are known ligands, stabilisers and co-receptors (Delehedde *et al.*, 1996; Zhang *et al.*, 2001) of FGF-2, a growth factor essential to lens fibre differentiation (see section 1.1.2). Accordingly, targeted deletion of GAG-binding serines from the *Hspg2* gene, whose product, perlecan, is a FGF-2 binding heparan sulphate proteoglycan (HSPG) (Knox *et al.*, 2002), result in progressive lens fibre degeneration in mouse lenses (Rossi *et al.*, 2003). However, as perlecan also binds laminin and collagen IV via its heparan sulphate chains, the disruption of perlecan weakens the lens capsule and it is not clear whether the degeneration of fibres occurs independently or because of an increased posterior capsule permeability. A very similar phenotype was obtained in the secreted protein, acidic and rich in cysteine (Sparc) -null mouse lens (Yan *et al.*, 2002), which, as in the GAG-less perlecan mice lens, is characterised by post natal fibre swelling and degeneration, posterior capsule rupture and filopodia-like extensions from the lens cells into the capsule. These similarities suggest that both proteins be involved in a common pathway, with the GAG-less perlecan mouse phenotype also highlighting the important role of heparan sulphate chains in cell adhesion.

With age, the amount of proteoglycans in the lens capsule decreases (Winkler *et al.*, 2001), with deleterious effects on the capsule's strength and FGF binding ability. After-cataract does not bring any major changes in proteoglycans, which are still present in the matrix deposited on the posterior capsule and IOLs, although

keratan sulphate becomes detectable, in association with collagen fibrils (Saika *et al.*, 1998; Ishibashi *et al.*, 1995).

2.1.2. Collagen and capsule elasticity:

Collagen proteins belong to a large family, the members of which are essential to the formation of extracellular networks (Alberts *et al.*, 2002). Individual collagen proteins contain a long central helical domain of Gly-X-Y repeats, flanked by short non-collagenous N-terminal and C-terminal domains. Collagen assembles as homo- (as for collagen III) or hetero- (as for collagen I and IV) trimers, with initial trimerisation of the C-terminal non-collagenous domains of three separate collagen chains allowing alignment of the long helical domains and triple helix formation. Collagen trimers can further assemble either into fibrils, after C-terminal domain cleavage, or into meshworks, via dimerisation of the C-terminal trimers into hexamers and tetramerisation of the N-terminal domains (Krag and Andreassen, 2003; Sundaramoorthy *et al.*, 2002).

In the lens capsule, as in other basement membranes, collagen IV is predominant and assembles into a three-dimensional network (Krag and Andreassen, 2003). The outer layer of the lens capsule is the first to be laid down during development, between 10 to 11.5 days post-conception in the mouse, and is rich in the $\alpha 1$- $\alpha 1$- $\alpha 2$ trimer, and in the α 5- α 5-α 6 trimer (Kelley *et al.*, 2002). This deposition is retained throughout adulthood (Kelley *et al.*, 2002) and, thanks to the recoverable stretching of collagen IV helical regions (Fisher *et al.*, 1976), gives the

capsule elastic properties. This elasticity is needed during development to allow the lens capsule to stretch as the fibre mass volume increases and during accommodation when contraction of the ciliary fibres mediates an adjustable and recoverable reduction of the equatorial diameter and forward movement of the lens mass (Garner and Yap, 1997). To withstand the pressure exerted during accommodation, the less common and highly cross-linked α 3- α 4- α 5 collagen IV trimer is laid down later in life, from two weeks post-natal in the mouse, and increases the strength of the inner lens capsule layer (Kelley *et al.*, 2002).

Age greatly affects the lens capsule's collagen network. The original parallel lammellar deposition, though persisting in the equatorial regions, degrades into a more random arrangement, first at the posterior capsule in childhood and then at the anterior capsule in adulthood. This disorganisation reduces the capsule strength by preventing the parallel alignment of the collagen layer during deformation (Krag and Andreassen, 2003). The overall collagen content also decreases, as indicated by the hydroxyproline fraction (Peczon *et al.*, 1980), and the amount of non-enzymatic glycosylation (Cohen and Yu-Wu, 1983) and cross-linking (Krag and Andreassen, 2003) increase. The strength and elasticity of the capsule, respectively, are heavily affected by those changes and steadily decrease from 35 years onwards in human lens capsules (Krag *et al.*, 1997).

After-cataract also affects the collagen fraction of the lens capsule, but in a very different manner. The epithelial-mesenchymal transition that occurs in lens epithelial cells during after-cataract (Wormstone *et al.*, 2004) correlates with an

increase in prolyl-4-hydroxylase expression, an enzyme involved in the triple helix formation of the collagen chains, and in the synthesis of collagen I, III, V and VI (Saika *et al.*, 1998). These collagen types are not normally found in the healthy lens capsule and indicate a departure of the lens cells from the epithelial phenotype.

Other lens capsule components involved in ECM elasticity include fibrillin, a glycoprotein found in elastic fibres (Alberts *et al.*, 2002), which is present at the equatorial region of the lens capsule. At the peripheries of both anterior and posterior capsules, fibrillin fibres are arranged in a radial fashion, while a fibre meshwork encircles the equator, the point of contact between the lens and the zonular fibres (Mir *et al.*, 1998). This distribution suggests a role for fibrillin networks in the accommodative properties of the lens. With age, the zonular insertions into the capsule are displaced away from the elastic fibrillin belt at the equator towards the anterior capsule, which correlates with a decrease in accommodation (Farnsworth and Shyne, 1979).

2.1.3. Glycoproteins and cell adhesion:

The lens capsule's collagen IV network is reinforced by cross-links with a number of glycoproteins that also mediate cell-matrix adhesion.

2.1.3.1. Laminin and Fibronectin:

After collagen IV, the glycoproteins laminin, fibronectin and entactin are the most readily found components of the lens capsule (Cammarata and Spiro, 1985).

Laminin is an heterotrimer that assembles in the shape of an asymmetric cross and can bind to itself via its short arms and indirectly to type IV collagen via an entactin molecule (Alberts *et al.*, 2002). In the lens, it is present in equal amounts in the anterior and posterior capsules as a high-molecular weight, over 500kDa, unreduced form and as smaller, 200kDa and 400kDa, reduced forms. Outside the lens, laminin, but not collagen IV, fibronectin or entactin, can mediate cell adhesion via dystroglycan binding, which can itself link to the actin cytoskeleton (Ervasti and Campbell, 1993). Entactin, found in higher quantities in the anterior than in the posterior capsule, is present as a 150kDa form (Cammarata and Spiro, 1985).

Both exogenous laminin and fibronectin are promoters of lens epithelial cell adhesion and migration *in vitro* (Olivero and Furcht, 1993). *In vivo*, however, their adhesive properties are very different, as demonstrated by the inability of a lens-capsule-extracted laminin-fibronectin mix to improve lens epithelial cell adhesion (Cammarata and Spiro, 1985). Selective degradation of fibronectin is known to reveal anti-adhesive peptides and could explain these contradictory results (Fukai *et al.*, 1998), but they can also be explained by examining protein expression profiles in the lens.

Fibronectin is a large heterodimer formed by two multidomain polypeptide chains translated from splice variants of the same mRNA and linked at one end by

disulphide bonds. In the rat lens, fibronectin deposition in the capsule is detectable only until day 19 of embryonic development, after which laminin is the main glycoprotein in direct contact with the cells. Accordingly, the adhesion of rat embryonic lens epithelial cells to fibronectin is progressively lost from day 16 of embryonic development onwards, while adhesion to laminin is maintained (Parmigiani and McAvoy, 1991). It is therefore expected that adult, healthy lens epithelial cells shouldn't adhere significantly to fibronectin. With cataract and after-cataract, however, lens epithelial cells can undergo an EMT (detailed in section 1.3.2) that might affect their adhesive abilities. Fibronectin is known to be up regulated by TGF-β and is indeed found in capsular bags in after cataract and anterior polar cataract (Tanaka *et al.*, 2002; Joo *et al.*, 1999). It seems logical that an increase in fibronectin expression in these pathological conditions be accompanied by an increase in its ability to promote cell adhesion and migration.

2.1.3.2. SPARC:

Osteonectin/SPARC is a Ca^{2+}-binding secreted glycoprotein, whose expression is wide during embryonic development, but limited in adult tissues. Strictly speaking, SPARC is not a structural ECM glycoprotein, and is not found in the lens capsule itself (Yan *et al.*, 2003), although it regulates FGF-2's binding and TGFβ-1's expression in other cell systems and should therefore be important in cell proliferation. Its role in cell adhesion is less well-defined, as SPARC's binding of collagens I, II, III, IV, V, VIII and vitronectin suggest a promotion of cell adhesion,

while its induction of focal adhesion complex dissolution should reduce adhesion to the substrate (Yan and Sage, 1999).

In the adult lens, SPARC is restricted to the lens epithelium and the basal end of the secondary lens fibres (Kantorow *et al.*, 2000). The increase in SPARC expression in peripheral epithelium that is reported in human lenses (Kantorow *et al.*, 2000) and the known cell-cycle –inhibitory function of SPARC (Yan and Sage, 1999) would suggest a role for SPARC in the cell cycle exit during lens epithelial cell differentiation. However, the reverse pattern of expression of SPARC, i.e. higher in the central lens epithelium than at the bow region, in murine lens cells (Yan *et al.*, 2003) contradicts this hypothesis. The most direct evidence of SPARC function in the lens can be seen in the Sparc-null mice (Yan *et al.*, 2002; Gilmour *et al.*, 1998). Lenses of Sparc-null mice are characterised by numerous cell extensions into the lens capsule which result in cleft formation and capsule rupture. This phenotype suggests either that SPARC ensures cell adhesion and that in its absence the cells have to increase their contact area with the capsule to stay attached, or that SPARC prevents active ECM degradation by the cells and its absence results in increased cell invasion into the matrix. The lenses of Sparc-null mice are also characterised by swollen lens fibres and cataract. With human cataract, SPARC expression increases (Kantorow *et al.*, 2000) which is compatible with an increase in collagen I and TGFβ-1 expression in cataractous lens epithelial cells (Joo *et al.*, 1999; Saika *et al.*, 2001, respectively).

2.2. Aims:

In this Chapter, I wanted to investigate whether the presence of the lens capsule could maintain lens epithelial cell viability in the presence of stress, as has been described for basement membranes in other systems (Rintoul and Sethi, 2002; Judware *et al.*, 1998; Aharoni *et al.*, 1997). I also wanted to test whether any protection granted to the lens cells by the lens capsule could be extended to non-lens cell lines, i.e. whether the protection be lens-specific.

2.3. Experimental procedure:

To address the aims cited above, I established an *in vitro* model by which a number of lens and non-lens cell lines were to be cultured onto bovine lens capsules. Lens capsules were preferred to artificial matrices as lens capsules are the *in vivo* substrate of the lens epithelial cells. Cell viability was assayed using a colorimetric measurement of metabolic activity (CellTiter 96^R Aqueous Non-Radioactive Cell proliferation Assay kit (Promega, Madison, USA)) (O'Flaherty *et al.*, 2005; Tighe and Talmage, 2004), in the presence of staurosporine, a well-characterised protein kinase inhibitor (Couldwell *et al.*, 1994).

2.3.1. Bovine eye dissection:

To establish the lens capsule culture model, bovine eyes were collected from

Northern Counties Meat Group, Sunderland, and used in accordance with the 'Meat Hygiene Service regulations for the despatch of serious risk material for research purposes'. Donor cows were culled at 30 months of age. Eyes from both male and female animals of a range of breeds (including Simmental, Charolais, Friesan, Limousin and Belgian Blue) were used within each experiment. I consider that using animals of both sexes from different breeds is an advantage for my experiments, as results that are conserved across sex and breed are more likely to also be conserved in the whole population and therefore more useful for potential clinical applications.

Lenses were dissected from the eye by cutting with a major surgical blade (Swann-Morton, Sheffield, UK) through the sclera into the posterior chamber, well far back from the lens. The vitreous was removed and the eye inverted to expose the lens and the ciliary muscles. The lens was freed by gently cutting the end of the suspensory ligaments of the ciliary muscles, taking care not to touch the lens capsule itself. The lens was then put in Dulbecco's minimun eagle's medium (DMEM) (Sigma-Aldrich, Poole, UK) in a 50 ml tube overnight at 37^0C. On the next day, lenses were taken out of the culture media and cleaned from any remaining ciliary fibres with a pair of fine forceps. They were then put in a cell culture 12-well plate (1 lens/well) coated with Sylguard (Dow Corning, Wiesbaden, Germany), the anterior side of the lens facing down, as it is the anterior side of the lens capsule that was to be used as culture substrate. 12-well plates were used as, once dissected, the lens capsule almost exactly covers the whole surface of the well, thus minimising the number of cells liable to grow onto the Sylguard. Using a surgical knife, a long single cut was

made with the posterior side of the lens facing up. Pins were inserted along these cuts to push outwards the capsule, pin it down to the Sylguard and expose the fibre cell mass. The fibre cell mass was removed with fine forceps to reveal the internal side of the anterior capsule, after which cell culture medium was poured over the capsule to keep it moist.

2.3.2. Lens capsule cleaning:

As human cells were meant to be cultured onto the lens capsules, the capsules were thoroughly cleaned to get rid of all primary bovine lens cells. The dissected and pinned-down capsules were trypsinised for 40 min in trypsin-ethylene diamine tetra acetate (EDTA) (Sigma-Aldrich, Poole, UK), then washed once in 70% (v/v) ethanol for 10 min. The trypsin-EDTA was used to detach most primary cells present on the lens capsule, while the ethanol wash was expected to kill all remaining cells and turn them white, making them highly visible and allowing their scraping off the capsule with a pair of large forceps. The use of trypsin-EDTA was previously shown not to have any adverse effects on cell culture on the lens capsule (Gribbons, 2003). Three washes in phosphate-buffered saline (PBS) followed, to remove the detached primary bovine cells. The capsules were then left overnight in the third PBS wash to be used the next day as a substrate for cell culture.

To check the effectiveness of the cleaning process, a preliminary experiment was conducted with pictures of lens capsules being taken before and after the cleaning process, to visually demonstrate the removal of the primary bovine cells. Those same

capsules were then left in 10%FCS-supplemented cell culture media for 96h, which corresponds to the total duration of the viability experiments to be conducted with the human cells. The cell viability of the cleaned capsules was then determined using the CellTiter 96^{R} Aqueous Non-Radioactive Cell proliferation Assay kit (Promega, Madison, USA). The presence of viable cells immediately after cleaning was not checked as the measurement of cell viability at the 96h time point was judged more relevant to the experimental set-up. This time point was chosen to check whether, in the event of even a small number of primary cells remaining on the capsule after cleaning, these primary cells could subsequently proliferate and result, by the end of the viability experiment, in a primary cell population big enough to interfere with the quantification of human viable cells on the lens capsule. This experiment was repeated three times independently. Representative pictures of un-clean and cleaned capsules, as well as cell viability graphs at 96h are shown in Figure 2.1.

2.3.3. *Cell culture:*

A variety of human cells were cultured onto both plastic and cleaned lens capsules. The cell lines used during this study are presented in Table 2.1. All cell lines were cultured in 1ml of DMEM supplemented with at 10% (v/v) foetal calf serum (FCS) and a 1% (v/v) L-glutamine-penicillin-streptomycin mix (all from Sigma-Aldrich, Poole, UK). All cells were used between passage 8 and passage 12, with the exception of cells used during the early vs late viability experiment (see Figure 2.7), when cells of passage over 20 were used. Cells were plated in 12-well plates, either on cleaned lens capsules or on plastic, and were left at 37^{0}C for 48h.

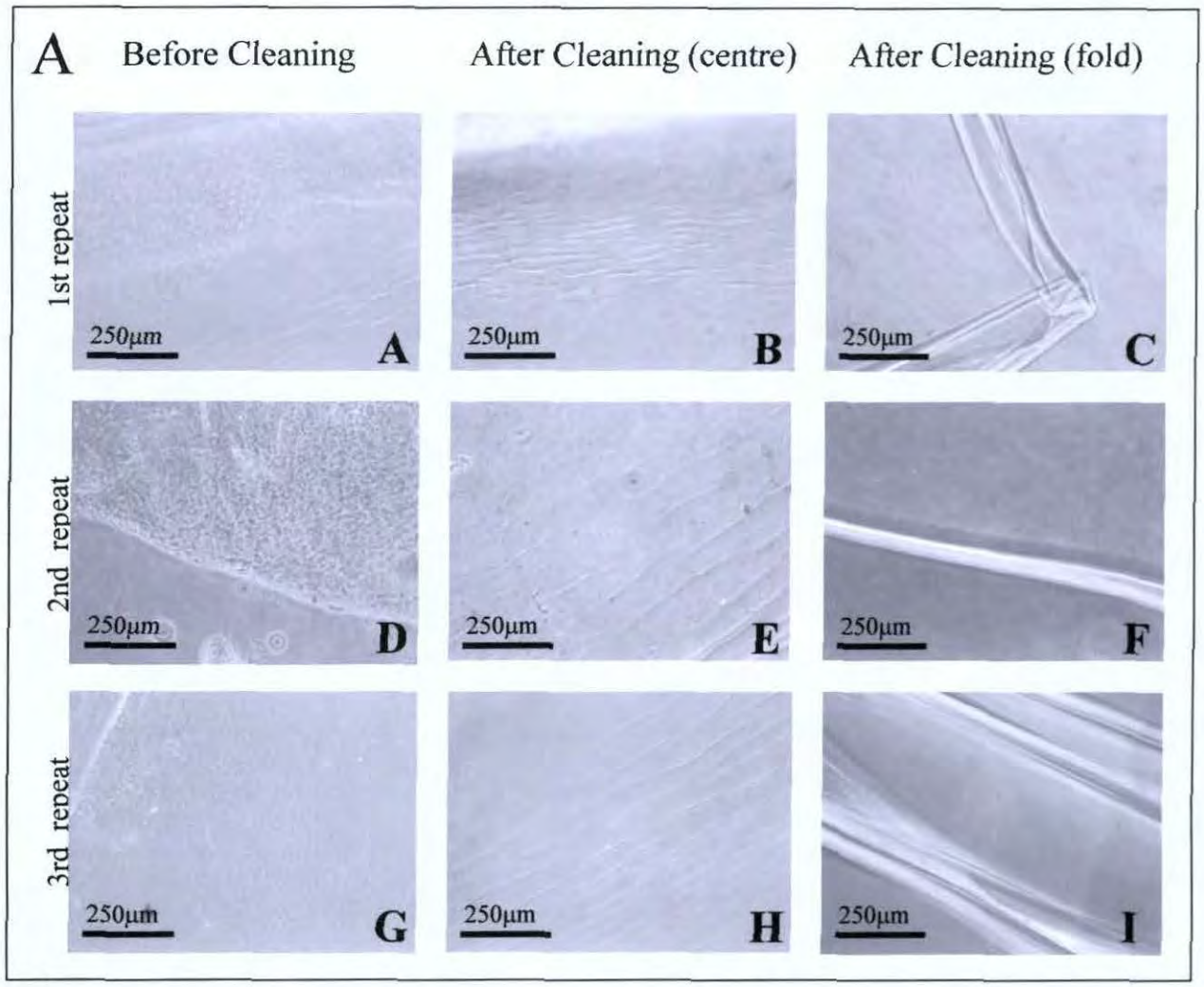
This duration was chosen to allow any potential interactions of the cells with the lens capsule to occur before proceeding to the next experimental step.

Consistency in the number of cells plated was insured by first determining the number of cells present in a fixed volume of a confluent $75cm^2$ flask following complete cell detachment by friction with sterile glass beads (Sigma-Aldrich, Poole, UK). This cell number was determined by haemacytometry. Each cell line was grown to confluence in a $75cm^2$ flask, before the cells were fully detached from the plastic substrate by friction with autoclaved glass beads and resuspended in 20ml of DMEM. Single samples of 500µl, 200µl, 100µl, 50µl and 10µl were then taken from the flask and pipetted into eppendorf tubes. 10µl from each tube were diluted 1:1 in a 0.25% Trypan Blue solution (BDH Chemicals Ltd., Poole, UK) after which they were pipetted onto the haemacytometer (Hawksley, London, UK). Viable cell (bright against a dark blue background) numbers were recorded for all nine squares and averaged. This average was then converted into viable cells number per 500µl, 200µl, 100µl, 50µl and 10µl of original sample. Standard curves of the viable cell number as a function of original sample volume were constructed and the Pearson product moment correlation coefficient (r), which indicates closeness to a linear fit (Zolman, 1993), calculated. The experiment was repeated three times independently for each cell line (see Figure 2.2).

Figure 2.1 Effectiveness of the lens capsule cleaning process.

A-Pictures of freshly dissected lens capsules before (A, D and G) and after (B, C, E, F, H and I) cleaning were taken with a Nikon Coolpix 5000 camera via a Nikon Eclipse TS100 microscope. Cleaning consisted of one 40 min trypsin-EDTA wash, one 10min 70% ethanol wash and three PBS washes. Six capsules were used for each experiment. The experiment was repeated independently for a total of three times. Representative pictures of total or partial coverage of primary bovine lens epithelial cells (A,D and G), cleaned central capsule (B, E and H) and cleaned peripheral capsular folds (C, F and I) are shown. Wrinkles and folds in the lens capsule were chosen as a focus point to confidently identify the lens capsule. Magnification 20X.

B-Cell-free plastic wells and cleaned lens capsules were left in 10%FCS-supplemented DMEM for 96h, after which cell viability was measured using the CellTiter 96^{R} Aqueous Non-Radioactive Cell proliferation Assay. Absorbances were not translated into cell numbers as no appropriate standard curve can be chosen for testing the absence of cell. Absorbance values were not significantly different ($P>0.05$ for all three repeats) between the plastic wells and the cleaned capsules. The experiment was repeated three times independently with six samples used for each treatment of each repeat. ANOVAs between independent repeats within a treatment were also conducted. Exact t-test and ANOVA P values are presented in Appendix 2.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After Cleaning (centre)
After Cleaning (fold)
1st repeat
2nd repeat
3rd repeat
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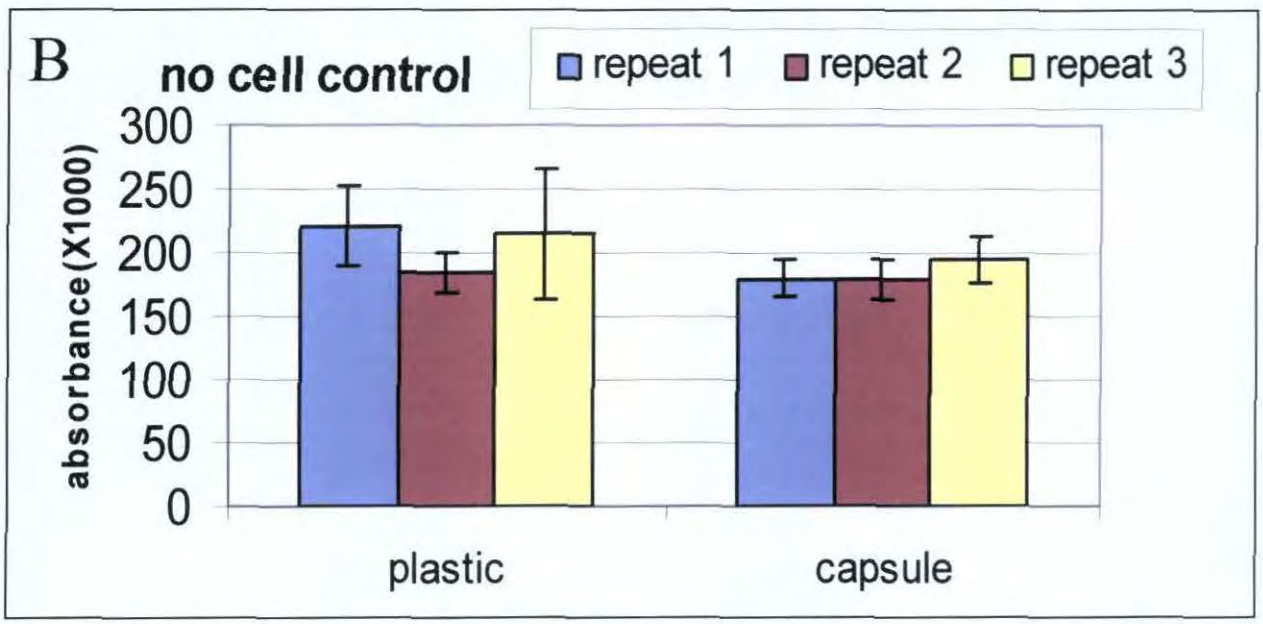
A
B
C
D
E
F
G
H
I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200
150
100
50
0
plastic
capsule

Name	Type	Origin	Reference
H36LE2	Human, wild-type lens epithelial cell line	A.R. Prescott, Dundee University	Reddan *et al.*, 1999.
9MaB	Human, αB-crystallin 450Δ, lens epithelial cell line	A.R. Prescott, Dundee University	Berry *et al.*, 2002
U373	Human astrocytoma cell line	HTB-17 (ATCC)	De Ridder *et al.*, 1987
MCF7	Human breast epithelial carcinoma cell line	HTB-22 (ATCC)	Soule *et al.*, 1973.
SW13 vim+	Human adreno carcinoma cell line, stably transfected with mouse *vimentin*-expressing plasmid	Derived from SW13 vim-	Sarria *et al.*, 1992
SW13 vim-	Human adreno carcinoma cell line, lacking expression of all intermediate filaments	CCL 105 (ATCC)	Leibovitz *et al.*, 1973.

Table 2.1: List of all cell lines used during this study.

Figure 2.2 Quantification of cell numbers in confluent cultures.

All six cell lines used in this study (H36LE2, 9MaB, U373-MG, MCF7, SW13vim+ and SW13vim-) were cultured in 10%FCS-supplemented DMEM in 75cm^2 plastic flask to confluence. Cells were then detached by friction with autoclaved glass beads and resuspended in 20ml of 10%FCS-supplemented DMEM. Single samples of 10µl, 50µl ,100µl, 200µl and 500µl were taken from the 75cm^2 flask and pipetted into eppendorf tubes. 10µl of each sample was then diluted 1:1 with trypan blue (0.25% (w/v) in PBS) and pipetted onto an haemacytometer. Viable cells (bright against a dark blue background) were counted in all nine squares of the haemacytometer. Viable cell numbers for each square were averaged and converted into viable cell numbers per sample. To do so, the average viable cell number per square was multiplied by two to account for the trypan blue dilution, divided by 0.0125, which is the volume in µl of one square of the haemacytometer and multiplied by the sample volume in µl. Viable cell numbers were then plotted against the sample volume and the Pearson product moment correlation coefficient, r, was calculated as an indication of the goodness of the linear fit. Standard deviations are not indicated on the graph to preserve clarity. This experiment was repeated three times independently. ANOVAs between independent repeats at each sample volume were calculated to give an indication of the reproducibility of the results. Exact r and ANOVA P values are presented in Appendix 2.

The viable cell number of 5.10^4 was then chosen as the fixed number of viable cells to be plated for all viability assays involving the lens capsule. Corresponding volumes to be pipetted from a confluent donor flask were calculated for each cell line and are presented in Table 2.2. The number of 5.10^4 cells was chosen at it is low enough to prevent the cells from reaching confluence before the end of the experiment. It is within the recommended cell density range for the cell viability used in this study (Promega technical bulletin No. 169). It is also within the range of cell density used by others for cell lines relevant to my study (human lens epithelial cells: Marcantonio and Reddan, 2004; Liu *et al.*, 2004; MCF7 cells: O'Flaherty *et al.*, 2005; U373-MG cells: Zupanska *et al.*, 2005; Dunlop *et al.*, 1999; SW13 cells: Macioce *et al.*, 1999).

2.3.4. Staurosporine exposure:

After being left for 48h at 37^0C, half the cells grown on plastic and lens capsules were exposed to 500nM of staurosporine for a further 48h. Staurosporine was chosen as it has been shown to promote apoptosis in *in vitro* cultured cells relevant to my study (lens epithelial cells: Gribbon, 2003; Ishizaki *et al.*, 1993; MCF7 cells: Vasaturo *et al.*, 2005; U373-MG: Begemann *et al.*, 1996). Staurosporine induces apoptosis via the mitochondrial pathway and subsequent caspase-3 activation (Zhang *et al.*, 2004; Giuliano *et al.*, 2004; Xiao *et al.*, 2004). The concentration used was chosen after conducting experiments on the dose-dependent effect of staurosporine on the viability of the cell lines used in this study when cultured on

plastic. These experiments, which were repeated three times independently for each cell line, are presented in Figure 2.3. The duration of exposure, which is comparable to other studies using staurosporine (Zhang et al., 2004; Xiao et al., 2004), was chosen to constitute a robust test of the protective ability of the lens capsule.

2.3.5. *Cell viability assay:*

Although staurosporine is a known apoptogenic substance, I chose to study cell viability and this for two reasons. Firstly, the effects of staurosporine on apoptosis induction on the lens capsule have already been characterised by a previous member of my lab using TUNEL labelling (Gribbon, 2003). Overall cell viability, however, depends on the balance between cell death and cell proliferation in the cell population, and had not been addressed in this previous study. Secondly, PCO is the result of the abnormal behaviour of the viable cells remaining in the capsular bag after surgery, irrespectively of how many other cells might have been removed during surgery. I was therefore more interested in observing, and eventually reducing, cell viability on the lens capsule, as it is cell viability, rather than cell death, that leads to PCO development.

Cell viability was assessed using the CellTiter 96^{R} Aqueous Non-Radioactive Cell proliferation Assay kit (Promega, Madison, USA)(Roehm, 1991; Scudiero *et al.*, 1988; Mosmann *et al.*, 1983). This assay relies on the reduction by mitochondrial dehydrogenase enzymes from viable cells of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetratzolium (MTS) into formazan,

which is a compound soluble in cell culture medium and which absorbance can be recorded at 490nm (Promega technical bulletin No 169). This assay was chosen because it is quick, easy to use, and allows for a high number of samples to be processed at the same time.

After 48h of exposure of the cells to staurosporine, the cell culture medium was changed and a 100µl of luminometric reagent from the CellTiter 96^{R} Aqueous Non-Radioactive Cell proliferation assay per 0.5ml of new media was added to each well. This change of medium was done to insure that the volume of cell culture medium was known and similar for each well. After 90min exposure to the reagent, 100µl of cell culture medium was taken from each well and individually transferred to a 96 well-plate, after which the absorbance value at 490nm for each sample was quantified using an anthos lucy I microplate luminometer (Anthos Labtec Instruments, Salzburg, Austria). Absorbances were then converted into live cells' numbers using a cell-specific standard curve.

At the very start of my study, I also tested the discarded cell culture medium for evidence of cell viability, which would have resulted from the presence of viable detached cells in the cell culture medium. After 90 min exposure to the luminometric reagent, no colour change was recorded, indicative of the absence of viable cells in the cell culture medium. This initial result was in accordance with results from others that cell detachement from the culture substrate leads to cell death rather than allows the maintenance of cell viability (Hamada and Utiyama, 2005; Fouquet et al., 2004;

	Volume used in µl for 5.10^4 cells	Corresponding cell number with confidence interval
H36LE2	100	50725 ± 3019
9MaB	100	48890 ± 2871
U373-MG	200	49555 ± 2528
MCF7	100	49555 ± 4547
SW13vim+	20	51987 ± 2604
SW13vim-	30	50953 ± 5216

Table 2.2 Volumes to be taken for 50000 viable cells.

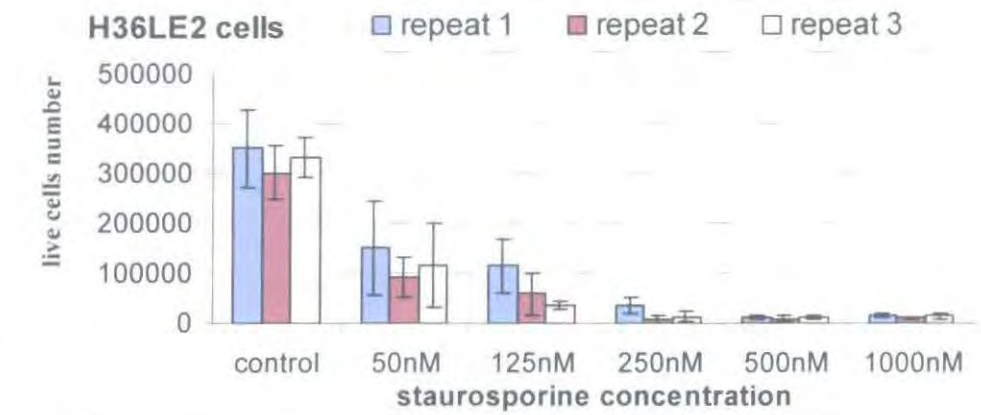
The volumes of resuspended detached cells to be used for cell seeding at the beginning of the cell viability assays were chosen based on the haemacytometry results presented in Figure 2.2. For each independent repeat presented in Figure 2.2, the FORECAST function (Microsoft Excel software) was used to determine the viable cell number corresponding to a given volume. When a volume was found that gave a corresponding viable cell number close to 5.10^4 for all three repeats, the corresponding viable cell numbers were averaged and a confidence interval (significance level=0.05, sample size=3) was calculated.

Figure 2.3 Determination of staurosporine concentration to be used in cell viability assays.

For each cell line used in this study (H36LE2, 9MaB, MCF7 and U373-MG) ,5.10^5 cells were seeded in each well of three 12-well plates in 10%FCS-supplemented DMEM.

After 48h, each six wells received either 0nM, 50mM, 125nM, 250nM, 500nM or 1μM of staurosporine. After a further 48h, colorimetric reagent from the CellTiter 96^R Aqueous Non-Radioactive Cell proliferation Assay kit was added. After 90min exposure to the reagent, viable cell numbers were calculated by measuring each well's absorbance at 490nm and converting it into viable cell numbers using the standard curves obtained in Table 2.3.

The viable cell numbers within a treatment were averaged and plotted against staurosporine concentration, with standard deviation. The significance in average viable cell number decrease with staurosporine addition was determined by two tailed independent t-test. This experiment was conducted independently three times. ANOVAs between independent repeats within a treatment were also conducted. Exact t-test and ANOVA P values are presented in Appendix 2.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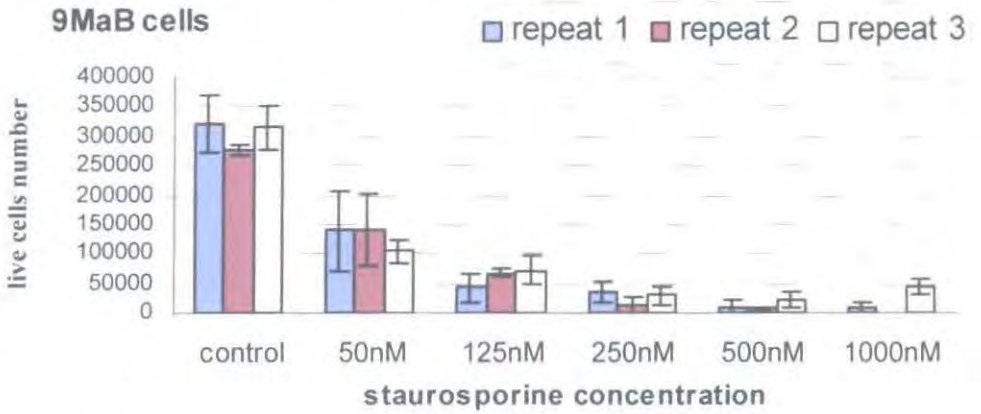
staurosporine concentration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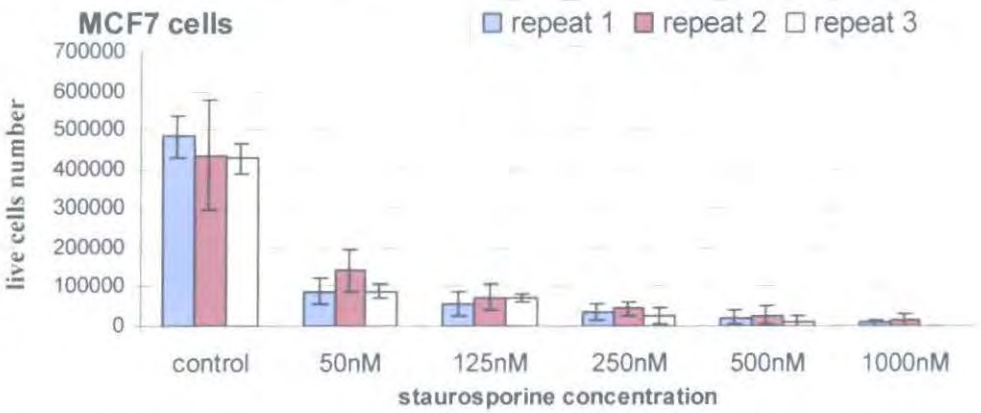
staurosporine concentration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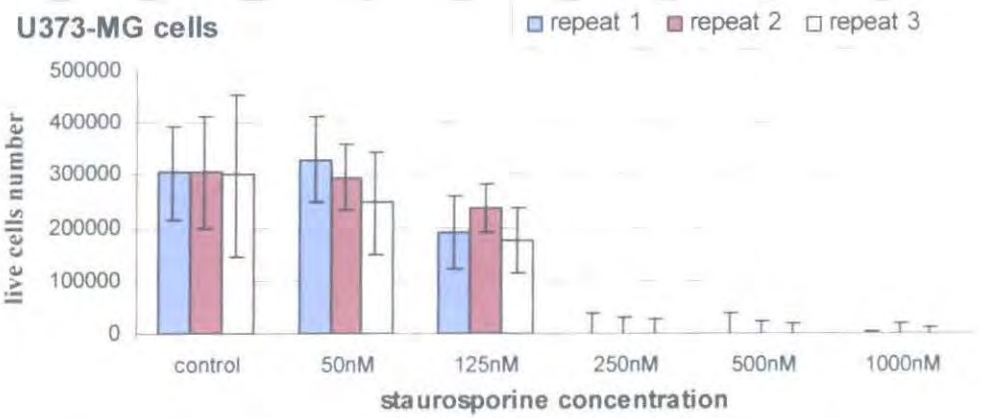
1000nM
staurosporine concentration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repeat 3
live cells number
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400000
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200000
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0
control
50nM
125nM
250nM
500nM
1000nM
staurosporine concentration

Marco et al., 2003; Findlay et al., 2002). Therefore, for the rest of my study, the investigation of cell viability in the cell culture medium alone was not conducted.

Standard curves were constructed in advance for each cell line. A range of 10^4 to 10^6 cells, chosen to encompass the starting cell number while allowing for potential increase or decrease in cell number during the viability assay, were plated and left for 2h to allow re-attachment. Six wells of a 12-well plate were used for each density. The luminometric reagent from the CellTiter 96^R Aqueous Non-Radioactive Cell proliferation Assay kit (Promega, Madison, USA) was then added and, after 90 min, the corresponding absorbance at 490nm was read. This experiment was repeated independently three times for each cell line, with the results being presented in Figure 2.4. The absorbance values obtained at each cell number were averaged over the three independent repeats for each cell line, with the mean absorbance values being used as standards for the subsequent cell viability assays (see Table 2.3)

2.3.6. Special considerations for co-culture:

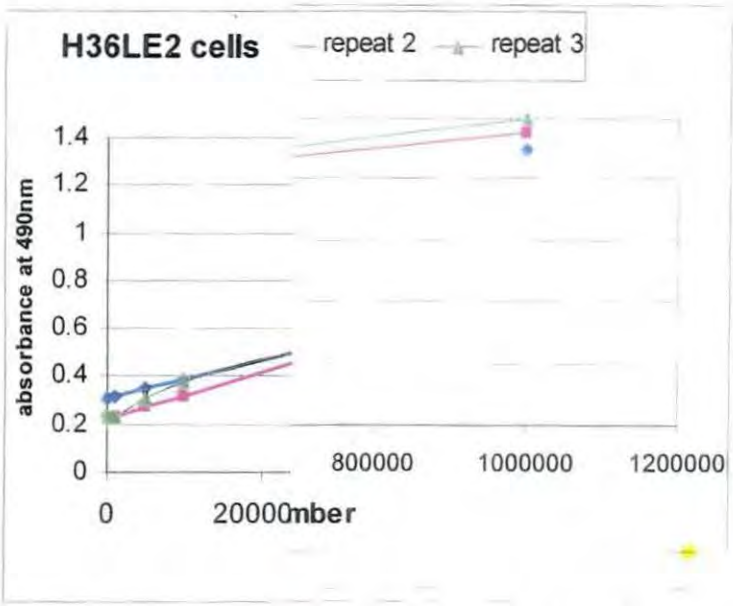
Following a first series of cell viability experiments, non-lens cells were cultured in co-cultured inserts with either cell-free plastic or cell-free lens capsule at the bottom of the well. This was to test the hypothesis that the decreased viability of the non-lens cells when cultured directly onto the bovine lens capsule (see Figure 2.5) was due to incompatible attachment factors. MCF7 and U373-MG cells were grown on a Millicell^R culture plate insert (Millipore, Molsheim, France; pore size: 12μm), with either cell-free plastic or a cell-free lens capsule at the bottom of the culture plate

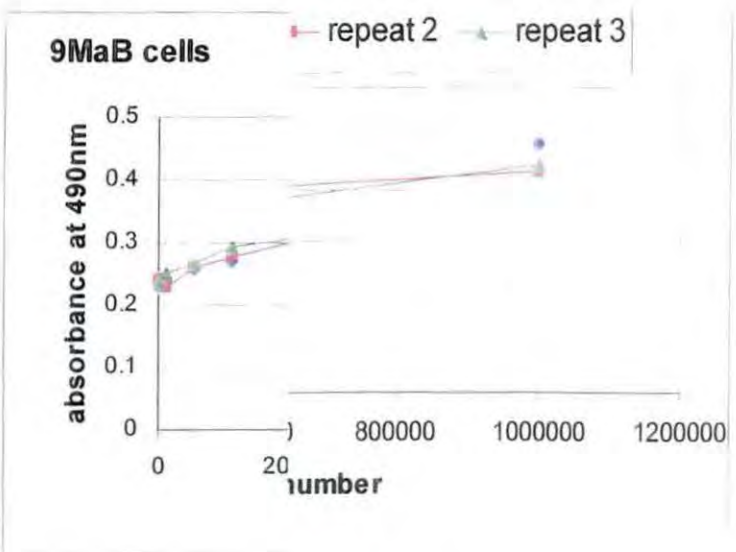
Figure 2.4 Standard curves for conversion of absorbance values into viable cell numbers.

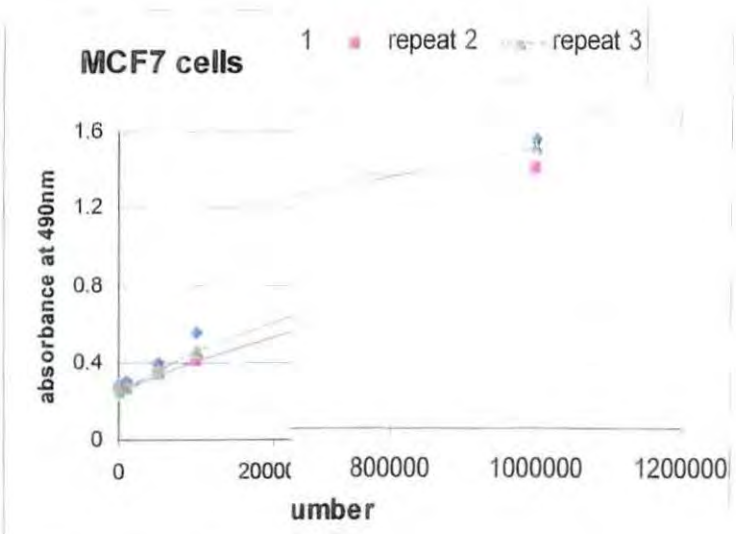
For each cell line used in this study (H36LE2, 9MaB, MCF7, U373-MG, SW13vim+ and SW13vim-) ,10^4 , 5.10^4 , 10^5, 5.10^5 and 10^6 viable cells were seeded in six wells of a 12-well plate in 10%FCS-supplemented DMEM. The range of cell numbers plated was chosen so that the highest cell number approximates the cell number present at confluence in a well of a 12-well plate.

2h after plating the cells, colorimetric reagent from the CellTiter 96^R Aqueous Non-Radioactive Cell proliferation Assay kit was added to each well. The time period of 2h was chosen to allow the cells to attach to the substrate. After 90min exposure to the reagent, the absorbance at 490nm was measured for each well.

The absorbance values within a treatment were averaged and plotted against the viable cell number plated. This experiment was repeated three times independently. ANOVAs between independent repeats at each viable cell number were calculated to give an indication of the reproducibility of the results. Exact ANOVA P values are presented in Appendix 2.


H36LE2 cells
repeat 2
repeat 3
absorbance at 490nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
800000
1000000
1200000


9MaB cells
repeat 2
repeat 3
absorbance at 490nm
0.5
0.4
0.3
0.2
0.1
0
0
800000
1000000
1200000


MCF7 cells
repeat 2
repeat 3
absorbance at 490nm
1.6
1.2
0.8
0.4
0
0
800000
1000000
1200000

Cell number	0	10^4	5.10^4	10^5	5.10^5	10^6
Standard absorbance value for H36LE2 ☝ standard deviation	0.234 ☝ 0.010	0.238 ☝ 0.008	0.306 ☝ 0.033	0.383 ☝ 0.063	0.886 ☝ 0.019	1.281 ☝ 0.063
Standard absorbance value for 9MaB☝ standard deviation	0.238 ☝ 0.005	0.240 ☝ 0.011	0.261 ☝ 0.006	0.280 ☝ 0.011	0.373 ☝ 0.019	0.418 ☝ 0.045
Standard absorbance value for U373-MG☝ standard deviation	0.251 ☝ 0.007	0.283 ☝ 0.030	0.410 ☝ 0.041	0.587 ☝ 0.071	1.616 ☝ 0.084	1.924 ☝ 0.130
Standard absorbance value for MCF7☝ standard deviation	0.261 ☝ 0.017	0.280 ☝ 0.021	0.364 ☝ 0.030	0.474 ☝ 0.076	1.016 ☝ 0.105	1.299 ☝ 0.096
Standard absorbance value for SW13vim+☝ standard deviation	0.258 ☝ 0.015	0.273 ☝ 0.017	0.303 ☝ 0.015	0.342 ☝ 0.023	0.623 ☝ 0.046	0.871 ☝ 0.050
Standard absorbance value for SW13vim-☝ standard deviation	0.260 ☝ 0.020	0.267 ☝ 0.011	0.285 ☝ 0.009	0.303 ☝ 0.020	0.374 ☝ 0.020	0.46 ☝ 0.027

Table 2.3 Absorbance values used as standards for viable cell quantification.

The absorbance values to be used a standards for the cell viability assay for each cell line were determined based on the results presented in Figure 2.3. The mean absorbance value obtained for each independent repeat for one given cell number were averaged to give the value to be used a standard for the cell viability assay. The standard deviation between the repeat means was calculated to give an indication of the variabiltiy of the mean absorbance values between independent repeats.

well. MCF7 and U373-MG cells were also cultured directly on the plastic or lens capsule substrates as a control. The number of cells plated on the insert was lowered to one quarter of the cell number plated at the bottom of the well. This was to account for the difference in area between the well and the insert, so that the cell number to substrate area ratio was similar for both. After 96h, the inserts were moved to a new 12-well plate, after which the cell culture medium was changed and the CellTiter 96^{R} Aqueous Non-Radioactive Cell proliferation Assay (Promega, Madison, USA) reagent added. Data was then collected as described in section 2.3.4. Data was collected for the inserts and all wells used, so that cell viability was also measured for the cell-free wells that had originally contained the inserts. This was done to provide a negative control. Six wells were used per treatment and the experiment was conducted three times independently for both MCF7 and U373-MG cells. A diagram of the method used is presented in Figure 2.5.

2.3.7. Data analysis:

The live cells numbers obtained for each well within a treatment were averaged to give the final live cell numbers for each treatment. A number of six wells were used for each treatment. The average live cell numbers for each treatment were then plotted against the nature of the treatment and standard deviation calculated for each treatment. Standard deviation, which is a measure of the variability in the samples values within a treatment, was preferred to the standard error of the mean, which is a measure of the variability between the sample mean and the population

mean (Zolman, 1993; see Appendix 1 for excerpt). As the standard error of the mean is calculated by dividing the standard deviation by the root square of the number of samples used (Zolman, 1993), it is also smaller than the standard deviation. When plotted on a graph, this constitutes, in my opinion, an artificial way of making one's data look better by hiding the true variability of the data, and should be discouraged.

Also, data gathered from different repeats were not pooled to allow to differentiate between within-repeat variability and between-repeat variability, as well as to provide visual proof that three independent repeats were done for each experiment.

The significance in the difference between treatments' means was determined by independent t-test. The independent t-test was chosen as the experimental set-up did not conform to a paired t-test analysis, i.e a before-after effect, and the treatments used were not random enough to necessitate an ANOVA. A two-tailed test was used to minimise the occurrence of Type I error, i.e a difference in means being falsely calculated as being significant. The critical probability value for significance was arbitrarily set at 0.05, as it is widely used in the scientific literature. The variability within a treatment between independent repeats was calculated using ANOVA, as it is quicker than three consecutive t-test (to test differences between three repeats). Exact probability values are presented in Appendix 2.

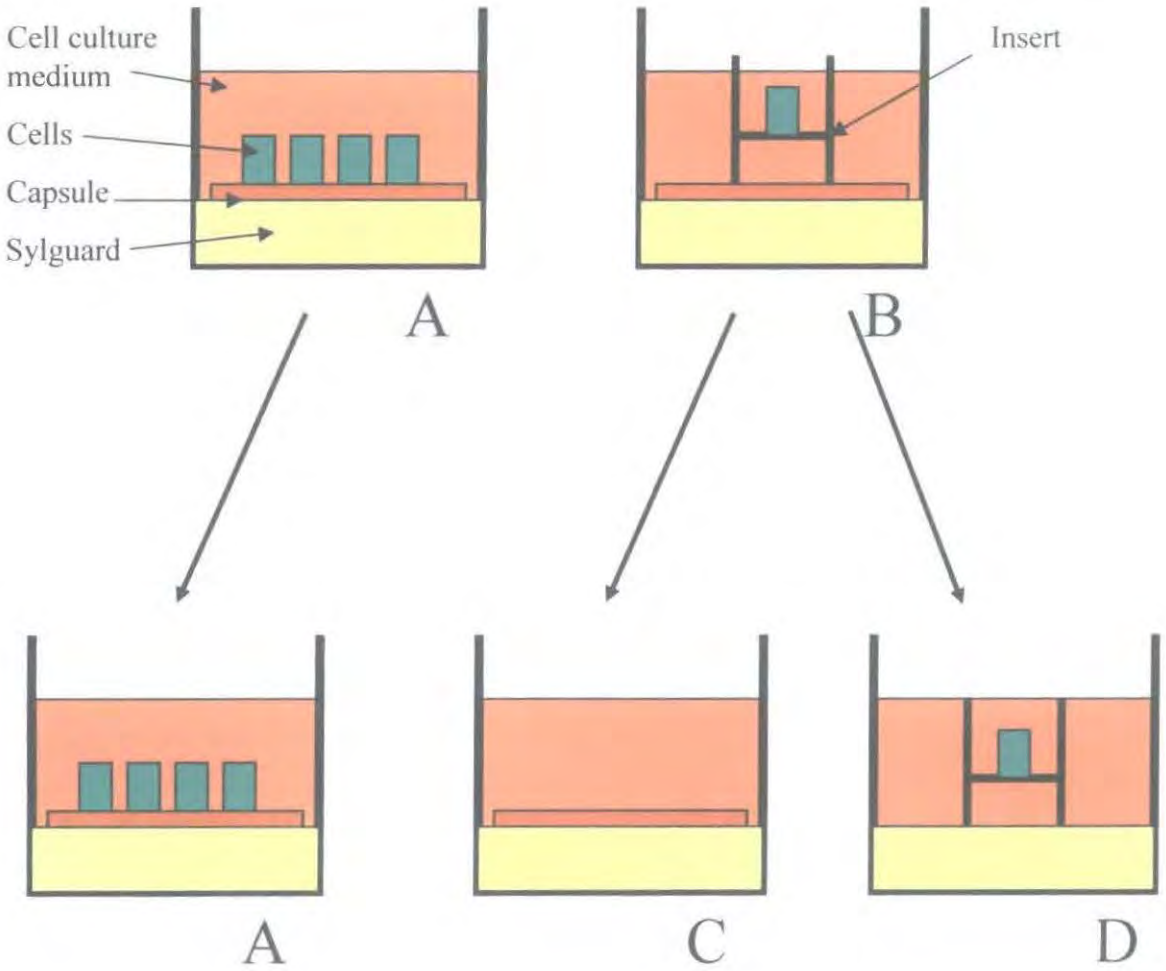


Figure 2.5 Co-culture set-up explained.
MCF7 and U373-MG cells were grown on a Millicell^{R} culture plate insert (Millipore, Molsheim, France; pore size: 12μm), with either cell-free plastic or a cell-free lens capsule at the bottom of the culture plate well (B). MCF7 and U373-MG cells were also cultured directly on the plastic or lens capsule substrates as a control (A). After 96h, the inserts were moved to a new 12-well plate (D), allowing the use of the cell-free wells that had originally contained the inserts (C) as a negative control for cell viability.

Example shown here with the lens capsule.

2.4. Results:

2.4.1. The lens capsule maintains lens epithelial cells' viability in the presence of staurosporine:

To first test whether the bovine lens capsule had any effect on cells viability, I cultured a range of cell lines on either a plastic or a bovine lens capsule substrate and exposed half of each group to staurosporine (as described in section 2.3.4).

As shown in Figure 2.6, when cells were cultured on the plastic substrate, all four cell lines examined showed a significant decrease in cell viability when staurosporine was added.

In the absence of staurosporine, changing the culture substrate from plastic to the lens capsule resulted in a small decrease in cell viability in H36LE2 and 9MaB cells. This decrease was not consistently significant. However, the same conditions resulted in a significant decrease in cell viability for both MCF7 and U373-MG cells. In the presence of staurosporine, changing the culture substrate from plastic to the lens capsule resulted in a significant increase in cell viability in both H36LE2 and MCF7 cells. No such significant increase in cell viability was recorded for both MCF7 and U373-MG cells.

When considering cells cultured on the lens capsule, the addition of staurosporine did produced a consistently significant decrease in cell viability for both H36LE2 and 9MaB cells, but not for MCF7 and U373-MG cells. However, it is worth remembering that the viable cells on the lens capsule in the absence of staurosporine for MCF7 and U373-MG are much less numerous than H36LE2 and 9MaB cells and therefore any decrease from these numbers is less likely to be significant.

2.4.2. The lens capsule protection decreases with the length of in vitro culture:

During the course of my study, I noticed that the cell line's passage number had a marked effect on cell growth and survival on the lens capsule. As shown in Figure 2.7, when H36LE2 and 9MaB cells at passage greater than 20 were cultured on the lens capsule, the cell viability both in the absence and presence of staurosporine were significantly decreased. This decrease was also more marked for the 9MaB cells than for the H36LE2 cells, even though the H36LE2 cells used were at a higher passage number than the 9MaB cells.

Figure 2.6 Culture on the bovine lens capsule significantly reduces the staurosporine-induced decrease in cell numbers in H36LE2 and 9MaB cells, but not in MCF7 and U373-MG cells.

H36LE2, 9MaB, U373-MG and MCF7 cells were seeded onto either plastic or bovine lens capsule substrates in 10% FCS-supplemented cell culture medium. After 48h, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, viable cells numbers were determined using the Aqueous 96 non-radioactive cell viability assay as described in section 2.3.4.

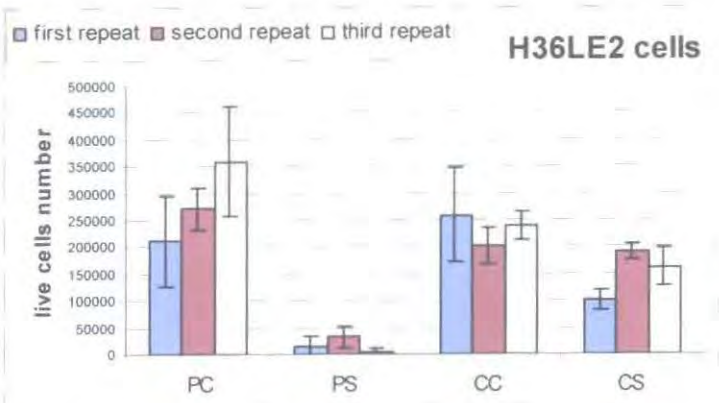
Note that culture of both lens epithelial cell lines on the bovine lens capsule resulted in a significant increase in cell viability in the presence of staurosporine (PS-CS: $P<0.05$ for all three repeats for both H36LE2 and 9MaB cells). Also, culture on the bovine lens capsule of both non-lens cell lines resulted in a significant decrease in cell viability in the absence of staurosporine (PC-CC: $P<0.05$ for all three repeats for both U373-MG and MCF7 cells).

Bars represent the average viable cell number over six samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 2.

Treatment legend:

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine


first repeat
second repeat
third repeat
H36LE2 cells
live cells number
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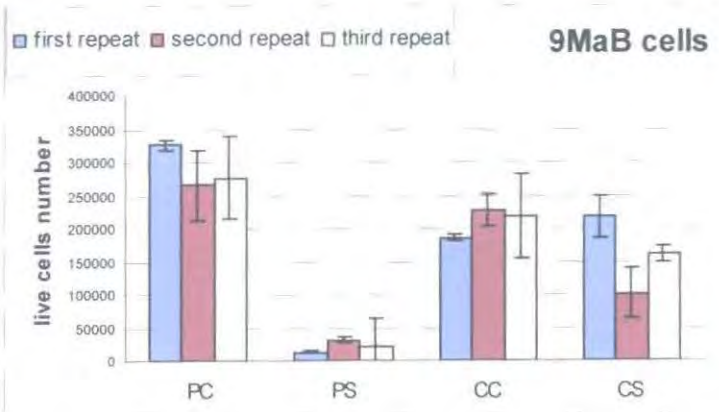
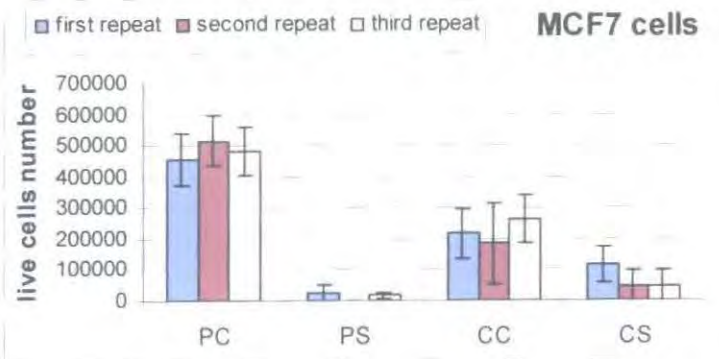
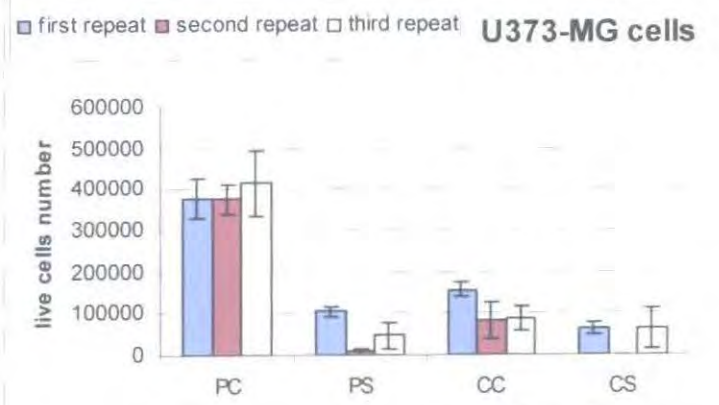
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
PC
PS
CC
CS


first repeat
second repeat
third repeat
9MaB cells
live cells number
400000
350000
300000
250000
200000
150000
100000
50000
0
PC
PS
CC
CS


first repeat
second repeat
third repeat
MCF7 cells
live cells number
700000
600000
500000
400000
300000
200000
100000
0
PC
PS
CC
CS


first repeat
second repeat
third repeat
U373-MG cells
live cells number
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500000
400000
300000
200000
100000
0
PC
PS
CC
CS

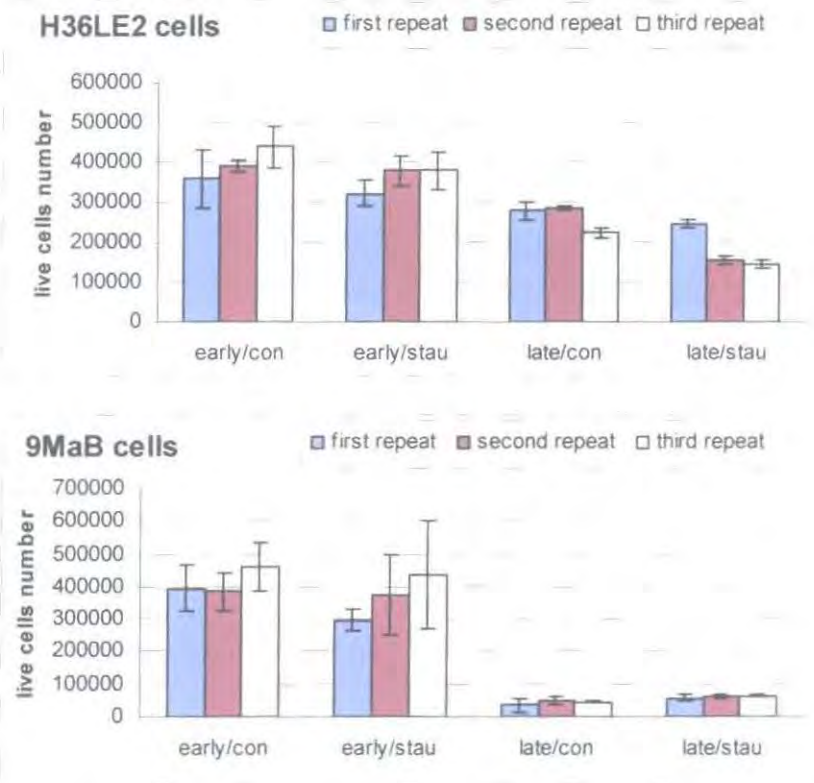

Figure 2.7 Increased length of *in vitro* culture decreases cell viability on the lens capsule for both H36LE2 and 9MaB cells.

H36LE2 and 9MaB cells of either an early passage (passages 10 and 9, respectively) or a late passage (both over passage 20) were seeded onto bovine lens capsule substrates and, after a 48h recovery period, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, live cells numbers were determined using the Aqueous 96 non-radioactive cell viability assay (Promega, Madison, USA) as described in section 2.3.4.

Note that for both H36LE2 and 9MaB cells, the later passages cells have a significantly decreased cell viability on the lens capsule, both in the absence and presence of staurosporine. Effect of passage number was not investigated on the plastic as I judged the lens capsule to be more important and interesting to my study.

Bars represent the average viable cell number over six samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 2.

Treatment legend:

early/con:early passage, no staurosporine early/stau: early passage, 500nM staurosporine

late/con:early passage, no staurosporine late/stau: early passage, 500nM staurosporine

2.4.3. *Soluble factors are released from the lens capsule that prevent non-lens cell growth:*

Next, I wanted to test whether the poor viability of MCF7 and U373-MG cells on the lens capsule was due to potentially incompatible attachment factors between the cells and their substrate. To this end, MCF7 and U373-MG cells were tested in a co-culture set-up, as described in section 2.3.6.

As seen in Figure 2.8, when either MCF7 or U373-MG cells were grown on the insert, there was a significant decrease in cell viability when cells were co-cultured with cell-free lens capsules compared to when cells were co-cultured with cell-free plastic ($P<0.05$ for all three independent repeats for both cell lines). When cells were cultured directly onto lens capsules, there also was a significant decrease in cell viability compared to when cells were grown directly on plastic ($P<0.05$ for all three independent repeats for both cell lines). I did not directly compared cells grown directly on the substrate to cells grown on co-culture due to the difference in starting cell number (see section 2.3.6).

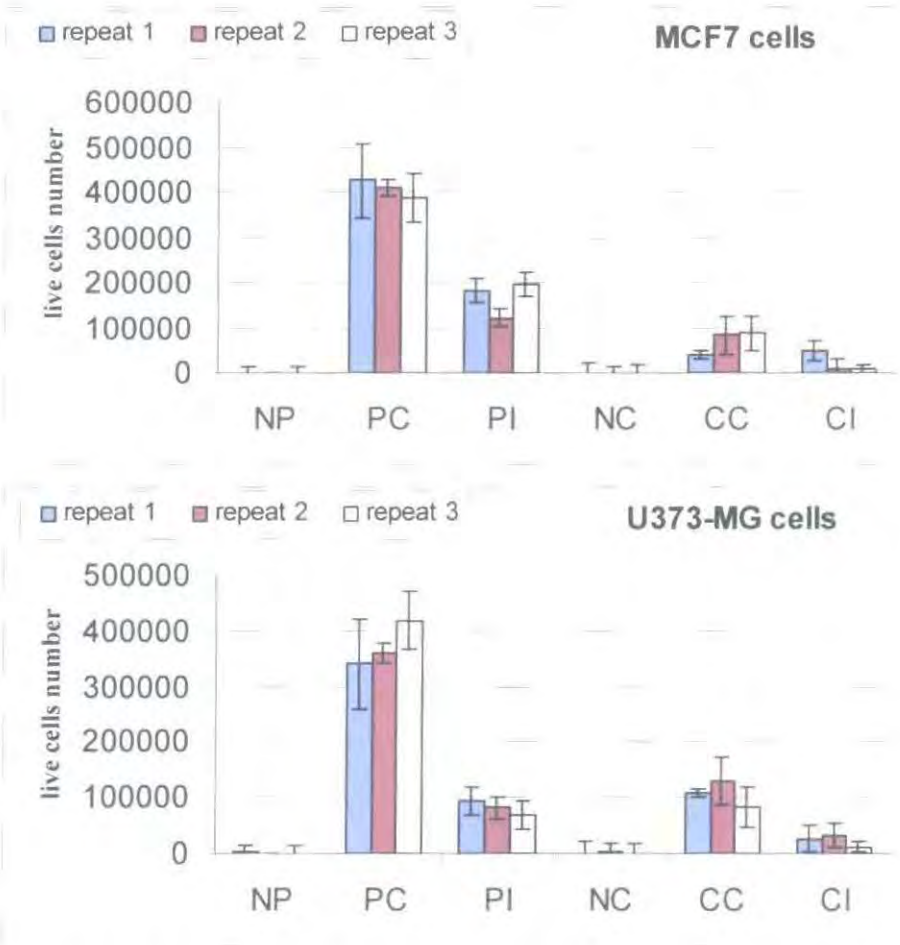

Figure 2.8 Co-culture of MCF7 and U373-MG cells with the lens capsule.

MCF7 and U373-MG cells were plated in a co-culture set-up in 12-well plates as described in 2.3.6. After 96h, viable cells numbers were determined using the Aqueous 96 non-radioactive cell viability assay (Promega, Madison, USA) as described in section 2.3.4.

Note that, for both MCF7 and U373-MG cells, when cells were grown on the insert in the presence of the lens capsule, there was a significant decrease in cell viability compared to when the cells were grown in the presence of plastic (PI,CI: $P<0.05$ for all three repeats for both cell lines).

Bars represent the average viable cell number over six samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 2.

Treatment legend:

NP: cell-free plastic used for co-culture NC: cell-freelens capsule used for co-culture

PC: cell-seeded plastic, no staurosporine CC: cell-seeded lens capsule, no staurosporine

PI: cell-seeded insert co-cultured with cell-free plastic, no staurosporine

CI: cell-seeded insert co-cultured with cell-free lens capsule, no staurosporine

2.4.4. *A role for the cytoskeleton in cell viability on the lens capsule:*

In the course of the viability experiments, an interesting fact was noticed. When using the adrenocarcinoma cell lines, SW13 vim- and SW13 vim+, the presence of type III intermediate filament vimentin correlated with growth on the lens capsule.

As can be seen in Figure 2.9, the number of viable SW13vim+ cells was not significantly decreased on the lens capsule compared to the plastic substrate. However, the number of viable SW13vim- cells was significantly decreased on the lens capsule compared to the plastic substrate. SW13 cell viability on the lens capsule correlated with the presence of vimentin, which was checked by immunoblotting.

Cell viability experiments were also conducted in the presence of staurosporine but showed no difference between the SW13vim+ and the SW13vim- cells, i.e both had poor cell viability on the lens capsule in the presence of staurosporine. I chose not to include these results as they were only echoing the results for MCF7 and U373-MG cells on the lens capsule in the presence of staurosporine and therefore presented no novelty.

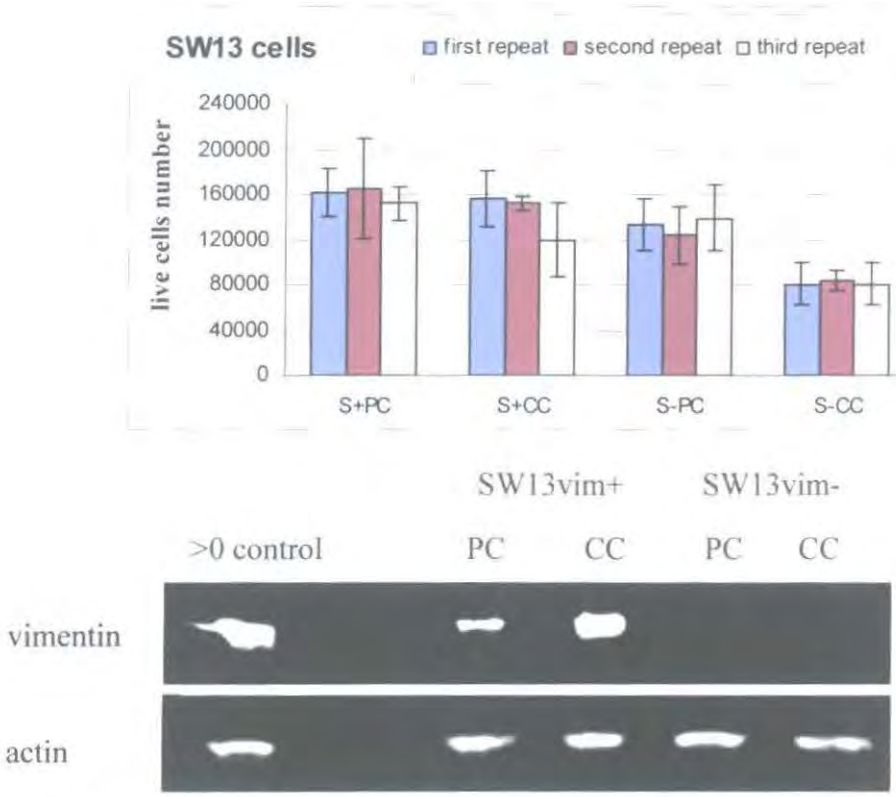

Figure 2.9 Vimentin-expressing SW13vim+ cells have an increased viability on the lens capsule compared to the vimentin-lacking SW13vim-cells.

SW13 vim+ and SW13 vim- cells were seeded onto either plastic or bovine lens capsule substrates. After 96h, viable cells numbers were determined using the Aqueous 96 non-radioactive cell viability assay as described in section 2.3.4.

Note that culture on the lens capsule resulted in a significant decrease in SW13vim- cell viability compared to SW13vim+ cells.

Bars represent the average viable cell number over six samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 2. Vimentin expression in the SW13 cells was checked by immunoblotting.

Treatment legend:

S+: SW13vim+ cells

S-: SW13vim- cells

PC: plastic substrate, no staurosporine

CC: capsule substrate, no staurosporine

2.5. Discussion:

2.5.1. *The lens capsule as a culture model:*

Studies of lens epithelial cells involvement and behaviour during PCO require the use of a culture model. *In vivo* models would be ideal as they test the cells in their natural environment, but the animals used are costly to maintain and their use might be restricted for ethical reasons. *In vitro* models, on the other hand, are easy to manipulate but might be an over-simplification of the *in vivo* processes. When studying lens epithelial cells, a middle way has been found with the capsular bags models, where lens epithelial cells are cultured directly on the lens capsule, their natural basement membrane, with the capsule itself being kept *in vitro*. Cultured in this way, lens epithelial cells proliferation and PCO formation can be accurately observed (Quinlan *et al.*, 1997), and therefore experimented upon.

Three experimental set-ups have been described. The latest models make use of either an internal ring (Saxby *et al.*, 1998) or an external adhesive holders (El-Osta *et al.*, 2003) to prevent collapse of the capsular bag and thus insure maximum access of the cells to the cell culture media. The earliest and simplest model (Liu *et al.*, 1996) consists of a lens capsule pinned down on a plastic substrate following anterior capsulorhexis. Although it has been criticised for not allowing expansion and culture media access to the peripheral capsule regions, this model might actually mirror the *in vivo* condition best. Indeed, after a cataract operation, the peripheral regions of the

anterior and posterior capsule do come in contact with each other under the posterior pressure of the vitreous and the lateral pressure of the IOL. *In vitro*, the capsular bags cultured in this way present the lens epithelial cells' migration and wrinkling of the posterior capsule that are characteristic of after-cataract (Quinlan *et al.*, 1997).

It is a variation of this early model that was chosen to perform our experiments. Bovine eyes were preferred to human eyes as a source of capsule material as they are inexpensive, larger and stronger than their human counterparts (Danielsen, 2004) and available in large quantities. The bovine lens capsule has previously been used as a culture substrate for both rabbit lens cells (McDonnell *et al.*, 1985) and human keratinocytes (Lenoir et al., 1985) with good results. Therefore, I judged it to be a good culture substrate for human lens epithelial cells. After dissection from the bovine eye, the lens was placed anterior face down and the posterior capsule was ruptured, peeled off and pinned down, so that the inside of the capsule is fully exposed and the anterior capsule, after removal of the lens fibres, is the main culture surface. The anterior capsule was chosen as it is more resistant than the posterior capsule and less likely to break during subsequent manipulations.

2.5.2. *The lens capsule as a promoter of lens cell viability:*

The most important result of this chapter is that culture of lens epithelial cells on the lens capsule maintains cell viability in the presence of staurosporine. Although I did not test whether this viability was the result of increased proliferation or reduced apoptosis, previous studies of cell-ECM systems in and outside the eye have shown

that the ECM does confer protection against apoptosis to the overlying cells (Gribbons, 2003; Rintoul and Sethi, 2002; Judware *et al.*, 1998; Aharoni *et al.*, 1996). Therefore it is possible that anti-apoptotic mechanisms are also present in my system.

The lens capsule's effect on cell viability, however, was reduced with the length of *in vitro* culture. This effect could not have been due to a variation in the capsule itself with age, as all animals used were culled at the same age (see section 2.3.1).

When comparing the viability of early and late passage lens cells on the lens capsule, the decrease in cell viability for the 9MaB cells, can be partially explained by the genetic instability inherent to this cell line. Knocking-out αB-crystallin is known to result in genetic instability in mice lens epithelial cells, and although some αB-crystallin$^{-/-}$ cells proliferate faster than the wild-type initially, their long-term apoptotic resistance is greatly decreased (Andley *et al.*, 2001). Also in the 9MaB cell line, the 450Δ mutation in αB-crystallin results in a frameshift and a complete change in the amino acid sequence of the C-terminal tail (Berry *et al.*, 2001). This mutation is expected to greatly affect the solubility of αB-crystallin and hence impair its chaperone function, resulting in increased protein unfolding and decreased cell viability with time and stress.

In H36LE2 cells, however, no such mutation could explain the decreased cell growth and resistance seen in our results. Long-term studies of changes in protein

expression with *in vitro* culture have been conducted, not on lens epithelial cells, but on the neighbouring retinal pigment epithelial cells (Wang *et al.*, 2004). A number of genes were found to be down-regulated and, amongst them, αB-crystallin, which expression was decreased over 6 folds. It is conceivable that this decrease might also happen in H36LE2, with again deleterious effects on protein solubility and cell viability. These changes in protein expression, which in retinal cells also included a decrease in E-cadherin, indicate that the cell line is gradually losing its strict epithelial phenotype.

The fact that the lens epithelial cells' viability on the capsule decreases as the length of *in vitro* culture increases suggests that a strict maintenance of the epithelial phenotype is required for maintenance of cell viability on the lens capsule. Therefore, the hypothesis can be formulated that the cell viability pathways operating when lens epithelial cells are grown on the lens capsule should involve lens epithelial-specific proteins.

2.5.3. *The lens capsule as an inhibitor of non-lens cells viability:*

When repeating my viability experiments with non-lens cells lines my results indicated that culture of non-lens cells on the lens capsule was deleterious to their viability, which is a very different outcome from what is observed in lens epithelial cells. That a cell line's ability to remain viable on the capsule varies with its origin suggests that the factors involved be not widely expressed and strengthens the hypothesis that they might be preferentially expressed in the lens.

The ability of a cell-type to bind to and grow on a given substrate depends on which integrin dimers it expresses, as integrins bind specifically to individual ECM components (Zhang *et al.*, 2002; Fukai *et al.*, 1998). In my study, the anterior lens capsules used as a culture substrate were obtained from healthy, young cows and should therefore contain mainly collagen IV and laminin, but also a range of proteoglycans and some fibronectin. The MCF7 and U373-MG cell lines are both known to adhere to laminin- collagen IV-fibronectin matrices *in vitro* (Goldbrunner *et al.*, 1996; Terranova *et al.*, 1983). Their reduced cell numbers on the lens capsule, even in the absence of staurosporine, might appear surprising. However, the adhesive properties of MCF7 cells and glioma cells other than U373-MG has also been shown to depend on the proteoglycan sulfation levels within the ECM (Liu *et al.*, 2000; Mendes de Aguiar *et al.*, 2002). As the lens capsule is rich in proteoglycan, this might be the limiting factor for U373-MG and MCF7 cells' adhesion and subsequent growth. Other cell-specific anti-adhesive factors present in the lens capsule include perlecan (Klein *et al.*, 1995), SPARC (Yan and Sage, 1999) and thrombospondin-1 (Chandrasekaran *et al.*, 1999; Saika *et al.*, 2004) and all three might induce decreased adhesion of U373-MG and MCF7 cells onto the capsule. In turn, this impaired adhesion might also increase the cells sensitivity to staurosporine-induced apoptosis.

That non-lens cells might have an impaired adhesion to the capsule makes it unclear to determine whether their poor viability on the lens capsule is due to their generally impaired adhesion, which would disrupt integrin-mediated survival, or to their lack of lens-specific survival factors.

A co-culture experiment was used to determine whether attachment or soluble factors were involved in the reduced viability of MCF7 and U373-MG cells on the lens capsule. If attachment factors alone were indeed involved, then the viability of cells grown on an insert would not be expected to vary with the substrate placed at the bottom of the well, be it plastic or the lens capsule.

An unexpected result from this experiment was the decrease in both MCF7 and U373-MG cells viability in the presence of the lens capsule, even though the cells were physically separated from it. This suggested the presence of soluble factors released from the lens capsule, which actively impaired non-lens cells' growth. Soluble factors could be initially released directly from the cells. These factors would induce the release of further factors from the lens capsule that are deleterious to MCF7 and U373-MG cell viability. Alternatively, proteases present in 10% FCS-supplemented cell culture medium could release factors from the lens capsule, without the intervention of the cells cultured on the insert. Although I did not address this question in my experiments, it could be answered by repeating a co-culture experiment in serum-free conditions, while increasing the cell density to prevent the absence of serum itself from reducing cell viability.

2.5.4. *A role for the cytoskeleton in cell growth on the lens capsule:*

Another interesting result of my cell viability experiments was the inability of SW13 vimentin-lacking cells to be cultured on the lens capsule as efficiently as their

vimentin-expressing counterparts, and this in the absence of staurosporine. These results suggest that the presence of vimentin, and potentially of an intact cytoskeleton, be a requirement for cell viability on the lens capsule.

Vimentin is a type III intermediate filament characteristic of mesenchymal cells (Hermann and Aebi, 2000). Intermediate filaments, so called due to their 10nm diameter, constitute, with microfilaments and microtubules, the cytoskeleton. Intermediate filaments are separated into six groups, with the cytokeratins and keratins being type I and type II, vimentin, desmin, GFAP and peripherin being type III, neurofilament proteins being type IV, lamins being type V and nestin, synemin and paranemin being type VI (Paramio and Jorcano, 2002). Vimentin monomers are constituted of an N-terminal non-helical head domain, a central α-helical rod domain and a C-terminal non-helical tail domain (Herrmann *et al.*, 1996). Assembly of vimentin filaments occurs in stages. First, the rod domains of two vimentin proteins come together to form a parallel α-coiled-coil (Lowrie *et al.*, 2000). The resulting homodimers interact as anti-parallel pairs to form tetramers, which associate with each other to give rise to unit-length filaments. Longitudinal linking of these unit-length filaments results in the formation of full-length vimentin filaments (Mucke *et al.*, 2004). The vimentin filament network is organised radially within the cell, from the nucleus to the plasma membrane and partially co-localises with the microtubule network. This association with microtubules mediates the motility of vimentin filaments within the cell (Clarke and Allan, 2002). Vimentin participates in cell processes such as the maintenance of nuclear shape (Sarria *et al.*, 1994) and lipid synthesis (Gillard *et al.*, 1998; Sarria *et al.*, 1992). However, for the purpose of this

study, it is vimentin's involvement in cell adhesion that will be examined most closely.

The involvement of cytoskeletal proteins in cell-ECM adhesion is best examplified at focal adhesion sites, where ECM-bound integrins link to the actin network via a talin, α-actinin, filamin or integrin-linked kinase (ILK) complex (Brakebusch and Fassler, 2003) (For a more detailed presentation of the role of integrins in cell viability, see section 4.1.1). Vimentin is also present at focal adhesions, where it can interact with α2β1 integrin (Kreis *et al.*, 2005), αVβ3 integrin (Gonzales *et al.*, 2001) and possibly actin (Correia *et al.*, 1999). The importance of this association was demonstrated by knocking out vimentin from fibroblasts, which resulted in disturbed actin and focal adhesion organisation, with deleterious consequences on cell motility (Eckes et al., 1998). Vimentin also participates in hemi-desmosomes via plectin binding of α6β4 integrin (Homan *et al.*, 2002; Rezniczek *et al.*, 1998). α2β1 integrin (Languino *et al.*, 1989) and α6β4 integrin (Homan *et al.*, 2002) are both involved in laminin binding in epithelial cells. In the same way as the disruption of the integrin-actin link by targeted deletion of talin or ILK in *Drosophila* result in cell detachment *in vivo* (Brakebusch and Fassler, 2003), it is conceivable that disruption of the integrin-vimentin link on the laminin-rich lens capsule might also impair cell attachment. In a laminin-rich substrate such as the lens capsule, this impaired attachment would have greater consequences than on plastic and could ultimately lead to anoikis.

2.6. Chapter's conclusions:

This aim of this Chapter was to determine whether the lens capsule had an effect on lens epithelial cell viability and whether any effect it had could be extended to non-lens cells. By the end of this chapter, I have presented evidence that the lens capsule is able to maintain lens cell viability, even in the presence of staurosporine. This effect also appears to be lens-specific, as it is not conserved in MCF7 and U373-MG cells or in late passage lens cells. I therefore formulate the hypothesis that intra-cellular lens-specific factors are involved in mediating the lens capsule-induced cell viability.

3. The lens capsule promotes α-crystallin protein expression in lens epithelial cells:

In chapter 2, I showed that lens epithelial cells have an increased viability compared to non-lens cells when cultured on the lens capsule in the presence of staurosporine. In this chapter, I wanted to investigate whether cell viability on the lens capsule correlated with lens-specific protein expression on the lens capsule. To address this question it is first necessary to examine the characteristics of lens-specific protein expression.

3.1. Introduction: Characteristics of lens-specific protein expression:

Many proteins perform functions necessary to all cells and are therefore ubiquitously found in an organism. Within every organ, however, cell populations also express a specific subset of proteins that control their morphological and functional uniqueness. The lens is made of two cell populations, the epithelial lens cells and the lens fibre cells. Here, I will describe a number of proteins, the expression of which defines the lens phenotype, with some also allowing for the distinction between lens epithelial and lens fibre cells.

3.1.1. At the source of lens-specific protein expression: The transcription factors

A lens-specific protein expression relies on the presence and activity of lens-specific transcription factors.

3.1.1.1. Lens epithelial specificity: Pax6, FoxE3 and LEDGF

Pax6 is well known as a master gene in the development of both vertebrate and invertebrate eyes (Gehring and Ikeo, 1999). In the adult lens, its expression is limited to the epithelial layer (Kondoh, 1999), where it induces the expression of αA-crystallin, αB-crystallin (Cvekl and Piatogorsky, 1996) and the taxon-specific δ1-crystallin (Cvekl *et al.*, 1995) and ζ-crystallin (Richardson *et al.*, 1995). Pax6 also represses the expression of β-crystallins in the lens epithelial cells (Kondoh, 1999). However, although crystallins expression is mainly lens-specific, Pax6 expression is not. Pax6 is widely expressed in the developing central nervous, visual and olfactory systems during embryogenesis, and is still readily detectable in the adult (Callaerts *et al.*, 1997). In the adult eye alone, Pax6 expression is present in the ganglion cells, bipolar nerve layer and amacrine neurons of the neuroretina (Callaerts *et al.*, 1997), as well as the conjunctiva, iris and corneal epithelium (Koroma *et al.*, 1997). Therefore, Pax6 does not constitute a good lens-specific marker.

FoxE3, on the other hand, is strictly restricted to the lens epithelium in the adult organism (Blixt *et al.*, 2000), and is a good example of lens epithelial-specific protein expression. Three other Fox family members, FoxC1, FoxC2 and FoxL2, are also present in the eye (Lehmann *et al.*, 2003), and conserved sequences between them and FoxE3 (Kaestner *et al.*, 2000) must be taken into account when designing antibodies or polymerase chain reaction (PCR) primers, to avoid false-positive detection. FoxE3 prevents early fibre differentiation by repressing the Cdkn1c-inducing Prox1 and αA-crystallin expression (see section 1.1.1). It also induces PDGF-A receptor expression, which maintains PDGF-induced cell proliferation in the epithelium, but is by no means lens-specific.

LEDGF is a proliferation and survival lens factor and induces AOP2, HSP27 and αB-crystallin expression under stress conditions (detailed in section 1.4.5). Despite its name, LEDGF is not truly lens-specific, although, within the lens, it is restricted to the lens epithelial layer (Kubo *et al.*, 2003). In the eye, LEDGF is expressed by RPE cells but absent from the cornea (Matsui *et al.*, 2001; Kubo *et al.*, 2003). It has also been detected in a number of non-ocular cell lines and tissues under its earlier names of PC4-interacting protein, p75 and DFS70 auto-antigen (Cherepanov *et al.*, 2003). Accurate detection of LEDGF in the lens epithelial cells is also complicated by the fact that human lens epithelial cells *in vitro* express several HDGF family members other than LEDGF, including the LEDGF splice variant, p52 (Singh *et al.*, 2000). Again, the presence of N-terminal conserved sequences within the HDGF family means that care must be taken when designing antibodies or PCR primers to avoid false-positive detection.

3.1.1.2. Lens fibre specificity:

Lens fibre-associated transcription factors are essentially factors that lens fibre formation releases from Pax6- or FoxE3-mediated repression (see section 1.1.2). Examples include the γ-crystallin-upregulating Sox1, Sox2 and Sox3, and the α-crystallins-upregulating c-Maf, also called maf-2, not to be mistaken for MafB, or maf-1, which is present in the lens epithelium (Kondoh, 1999). Neither of them, however, is strictly lens-specific (Pevny and Lovell-Badge, 1997; Christodoulopoulos *et al.*, 2001).

3.1.2. *Visual function in the lens: The crystallins*

Be it by their overwhelming abundance in the lens, where they constitute 90% of the protein fraction (Rao *et al.*, 1995) or by their essential roles in the lens' visual functions, crystallins are most often used to exemplify lens-specific protein expression. Seven classes of crystallins are known, three of which, the α-, β- and γ-crystallins, are found in all vertebrate lenses, while the other four, the δ-, τ-, ε-, ρ-crystallins, are restricted to specific classes or orders of vertebrates and are never found in the mammalian lens (Wistow and Piatigorsky, 1988).

3.1.2.1. α-crystallin:

α-crystallin is found as a high molecular complex of 800 kDa composed of an arrangement of αA-crystallin and αB-crystallin in a 3 to 1 ratio (Wistow and Piatigorsky, 1988). Several structure models have been proposed for α-crystallin. The double-annular model is particularly in accordance with the known properties of α-crystallin, as the peripheral C-terminal hydrophilic tails can maintain protein solubility, while the central N-terminal hydrophobic domains are involved in substrate binding (Carver and Lindner, 1998).

It is α-crystallin chaperone activity that is essential to the maintenance of lens fibre cell transparency (detailed in section 1.1.4), with the anti-apoptotic properties of both αA- and αB-crystallin being involved in lens epithelial cell proliferation and survival (detailed in section 1.4.2.3).

αA- and αB-crystallin are found in both lens epithelium and lens fibres, under the regulation of Pax6 and cMaf, respectively (see section 1.1.1 and Kondoh, 1999). αA–crystallin expression is mostly restricted to the lens, although it is also found in the thymus (Kato *et al.*, 1991), while αB-crystallin is expressed in the lens, retina (Wang *et al.*, 2004), heart, kidney and skeletal muscle, and at a lesser degree, in the brain and lungs (Krausz *et al.*, 1996; Bhat and Nagineni, 1989). Therefore, αA–crystallin is a better lens-specific marker than αB–crystallin, although it is worth remembering that its expression might be reduced in the lens epithelial cells due to the down-regulatory action of FoxE3 (see 1.1.1.1).

3.1.2.2. β- and γ-crystallins:

The β-crystallins and the γ-crystallins are fibre-specific crystallins (section 1.1.2) and are essential to the refractive index of the lens (section 1.1.5). Their structural modifications, due to congenital mutations (section 1.2.3) or age-related degradation (Hanson *et al.*, 1998; Srivastava and Srivastava, 1998), are linked with loss of transparency and cataract formation.

The β-crystallins and γ-crystallins are grouped together in the βγ-superfamily, on the basis of their structural similarities. Both β-crystallins and γ-crystallins are composed of two symmetrical domains separated by a connecting peptide. Each domain is constituted of two greek key motifs, each formed by four anti-parallel β-strands (Wistow and Piatigorsky, 1988). The flexibility of the γ-crystallins' connecting peptide allows association of the two main domains, resulting in stable γ-crystallins monomers. In contrast, the β-crystallins' connecting peptide is less flexible and stability is achieved by domain association between two β-crystallin molecules, forming a dimer (Fu and Liang, 2002).

All β-crystallin isoforms are found throughout the fibre mass (Fu and Liang, 2002), although they are not strictly lens-specific, as they have also been detected in neural cells (Coop *et al.*, 1998). γA- to γE-crystallins isoforms, on the other hand, are preferentially found in the nuclear fibres, while γS-crystallin, which present properties from both families, concentrates in the cortex (Carver *et al.*, 1999). Therefore, and as

previously suggested (Piatigorski, 1981), a careful detection of γ -crystallin isoforms should allow the identification of specific regions within the fibre mass.

3.1.3. Lens-specific cytoskeleton: the beaded filament:

Filensin/CP115/CP95 and phakinin/CP49/CP47 are lens-specific, atypical members of the intermediate filament protein family. Phakinin has been classed as a type I cytokeratin on the basis of its intronic sequence, although it lacks a C-terminal tail and has three amino acids substitutions in the Leu-Asn-Asp-Arg motif, which in other cytokeratins prevents filament assembly (Hess *et al.*, 1998). Filensin, with 29 a.a missing from its central rod domain, has proved even harder to classify and is considered to form a new class of intermediate filaments (Hess *et al.*, 1998).

In the lens, phakinin and filensin co-assemble into a structure called the beaded filament. Although beaded filament structures can be formed by filensin and phakinin on their own *in vitro* (Goulielmos, 1996), the beaded filament found in the lens consists of a phakinin-filensin filamentous core decorated with α-crystallin beads (Prescott *et al.*, 1996; Carter *et al.*, 1995). It is found exclusively in the mature lens fibres, where it is the only cytoskeletal structure present, as actin, vimentin and microtubule networks disappear during fibre cell differentiation (Hess *et al.*, 1998). The exact function of the beaded filament is unclear, although its disruption results in cataract formation *in vivo* in both humans and animal models (Alizadeh *et al.*, 2003; Alizadeh *et al.*, 2002; Carter *et al.*, 2000).

The fibre-specificity of filensin is linked to two lens-specific enhancer elements present in its promoter, one of which is highly reactive to FGF-2 (Masaki *et al.*, 2002) and would therefore be preferentially active in the developing fibres. Phakinin's promoter region also promotes lens-specific expression, and contains a Pax6 binding site (DePianto *et al.*, 2003), suggesting that the fibre-specific phakinin expression might be due to the release of Pax6-mediated promoter repression. Therefore filensin and phakinin can be used as markers of lens fibres. As filensin is also increasingly processed into its separate N-terminal-rod domains and C-terminal domains with fibre differentiation (Sandilands *et al.*, 1995), changes in immunoblotting patterns can also be used to distinguish between differentiation stages, with an increase in low molecular weight bands in more mature fibres.

3.1.4. Membrane transport proteins in the lens:

3.1.4.1. Aquaporin0:

Aquaporin0 (AQP0/MIP26) is a trans-membrane protein that tetramerises into a water channel. Each monomer consists of six α-helical trans-membrane folds, which assemble into an hourglass-shaped, water-specific channel (Harries *et al.*, 2004).

In the lens, AQP0 is found exclusively in the lens fibres, where it is the most abundant membrane protein (Yu and Jiang, 2003). In a chicken model, It has also been found to co-localise with the gap junction proteins Cx45.6 and Cx56 in the

elongating, but not the mature, lens fibres (Yu and Jiang, 2004). AQP0 functions in the lens are thought to be linked to cell-cell adhesion and the decrease in extracellular space between the lens fibres, as well as water transport away from the lens fibres and establishment of the refractive index gradient (see sections 1.1.3 and 1.1.5). Its importance in lens transparency is indicated by the existence of human congenital cataract associated with two separate AQP0 mutants, E134G and T138R. These substitutions are predicted to affect water molecule binding within the water channel and have been shown to result in a cytoplasmic, rather than membranous, distribution of AQP0 (Francis *et al.*, 2000).

The fibre-specificity of AQP0 is insured in a manner rather similar to filensin, via a lens-specific promoter with an element activated by FGF2-mediated ERK and JNK signalling (Golestaneh *et al.*, 2004).

3.1.4.2. channels isoforms:

The expression of other transport proteins differs within the lens and therefore can be used to differentiate between subsets of lens cells populations. The gap junction proteins Cx43, Cx45.6 and Cx56, in the chicken lens, are such an example. Cx43 is found in the lens epithelium and early differentiating fibre cells, while Cx45.6 and Cx56 are found only in the fibre cells (Jiang *et al.*, 1995). Therefore cells expressing Cx43 only, Cx45.6 and Cx56 only, or all three connexins can be identified as lens epithelial, mature lens fibre, or early lens fibre, respectively.

The same can be applied to isoforms of the Na,K-ATPase catalytic subunit (Garner and Kong, 1999). The three isoforms, α1, α2 and α3 (also called ATP1A1, ATP1A2 and ATP1A3), are all present in the lens, but the α2 isoform is only detectable in the fibre cells. Also, the α1 and α3 iosoforms, highly expressed in the lens epithelium, are polarised in the central lens epithelium, to basal and apical location, respectively, but not in the equatorial cells.

Therefore, a number of proteins are available to identify lens cells, some of which can also be used as either positive or negative controls to distinguish between the lens epithelial and lens fibre phenotypes.

3.2. Aims:

In this Chapter, I wanted to test the hypothesis formulated at the end of Chapter 2 that lens-specific viability on the lens capsule should be correlated with lens-specific cell protein expression. In addition, I wanted to determine whether protein expression in the SW13 cells on the lens capsule could be correlated with cell viability. To this end, I examined the expression profile of a range of factors chosen not only for their lenticular expression, but also for their known role in stress resistance. Depending on antibody availability, either mRNA expression or protein expression was examined.

3.3. Experimental procedure:

3.3.1. RT-PCR studies :

mRNA expression studies were conducted when an antibody to the target of interest was not readily available or judged too expensive.

3.3.1.1. Total RNA extraction:

Total RNA was extracted using the GenElute™ Mammalian Total RNA kit (Sigma-Aldrich, Poole, UK). After completion of the cell viability assay (described in 2.2.4), the remaining cell culture medium was removed and 250µl of lysis solution (provided in the kit) was added to the cells. After 2 min, the lysate was pipetted into a GenElute filtration column and centrifuged for 2 min at 12000 rpm in an Eppendorf 5417R centrifuge (rotor identification: UE005 F45-30-11) to remove cells debris and shear DNA. 250µl of 70% ethanol were added to the lysate and the mix was transferred into a binding column and centrifuged for 15 sec at 12000 rpm. 500µl of Wash solution 1 (provided in the kit) was then added to the column for a second round of centrifugation for 15 sec at 12000 rpm, after which the binding column was transferred to a collection tube. 500µl of Wash solution 2 (provided in the kit) were added, and a third centrifugation round was run (15 sec, 12000 rpm). An extra 500µl of Wash solution 2 was added and the column ran through another centrifugation for 2min at 12000 rpm. Lastly, the RNA was eluted by pipetting 50µl of elution solution

into the column, transferring the column to a new collection tube and running a last centrifugation round for 1min at 12000 rpm. The purity and quantity of the total RNA obtained was determined by spectrophotometry (Beckman DU640, Beckman Instruments Inc., Fullerton, USA). The ratio of absorbance values obtained at 260nm and 280nm, which detect nucleic acid and protein, respectively, was used to determine RNA purity. The absorbance value at 260nm was used to determine RNA concentration (See example in Table 3.1). Total RNA samples were then stored at -80^0C until later use.

3.3.1.2. cDNA first strand synthesis:

cDNA first strand synthesis was performed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Paisley, UK). RNA/primer mixes (5μg of total RNA (see Figure 3.1 for examples of equivalent volume), 1μl of oligodT (50ng/μl) (provided in the kit), 1μl of dNTP mix, up to 10μl with sterilewater) were prepared, incubated for 5min at 65°C and left to cool down on ice. OligodTs were used as they preferentially recognise and lead to the amplification of mRNAs. 9μl of reaction mixture (2 μl of 10xRT buffer, 4 μl of 25mM $MgCl_2$, 2 μl of 0.1M DTT, 1μl of RNaseOUT Recombinant Ribonuclease Inhibitor (all components provided in the kit)) was then added to each RNA/promoter mix, mixed gently and incubated for 10min at room temperature. 1μl of SuperScript™ II RT (provided in the kit) was then added to each sample and the samples left to incubate for 50min at 42°C. The reaction

Sample name	260nm/280nm absorbance ratio	Concentration in µg/ml	Volume used in µl for 5µg of RNA
H36LE2 PC	1.711	1311	3.8
H36LE2 PS	1.805	1643	3
H36LE2 CC	1.710	1211	4
H36LE2 CS	1.734	1429	3.5
9MaB PC	1.929	1588	3.1
9MaB PS	1.763	2559	2
9MaB CC	1.797	2830	1.8
9MaB CS	1.715	3131	1.6
MCF7 PC	1.577	1474	3.4
MCF7 PS	1.687	1408	3.5
MCF7 CC	1.702	2387	2.1
MCF7 CS	1.690	2178	2.3
U373-MG PC	1.790	3111	1.6
U373-MG PS	1.739	1988	2.5
U373-MG CC	1.662	1773	2.8
U373-MG CS	1.758	2035	2.4

Table 3.1 Example of quantification of RNA samples before cDNA synthesis.

was terminated by incubating the samples for 15min at 70°C, after which they were left to cool in ice before storing them at -20°C for later use.

No reverse transcriptase (RT) reaction and control RNA reaction were not run and this for the following reasons. The no RT reaction is intended to detect persistence of DNA and avoid false-positive results. This very role was insured by the positioning of the primers (see 3.3.1.3). The control RNA reaction is intended to provide proof that the cDNA synthesis was successful and avoid false-negative results. This was insured by the amplification of either actin or vimentin mRNA as a positive control for each cDNA sample examined. Because of this redundancy, I judged the use of the no RT reaction and the control RNA reaction unnecessary.

3.3.1.3. Polymerase Chain Reaction (PCR):

Primers were designed against LEDGF and vimentin, using the software available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. Whenever possible, the primers were positioned so that any product amplified from genomic DNA would be much longer than a product amplified from coding DNA, thus allowing the detection of genomic DNA contamination in the sample and preventing false positive detection. In the case of LEDGF, primers were also positioned to avoid detection of p52, the splice variant of LEDGF, which is expressed by lens epithelial cells (Singh *et al.*, 2000). As the DNA sequences used for primer

design are too long to figure in the main text of this thesis, they have been, together with the exact position of the primers designed, added in Appendix 3 at the end of the thesis. The exact sequence of all primer pairs designed and their corresponding predicted product sizes are presented in Table 3.2.

Both primer pairs were optimised by running them through a gradient PCR on a Hybaid MBS 0.2, for temperatures of 55.2^0C, 56.3^0C, 55.7^0C, 57.1^0C, 58.0^0C, 58.9^0C, 59.9^0C, 60.9^0C and 62.1^0C. For vimentin, plasmid cDNA was available for optimisation and 0.1μg of plasmid cDNA was used as a template at each temperature. Plasmid cDNA consisted of Vimentin cDNA cloned into the pEGFP C3 vector (GenBank accession code: U57607) (gift of Dr. Der-Perng, University of Durham). For LEDGF, no plasmid cDNA was readily available and therefore 2μl H36LE2 cDNA was used as a template at each temperature, as I judged H36LE2 to be the cell line most likely to express LEDGF mRNA. Identity of the PCR product was established by sending an aliquot of the PCR reaction, containing the PCR product, to be sequenced by the departmental sequencing facility, after which the sequence obtained (see Appendix 3) was checked by the Nucleotide-Nucleotide option of the Basic Local Alignment Search Tool (BLAST) software.

For the first round of primer optimisation, a range of PCR buffers was used (Roche Diagnostics Ltd., Lewes, UK; Sigma-Aldrich, Poole, UK; Bioline Ltd., London, UK), after which the Sigma PCR buffer was determined to work best and was therefore the only one used in all subsequent reactions. The exact composition of

Target	Accession number	Forward primer sequence	Reverse primer sequence	Optimised against	Expected product size
LEDGF human mRNA	AF-063020	GCCCTGTCCTTCA GAGAGTG	TGCTGCTCAGTTT CCATTTG	H36LE2 cDNA	375 bp
Vimentin human mRNA	NM-003380	TGAAGCTGCTAA CTACCAAGACAC	AGAAGTTTCGTTG ATAACCTGTCC	clone	319 bp

Table 3.2 PCR primers characteristics.

PCR mix	template cDNA: 2µl 10x PCR buffer (SIGMA):10 µl dNTPs mix (2mM):10 µl forward primer (100mM): 2 µl reverse primer (100mM): 2 µl Taq DNA polymerase: 0.4 µl Sterile H_2O: up to a 100 µl

Table 3.3 PCR mix composition.

Gradient PCR 55°C to 62°C (30 cycles)	Denaturation step: 3 min at 94°C. Stage 1 step 1: 30 sec at 94°C Stage 1 step 2: 30 sec at melting temperature (choice of 55°C to 62°C). Stage 1 step 3: 1 min at 72°C. Stage 2: 5 min at 72°C. Stage 3: hold at 4°C.

Table 3.4 Gradient PCR stages.

the PCR mix and the steps the gradient PCR program used are detailed in Table 3.3 and Table 3.4. After PCR, the products were run on a 1.4% (w/v) agarose gel to check for products of adequate size and to determine the optimal temperature for each primer pair. The results of both primers optimisation are presented in Figure 3.1.

PCRs for cDNA samples obtained as described in 3.2.1.1 and 3.2.1.2 were conducted using the same PCR mix and PCR machine as described above but at the temperature that was found to be optimal for each primer pair used. Namely, LEDGF primers were run at 56.3^{0}C and vimentin primers at 58^{0}C.

3.3.2. Protein samples studies:

Western Blotting was conducted to study changes in protein expression in the different cells lines in relation to their culture substrate and exposure to staurosporine.

3.3.2.1. Protein sample extraction:

After the cell viability assay, the remaining cell culture medium was sucked out of the cell culture plates and the cells washed twice in PBS before adding 150µl of 2 x sample buffer, which had been placed at –20^{0}C for 10min. Plates were then put on a shaker at 200rpm at 37^{0}C for 10 min to obtain a viscous cells-buffer mix, which was pipetted into an Eppendorf tube and homogenised with a 2.5ml syringe equipped

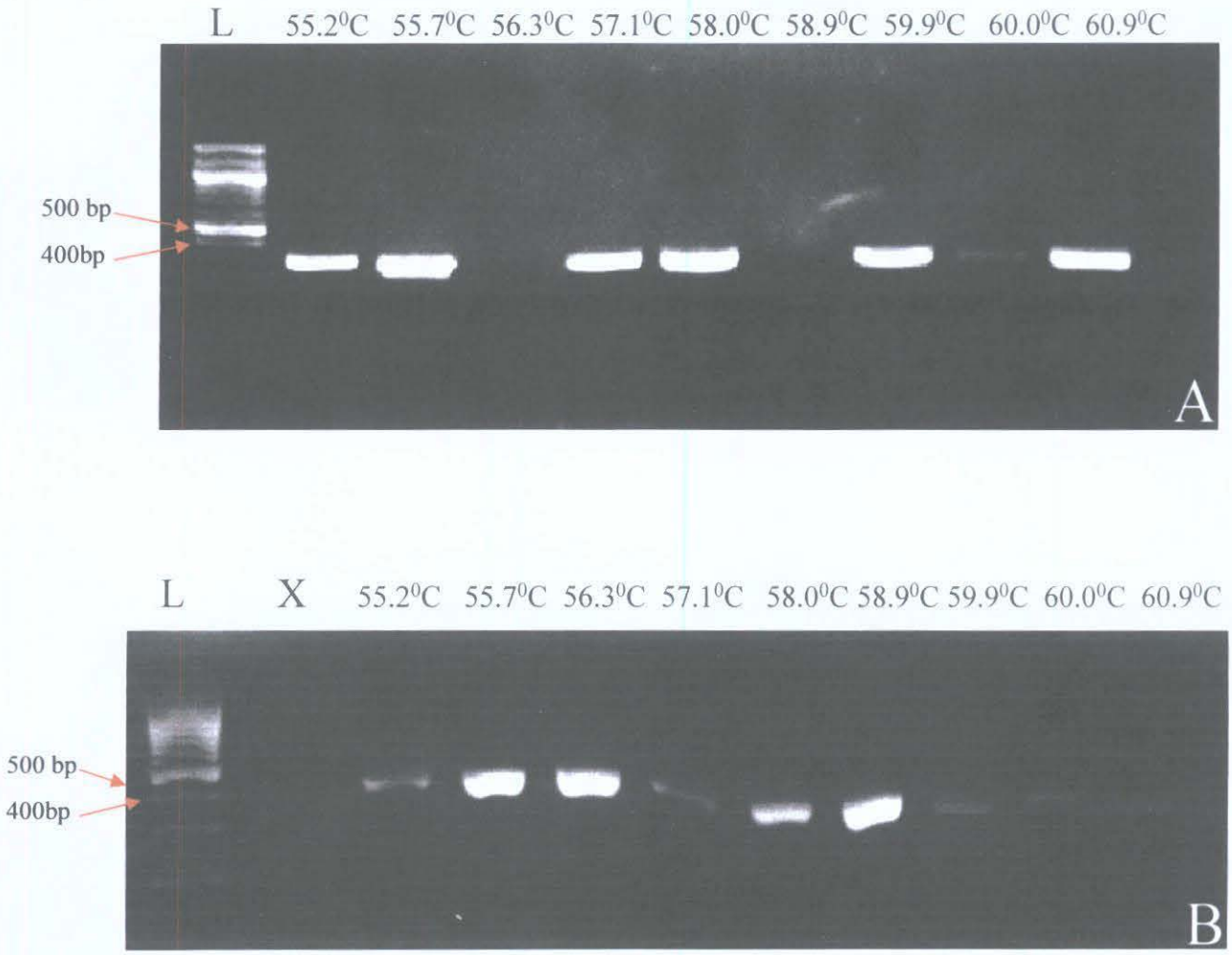

Figure 3.1 Primer pairs optimisation.

Primer pairs for vimentin (A) and LEDGF (B) were designed as described in section 3.3.1.3 and optimised against either a positive vector (vimentin) or H36LE2 cDNA (LEDGF). The identity of the products obtained was confirmed by DNA sequencing followed by BLAST. Expected product size: 319bp (vimentin) and 375bp (LEDGF).

with a 25G needle. Samples were then centrifuged for 5min at 4^0C and 12000rpm, after which they were stored at -20^0C for later use. Protein concentration of the cell extracts was quantified using the Bicinchoninic Acid (BCA) Protein Assay kit. 1µl of each protein sample were diluted with 49µl of distilled deionised (dd) H_2O and added to 1ml of Working Reagent (50 part reagent A to 1 part reagent B) and incubated at 60^0C for 30min, together with a set of bovine serum albumin (BSA) (2mg/mL) standards. The absorbance of each sample at 562nm was then measured on a Beckman DU640 spectrophotometer (Beckman Instruments, Inc., Fullerton, USA) and protein concentrations were calculated by comparison to the BSA protein standards. A new standard curve was generated for every BCA assay conducted. A representative quantification data, together with its BSA standard curve and the equivalent sample volume to be loaded for electrophoresis, are presented in Figure 3.2 and Table 3.5.

3.3.2.2. Western Blotting:

20µg of protein of each sample were run on a hand made sodium dodecyl sulphate (SDS) -polyacrylamide gel with a 4% (w/v) stacking layer and a 12% (w/v) separating layer at 220V for 45min. Positive control was provided by an HSP marker (gift of Dr Der-Perng, Durham University) containing recombinant vimentin, HSP70, GFAP, actin, HSP27, αA-crystallin and αB-crystallin, which was run alongside each set of protein samples.

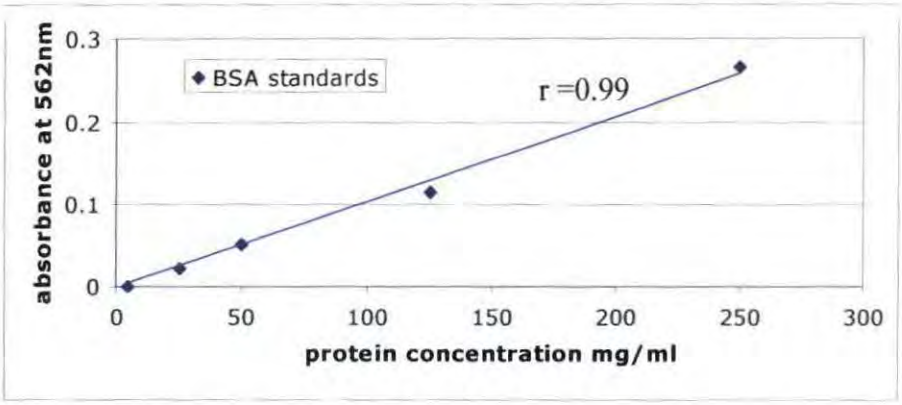

Figure 3.2 BSA standard curve used during the protein sample quantification below.

Sample name	Absorbance at 562 nm	Concentration in µg/ml	Volume used in µl for 20µg of protein
H36LE2 PC	0.1285	6651	3
H36LE2 PS	0.0634	3488	5.6
H36LE2 CC	0.0733	3993	5
H36LE2 CS	0.2820	13121	1.4
9MaB PC	0.1371	7041	2.8
9MaB PS	0.0843	4537	4.4
9MaB CC	0.1033	5462	3.6
9MaB CS	0.1115	5853	3.4
MCF7 PC	0.0617	3403	5.8
MCF7 PS	0.0646	3551	5.6
MCF7 CC	0.3658	16245	1.2
MCF7 CS	0.2825	13138	1.4
U373-MG PC	0.1255	6510	3
U373-MG PS	0.1019	5396	3.6
U373-MG CC	0.1971	9680	2
U373-MG CS	0.0987	5242	3.8
SW13+ PC	0.1038	5487	3.6
SW13+ CC	0.2088	10176	2
SW13- PC	0.1025	5422	3.6
SW13- CC	0.1655	8317	2.4

Table 3.5 Example of quantification of protein samples for immunoblotting.

Once run, the gel was blotted onto an Hybond^{TM}-C Extra nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) at 50mA for 2h on a semi-dry blotting machine. From bottom to top, the blotting stack was composed of six paper layers soaked in blotting buffer A, three paper layers soaked in buffer B, the nitrocellulose membrane soaked in buffer B, the SDS-polyacrylamide gel rinsed in buffer B and nine paper layers soaked in buffer C. After blotting, the nitrocellulose membrane was stained with ponceau red (0.2% g/ml ponceau, 3% trichloro-acetic acid) for 5min to check that the protein bands had adequately transferred onto the membrane, after which the membrane was cut as shown in Figure 3.3. Each membrane fragment was then rinsed in Tween 20-Tris-buffered saline (TTBS) once for 5min, then washed in blocking buffer for 1h on the shaker at room temperature. They were then rinsed twice for 5min in washing solution on the room temperature shaker, and left overnight at 4^0C in the primary antibody solution. The next morning, the membrane fragments were rinsed twice for 10min in washing solution on the room temperature shaker, incubated with the secondary antibody solution for 1h on a bacterial shaker (37^oC), and rinsed twice 10min in washing solution on the room temperature shaker. The membrane fragments were then incubated for 5min in an enhanced chemiluminescence (ECL) solution in the dark room and exposed by chemiluminescence for 2min to 15min in a Fujifilm Intelligent Dark Box II. Images were captured via the software LAS-1000. The composition of all the solutions used during protein extraction, SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting are detailed in Table 3.6 and Table 3.7. The characteristics of all the antibodies used during immunoblotting are presented in Table 3.8.

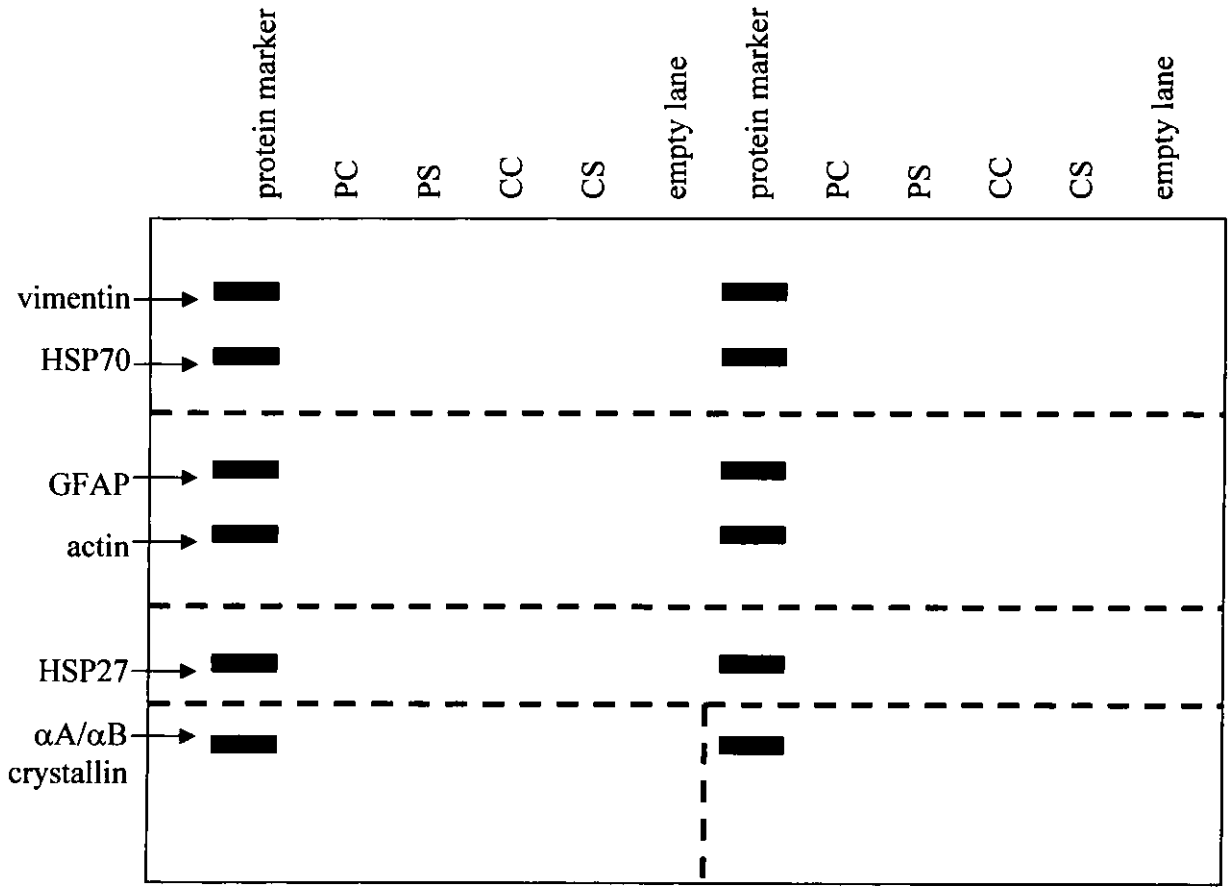

Figure 3.3 Cutting pattern for immunoblotting.

The diagram above represents the spatial arrangement following which gels used during immunoblotting were run and cut before blotting. The cutting pattern (indicated by the dashed lines) was designed to maximise the amount of results per gel run. Note that this results in the same actin control being used for HSP27 and either αA-crystallin or αB-crystallin.

In the case of the SW13 protein extracts the treatments were Sw13vim+ PC, SW13vim+ CC, SW13vim- PC, SW13vim- CC instead of PC,PS,CC,CS.

Lane legend:

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine

2 x Sample extracting buffer	2ml 0.5M Tris-HCl pH=6.8 4ml 40% (v/v) glycerol 40µl 0.5M EDTA, 2ml 10% (w/v) SDS made up to 10ml with sterile H_2O
5 x Sample loading buffer	2.25ml 1M Tris-HCl pH=6.8 5ml glycerol, 0.5g SDS 5mg bromophenol blue 2.5ml 1M DTT
Separating gel (for 2 gels, 12% (g/ml))	4.35ml of 40% (w/v) acrylamide 3ml of sterile water 2.5ml of 1.5M TrisHCl pH=8.8 100µl of 10% (w/v) SDS 50µl of 10% (w/v) APS 5µl of TEMED
Stacking gel (for 2 gels, 4% (g/ml))	1ml of 40% (w/v) acrylamide 6.35ml of sterile water 2.5ml of 1.5M TrisHCl pH=8.8 100µl of 10% (w/v) SDS 50µl of 10% (w/v) APS 10µl of TEMED
SDS gel Running Buffer	100ml Tris-Glycine (1l Tris-Glycine: 30.3g Tris 144.1g glycine up to 1l with ddH_20) 10ml 10% (w/v) SDS up to 1l with ddH_20

Table 3.6 List of the solutions used during SDS-PAGE.

Blotting buffer A	3.6g Tris, 20ml of 100% MeOH up to 100ml with dd H_2O, pH=10.4
Blotting Buffer B	0.3g Tris, 20ml of 100% MeOH up to 100ml with dd H_2O, pH=10.4
Blotting buffer C	0.3g glycine, 0.3g Tris, 20ml of 100% MeOH up to 100ml with dd H_2O, pH=9.4
Tris –buffered saline (TBS) (10x)	200ml of TrisHCl pH 7.4, 87.66g of NaCl up to 1l with ddH_2O
TTBS	1ml of Tween 20, 5ml of 10x TBS up to 500ml with ddH_2O
Blocking buffer	2.5g Marvel Milk up to 50ml of TTBS
Washing solution	15g of Marvel milk 250ml of TTBS, 25ml of 10x TBS up to 500ml with ddH_2O
Primary antibody solution	6xC ml of the antibody of interest (C: concentration of the antibody of interest) 100µl of BSA 5mg/ml up to 6ml with TTBS
Secondary antibody solution	6µl of mouse HRP-conjugated immunoglobulins (primary antibody monoclonal) up to 6mL with TTBS
ECL solutions (ECL I and ECL II are to be mixed just before addition to the membrane to give the final ECL solution)	ECL I: 44µl of coumaric acid, 100µl of luminol 1ml of 1M Tris HCL pH 8.5 up to 10ml with ddH_2O ECL II: 6µl of 30% H_2O_2 1ml of 1M Tris HCL pH 8.5 up to 10ml with ddH_2O

Table 3.7 List and composition of all immunobloting solutions used.

Target	Name	Origin	Source	Concentration used
αA-crystallin	anti aA-crystallin	monoclonal	P.Fitzgerald	1/1000
αB-crystallin	mono aB batch 4	monoclonal	R.A. Quinlan	1/10
HSP27	82p1.4	monoclonal	R.A. Quinlan	1/10
Actin	AC-40	monoclonal	SIGMA	1/1000
Vimentin	V9	monoclonal	SIGMA	1/1000
All primary monoclonals	Goat anti-mouse HRP	N/A	DAKO	1/1000

Table 3.8 Characteristics of all antibodies used for immunoblotting.

Commercial antibodies were obtained from Sigma-Aldrich, Poole, UK and from DakoCytomation, Olestrup, Denmark.

3.4. Results:

3.4.1. LEDGF mRNA expression:

cDNA samples, synthesised from total RNA extracts from H36LE2, 9MaB, MCF7 and U373-MG cells were subjected to PCR to determine whether the mRNA expression pattern of LEDGF could be correlated with the cell viability patterns observed in Chapter 2.

LEDGF was chosen as it is a known transcription factors of αB-crystallin and HSP27 genes (see section 1.4.2.3.2), themselves known for their anti-apoptotic properties expression was examined, as these crystallins are highly concentrated in the lens and known for their anti-apoptotic properties (see sections 1.4.2.3.3).

As shown in Figure 3.4, LEDGF mRNA expression was not detected on the lens capsule, and this for all four cell lines examined. LEDGF mRNA expression was also absent on the plastic substrate for both MCF7 and U373-MG cells, but was present on the plastic in the absence of staurosporine for both H36LE2 and 9MaB. With the addition of staurosporine on plastic LEDGF mRNA expression was still detected in 9MaB cells, but not in H36LE2 cells.

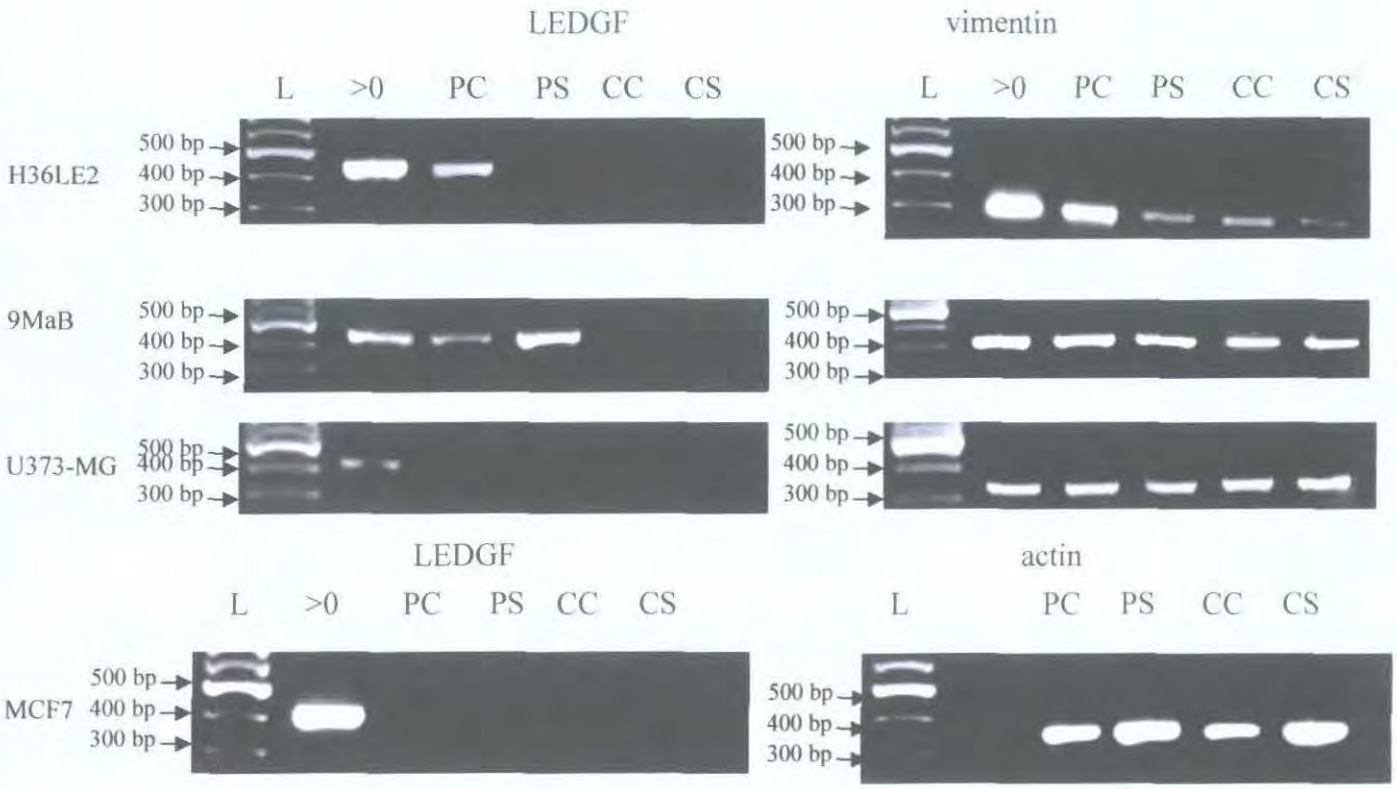

Figure 3.4 LEDGF mRNA expression in H36LE2, 9MaB, MCF7 and U373-MG cells.

H36LE2, 9MaB, MCF7 and U373-MG cells were seeded on either plastic or bovine lens capsule substrates and, after a 48h recovery period, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, mRNA extracts were collected and the corresponding cDNA was checked for LEDGF presence by RT-PCR (as described in section 3.3.1). For H36LE2, 9MaB and U373-MG cDNA, vimentin was used as a positive control. For MCF7 cDNA, actin was used as a positive control. Actin primers were a gift of Dr. M. Alvares-Reyes, University of Durham (expected product size 316 bp). As no positive control was used by the original investigator, the identity of the product obtained with the actin primers was confirmed by sequencing the product via the departmental sequencing facility, after which the sequence obtained was checked by the Nucleotide-Nucleotide option of the Basic Local Alignment Search Tool (BLAST) software.

Note that LEDGF cDNA is not detected on the capsule for all four cell lines. LEDGF cDNA is detected in both H36LE2 and 9MaB cells grown on the plastic but not in MCF7 or U373-MG cells.

The experiment was repeated three times independently. One representative result is shown. Note that the 9MaB and U373-MG PCR products shown were run on the same agarose gel, hence the same ladder was used for both sets.

Lane legend:

L: DNA 100bp ladder >0:positive control (either vimentin or H36LE2 cDNA)

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine

3.4.2. *Small heat shock proteins expression:*

Protein extracts from H36LE2, 9MaB, MCF7 and U373-MG cells were examined by immunoblotting to determine whether the protein expression pattern of small heat shock proteins could be correlated with the cell viability patterns observed in Chapter 2.

αA-crystallin and αB-crystallin expression was examined, as these crystallins are highly concentrated in the lens and known for their anti-apoptotic properties (see sections 1.4.2.3). HSP27 expression was also examined, as it has anti-apoptotic functions and can be expressed under the action of LEDGF (see section 1.4.2.3.2 and 1.4.2.3.3).

As seen in Figure 3.5, αA-crystallin expression was undetectable on the plastic for all four cell lines examined. It was also undetectable for MCF7 and U373-MG cells on the lens capsule. However, αA-crystallin expression was detectable on the lens capsule in both lens epithelial cells, although it did not consistently vary with the addition of staurosporine

As seen in Figure 3.6, αB-crystallin protein expression was, as for αA-crystallin protein expression, undetectable on the plastic for all four cell lines examined. It was also undetectable on the lens capsule in MCF7 cells. αB-crystallin

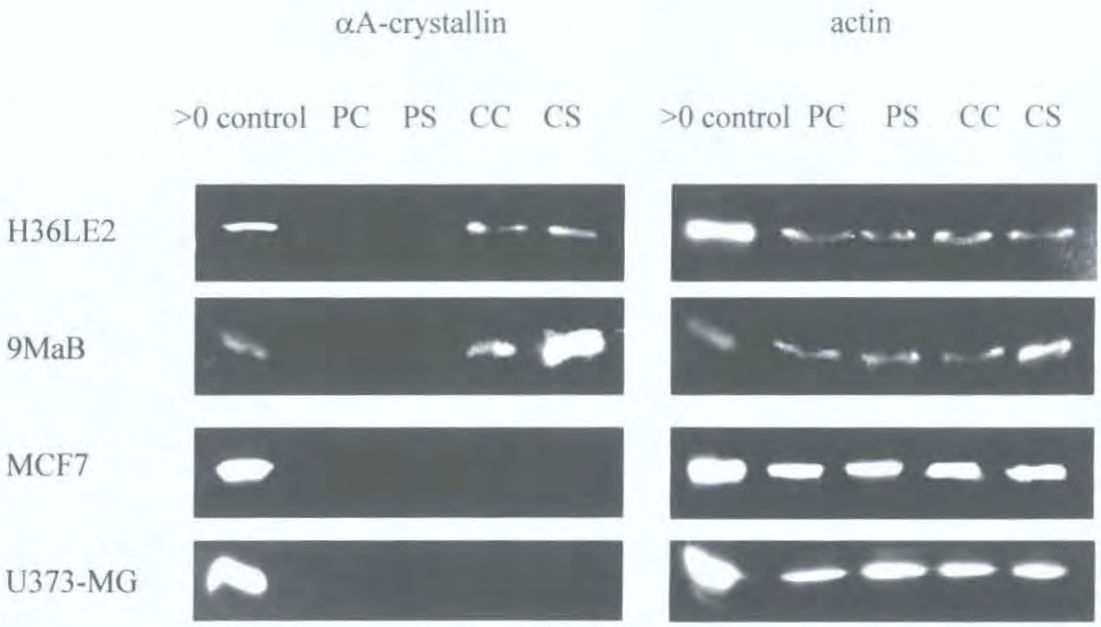

Figure 3.5 αA-crystallin protein expression is present in lens epithelial cells on the lens capsule.

H36LE2, 9MaB, MCF7 and U373-MG cells were seeded on either plastic or bovine lens capsule substrates and, after a 48h recovery period, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, whole cells extracts were collected and αA-crystallin expression was investigated by immunoblotting (as described in section 3.3.2).

Note that αA-crystallin expression is not detected on the plastic for all four cell lines. αA-crystallin expression is detected in both H36LE2 and 9MaB cells grown on the lens capsule but not in MCF7 or U373-MG cells.

The experiment was repeated three times independently. One representative result is shown.

Lane legend:

>0 control: recombinant protein marker

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine

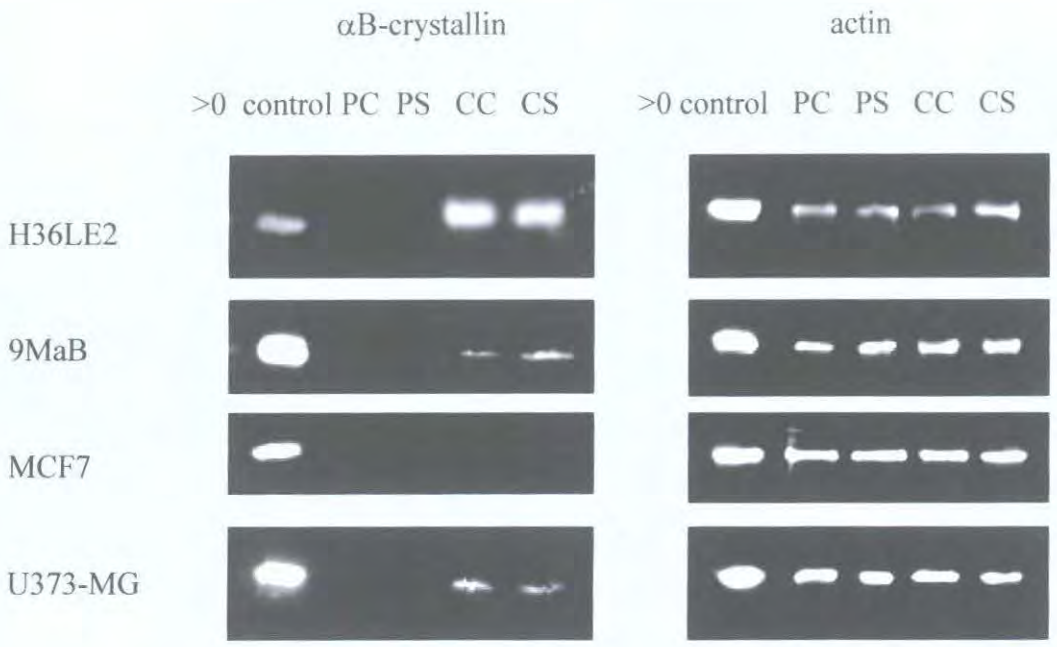

Figure 3.6 αB-crystallin protein expression is present in lens epithelial cells on the lens capsule.
H36LE2, 9MaB, MCF7 and U373-MG cells were seeded on either plastic or bovine lens capsule substrates and, after a 48h recovery period, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, whole cells extracts were collected and αB-crystallin expression was investigated by immunoblotting (as described in section 3.3.2).

Note that αB-crystallin expression is not detected on the plastic for all four cell lines examined. However, αB-crystallin expression is detected on the lens capsule in both H36LE2 and 9MaB cells, as well as in U373-MG cells, but not in MCF7 cells.

The experiment was repeated three times independently. One representative result is shown.

Lane legend:

>0 control: recombinant protein marker

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine

protein expression was detectable on the lens capsule in H36LE2, 9MaB and U373-MG cells, but did not consitently vary with the addition of staurosporine.

As seen in Figure 3.7, HSP27 protein expression was undetectable in all cells and for all four treatments, with the sole exception of MCF7 cells on the plastic in the absence of staurosporine, when HSP27 protein expression was detected.

3.4.3. HSP27 expression correlates with vimentin expression and cell viability on the lens capsule in SW13 cells:

In Chapter 2, I showed that the presence of vimentin correlated with SW13 cells viability on the lens capsule. In this Chapter, I examined whether the expression of small heat shock proteins could also be correlated with cell viability on the lens capsule in SW13 cells. Again, I examined the protein expression of αA-crystallin, αB-crystallin and HSP27.

As shown in Figure 3.8, both αA-crystallin and αB-crystallin expression was detected when SW13vim+ and SW13vim- cells were grown on the lens capsule, although αA-crystallin protein expression was very faint for both cell lines. HSP27 expression was also detected when the cells were cultured on the lens capsule, but only in the SW13vim+ cells.

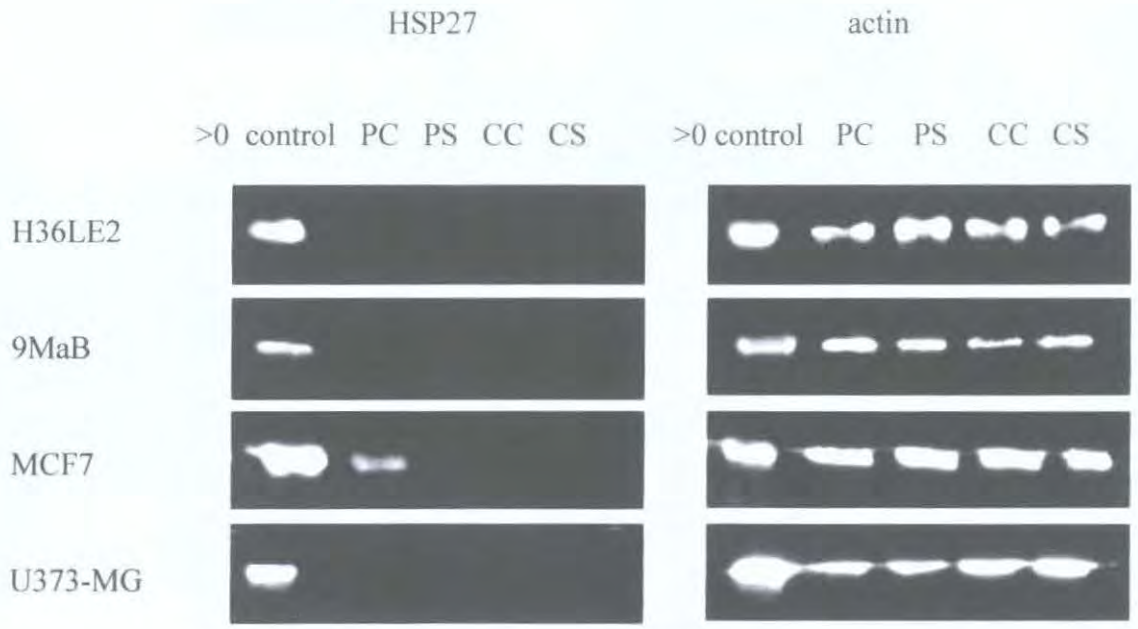

Figure 3.7 HSP27 protein expression is absent on the lens capsule.

H36LE2, 9MaB, MCF7 and U373-MG cells were seeded on either plastic or bovine lens capsule substrates and, after a 48h recovery period, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, whole cells extracts were collected and HSP27 expression was investigated by immunoblotting (as described in section 3.3.2).

Note that HSP27 protein expression is absent in all four cell lines both on plastic and on the lens capsule, with the only exception of MCF7 cells on plastic in the absence of staurosporine.

The experiment was repeated three times independently. One representative result is shown.

Lane legend:

>0 control: recombinant protein marker

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine

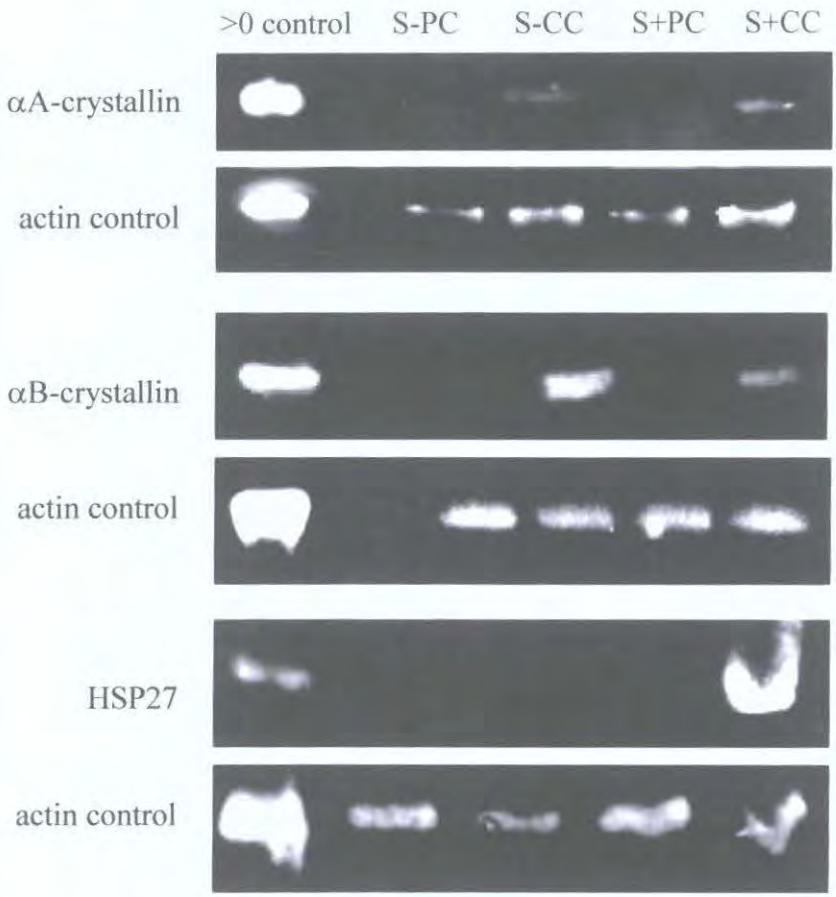

Figure 3.8 HSP27 protein expression differs between SW13vim+ and SW13 vim- cells on the lens capsule.

SW13vim+ and SW13vim- cells were seeded on either plastic or bovine lens capsule substrates. After 96h, whole cells extracts were collected and αA-crystallin, αB-crystallin and HSP27 protein expression was investigated by immunoblotting (as described in section 3.3.2).

Note that, although both cell lines express αA-crystallin and αB-crystallin when cultured on the lens capsule, SW13vim+ cells express HSP27 when grown on the lens capsule while SW13vim- cells do not.

The experiment was repeated three times independently. One representative result is shown.

Lane legend:

>0 control: recombinant protein marker

S+PC: plastic substrate, SW13vim+ cells S+CC: capsule substrate, SW13vim+ cells

S-PC: plastic substrate, SW13vim+ cells S-CC: capsule substrate, SW13vim+ cells

3.5. Discussion:

3.5.1. The case of the in vitro cell line:

Most of the proteins described in the introduction to this chapter were readily detected and characterised *in vivo* (see section 3.1) and, with care, can be used as lens-specific markers. In *in vitro* cell lines, however, the artificial culture conditions can affect protein expression patterns. The presence of serum decreases both αA-crystallin and αB-crystallin expression in bovine lens epithelial cells after as little as two weeks of culture (Kim *et al.*, 2004), and even the well-characterised HLE-B3 was shown to lack several proteins found in primary lens epithelial cells, notably αA-crystallin and HSP27 (Wang-Su *et al.*, 2003). Also, some lens epithelial cells *in vitro* have been shown to either drift towards a fibre phenotype, in low serum conditions, or an epithelial-fibre hybrid phenotype, in high serum conditions, as indicated by ATP1As and crystallins expression patterns (Ong *et al.*, 2003). These changes in protein expression might lead to an originally lens epithelial cell line being identified as having lost some lens specificity or acquired some fibre-specificity.

Therefore, the protein expression of the lens cell lines used in our study might not perfectly match the protein expression pattern expected from *in vivo* lens epithelial cells.

3.5.2. Culture on the lens capsule correlates with the expression of α-crystallins:

The main result of this chapter is that cell culture on the bovine lens capsule correlates with the expression of α-crystallins, even, in some cases, when the cultured cell line did not originate from the lens.

3.5.2.1. Protein expression pattern in lens epithelial cells:

Substrate-induced tissue-specific protein expression is well-documented, (Sultana *et al.*, 1998; Streuli *et al.*, 1991) and was therefore expected in the presence of a freshly-dissected lens capsule, which is the natural matrix of lens epithelial cells *in vivo*.

αA-crystallin and αB-crystallin expression was detected for both lens cell lines tested on the lens capsule, but not on the plastic substrate. Such up-regulation cannot be due to LEDGF activity, as LEDGF's detection pattern does not match with the crystallins'. Other potential mediators of this up-regulation include Pax6, which I was sadly unable to examine by PCR due to a failed primer optimisation stage. The absence of crystalline protein signal on the plastic can be explained, either by the effects of *in vitro* culture on tissue-specific protein expression, or by the amount of protein loaded in the gels, which might have been too low to allow detection.

HSP27 protein expression was examined, not for the purposes of lens-specificity, as HSP27 is a widely expressed protein (Ciocca *et al.*, 1993), but rather for its anti-apoptotic properties (detailed in section 1.4.2.3). However, no HSP27 expression was detected in any of the lens cell lines tested. This absence of HSP27 in lens cells cannot be due to defective antibodies, as HSP27 was detected in MCF7 cells, which was expected since HSP27 is expressed in their tissue of origin (Ciocca *et al.*, 1993). It can also not be due to defective protein extracts, as shown by the presence of bands when the same extracts were exposed to actin antibodies. It can only be suggested that the expression might have been lost with a lengthened *in vitro* culture, as has been demonstrated in other lens epithelial cell lines (see section 4.3.1).

3.5.2.2. Protein expression pattern in non-lens epithelial cells:

Protein expression of crystallins was detected in a number of non-lens cell lines. As the lens capsule used in our experiments are freshly dissected, the persisting expression of lens proteins might be due to native bovine lens cells remaining on the capsule after an incomplete trypsinisation. This, however, is unlikely, as the ethanol wash performed during the capsule clean-up, apart from being expected to efficiently kill any remaining cell, also make the cells turn white and therefore highly visible, allowing for their extensive removal by scraping. Therefore, I am confident in saying that protein expression of lens crystallins by non-lens cells when grown on the lens capsule is a true result and is not due to primary lens cells remaining attached to their substrate.

U373-MG cells are known to express αB-crystallin (Ito *et al.*, 2001; Tumminia and Russel, 1994), and its presence in my results is therefore not surprising. SW13vim+ and SW13vim-, however, were less expected to readily express crystallins proteins, even when grown on a lens substrate. Both crystallins are actually present in adrenal tissue, although at low levels (Kato *et al.*, 1991), and their detection on the lens capsule in the SW13 cells might be the result of the up-regulation of an already existing low-level expression, the means of which are still undetermined

These results suggest that culture on the lens capsule is able to up-regulate α-crystallins protein expression in non-lens cell lines. The exact nature of the lens capsule factors able to up-regulate such protein expression was not investigated. Structural components such as collagen, laminin or fibronectin are too commonly found in basement membranes to mediate such a specific protein expression. However, it can be suggested that growth or transcription factors present in the lens environment *in vivo* might have been stored in the capsule and be released during *in vitro* culture, thus re-creating an micro-environment favourable to lens protein expression.

3.5.3. *Are lenticular proteins involved in cell viability on the lens capsule?:*

As this study is primarily concerned with the viability of lens epithelial cells when grown on the lens capsule, the next important question is whether sHSP

expression can be correlated with cell viability both in the presence and absence of staurosporine.

All three sHSPs examined, αA-crystallin, αB-crystallin and HSP27 are well known for their anti-apoptotic activities (see section 1.4.2.3). Of course, any cell viability pathway is likely to involve a number of proteins greater than the ones covered in our study and the absence of a strict correlation between sHSP expression and viability in the absence or presence of staurosporine cannot be equated with them not being involved. If a cell line is viable in the absence of a given HSP, then this HSP can be described as not involved in the cell viability pathways. However, if a cell line is not viable in the presence of a given HSP it only means that this HSP is not sufficient to promote cell viability by itself. It can still be involved in the pathways, with the cell line lacking downstream factors.

Using this rationale, I can suggest that HSP27 is not involved in either lens cell viability on the lens capsule, as it is absent from all lens epithelial cells tested.

As for αA-crystallin and αB-crystallin, in the limited set of cell lines examined, the simultaneous expression of both α-crystallins correlated well with cell viability on the lens capsule, both in the absence and presence of staurosporine. The independent expression of αB-crystallin alone is also not sufficient to insure cell viability, as shown in U373-MG cells. The case of the SW13 vim cells is discussed later (see section 3.5.4).

To test the hypotheses that the presence of both α-crystallin is necessary to maintain cell viability on the lens capsule, it was my intention to selectively down-regulate their expression in the appropriate cell lines using small RNA interference (sRNAi) (Shi, 2003; Hasuwa *et al.*, 2002; Martinez *et al.*, 2002). Although I did succeed in optimising the sRNAi procedure on plastic, I lacked time to fully test it on the lens capsule and therefore no results are presented.

3.5.4. HSP27 expression correlates with vimentin expression and cell viability on the lens capsule:

As detailed in Chapter 2, when testing vimentin-expressing/vimentin-lacking pairs of SW13 cell lines, it was noted that the presence of vimentin correlated with cell viability on the lens capsule in the absence of staurosporine. To test whether this pattern could be further correlated with the expression of other proteins, I examined the expression of αA-crystallin, αB-crystallin and HSP27 in those same cell lines.

Although αA-crystallin and αB-crystallin protein expression patterns did not consistently differ between the two cell lines, a strong HSP27 expression, however, did correlate with SW13 cell viability on the lens capsule. As HSP27 is known to bind intermediate filaments *in vitro* (Perng *et al.*, 1999), its presence might reflect a chaperone function towards the vimentin network rather than a direct involvement in cell proliferation. However, HSP27 can affect cell-cell and cell-matrix adhesion independently of the vimentin network. Its over-expression leads to an increase in E-

cadherin expression (Aldrian *et al.*, 2003) and endogenous HSP27 can induce or inhibit actin polymerisation, depending on its phosphorylation state (Gerthoffer and Gunst, 2001), which will in turn affect focal adhesions (Schneider *et al.*, 1998). Although the resulting effect is not to increase cell proliferation (Kindas-Mugge *et al.*, 1998), it could increase cell adhesion to the substrate and between cells within the population, allowing a stable number of cells to be maintained on the lens capsule.

The absence of HSP27 expression in the lens cell lines tested, however, suggest that other mechanisms, independent of HSP27, must exist that promote lens cell viability on the lens capsule.

3.6. Chapter's conclusions:

The aim of this chapter was to determine whether protein expression could be correlated with cell viability on the lens capsule in the cell lines examined. By the end of this chapter, I can say that correlations between protein expression and cell viability can be made, but only when looking at reduced subsets of cell lines. When comparing H36LE2 and 9MαB cells to U373-MG and MCF7 cells, a correlation between the presence of both αA-crystallin and αB-crystallin and cell viability on the lens capsule can be made. When comparing SW13vim+ cells and SW13vim- cells, a correlation between the presence of HSP27 and increased cell viability on the lens capsule can be made.

4. The lens capsule as a store of releasable growth factors for lens epithelial cells:

In Chapter 3, I have shown that cell culture on the lens capsule correlates with increased crystallins protein expression. In the present Chapter 4, I will examine a number of extra-cellular factors, in an attempt to test their involvement in cell viability on the lens capsule. First, it is necessary to examine the range of extra-cellular factors available to lens cells on the lens capsule.

4.1. Introduction: Agents of cell-matrix interactions in the lens:

The extra-cellular matrix of the lens fulfils many roles. It is the adhesive base of both epithelial and early fibre cells, the physical barrier between all lens cells and the aqueous and vitreous humours and the elastic mediator of lens accommodation. Most of these functions have already been detailed in the introduction to Chapter 2. The present Chapter 4, however, is concerned with the interactions between the lens epithelial cells and the lens capsule specifically involved in maintaining cell viability.

4.1.1. The Integrins: attachment-dependent survival

Although integrin ligation in suspended cells can increase sensitivity to p53-mediated apoptosis (Lewis *et al.*, 2002), in adherent cell lines ECM adhesion via integrins is generally accepted to promote survival by a variety of pathways.

Fibronectin binding of β1-integrins induces membrane-bound FLIP long ($FLIP_L$) re-distribution as cytosolic $FLIP_L$, followed by competitive inhibition FADD binding, in the hematopoietic cancer cell line U937 (Shain *et al.*, 2002). Laminin binding of α6β1 integrins increases IRS-1 tyrosine phoshorylation following insulin binding and amplifies IGF signalling and its subsequent anti-apoptotic effects in murine mammary epithelial cells (Farelly *et al.*, 1999). Fibronectin binding to α5β1 or vitronectin binding to αvβ3 induce FAK-dependent activation of Ras, followed by PI3K-dependent AKT/PKB activation and Bcl-2 expression in CHO cells (Matter and Ruoslahti, 2001).

In the healthy lens, α3β1 is found in both lens epithelium and lens fibres, while α6β1 is restricted to the fibres cells and the α1 and α5 subunits are undetectable (Menko and Philip, 1995). Both α3 and α6 forms bind to laminin (Zhang *et al.*, 2000; Zuk and Hay, 1994), which is readily found in the lens capsule, while the β1 form suggests the existence of survival promotion in the lens cells via attachment to the lens capsule. With cataract, the integrin expression of the lens epithelial cells changes, with an increase in α2 and α5 sub-units (Zhang *et al.*, 2000), which is compatible with an increase in collagen I and fibronectin ECM deposition, respectively (Joo *et al.*, 1999). The formation α5β1-fibronectin adhesion might provide extra survival signals to the cataractous cells, and should be maintained during after-cataract as TGFβ-2, which is present in the after-cataractous capsular bag, increases the expression of the α5 integrin subunit in other cell types (Irving and Lala, 1995). Also, and as already discussed (see section 3.3.4), the presence of

laminin in the lens capsule might favour α6β4-mediumted cell adhesion and, indirectly, α6β4-mediumted cell survival via PI3K and ERK signalling (Zahir and Weaver, 2004).

4.1.2. Growth Factors and proteases: diffusible factors of proliferation

The presence of ECM components improves the effects of growth factors. As an example, addition of either laminin or fibronectin to neuronal cells greatly increases the survival effect of nerve growth factor (NGF) (Millaruello *et al.*, 1988). In the lens, capsule components can bind growth factors, thus affecting their activity and availability.

4.1.2.1. Binding of growth factors into the ECM:

Components of the lens capsule are able to bind a range of growth factors. The binding of a number of FGFs to heparin has been widely studied and both FGF-1 and FGF-2, bound to heparan sulphate proteoglycans, are readily detectable in the lens capsule (Schulz *et al.*, 1993). Collagen I, which is deposited in the lens capsule after cataract surgery (Saika *et al.*, 1998), can also bind FGF-2 *in vitro* (Kanematsu *et al.*, 2004). Fibronectin can bind IGF-1/IGFBP3 complexes (Gui and Murphy, 2001), while the proteoglycan decorin, which is expressed by lens epithelial cells after cataract surgery (Azuma and Hara, 1998) can bind TGFβs (Fowlkes and Winkler, 2002).

Binding of growth factors can affect the short-term proliferation of the overlying cells. This effect can be mediated by the stabilisation of an otherwise easily degradable growth factor, as in heparin binding of both FGF-2 and TGFβ-2, which prevents loss of their activity at body temperature (Caldwell *et al.*, 2004; Shroeder-Tefft *et al.*, 1997). ECM binding can also be necessary for the correct formation of cell receptor-growth factor complexes, as in heparin-mediated dimerisation and phosphorylation of FGFRs (Zhang *et al.*, 2001; Kan *et al.*, 1996). This participation of heparan sulphate proteoglycans in FGF signalling also presents a degree of cell-specificity, dependent on the sulphation levels of the heparan chains (Rossi *et al.*, 2003). Hence, cells might benefit from matrix-bound FGF only when grown on their original matrix.

For a delayed effect on cell proliferation and survival, growth factors can also be bound in ECM stores. Such stores have been found for latent TGF-β1 and BMP-2 in the bone matrix (Lalani *et al.*, 2003) and for FGF-1 and FGF-2 in the lens capsule (Lovicu and McAvoy., 1993; Jeanny *et al.*, 1987).

4.1.2.2. Release of growth factors from ECM stores:

For the cells to benefit from any growth factors stored in the ECM, they need to degrade the matrix components that cover or bind them. In the lens epithelial cells, a number of proteases are present which can effect this degradation.

MMPs are Zn^{2+}-dependent endopeptidases secreted as inactive zymogens. They are composed of an N-terminal pro-domain, which is cleaved to release the active MMP, a Zn^{2+}-binding active site, a hemopexin-like domain, and, in the transmembranous members of the family, a C-terminal transmembrane domain (Stetler-Stevenson and Yu, 2001). The collagenase MMP-1, the stromelysin MMP-3 and the gelatinases MMP-2 and MMP-9 are present in the lens after cataract surgery (Kawashima *et al.*, 2000) and taken together, are able to degrade collagens I to V, VII, X and XI, as well as fibronectin and laminin (Wong *et al.*, 2002). They also cleave known growth factors binding proteins, such as IGFBPs, the FGF-binding perlecan and the TGFβ-binding decorin (Fowlkes and Winkler, 2002). The only limitation to their activity in the lens is the presence of the tissue inhibitor of MMP (TIMP) -1 and -2 (Kawashima *et al.*, 2000).

Another Zn^{2+}-dependent protease, a disintegrin and metalloprotease (ADAM)-9, is also present in the lens (Lim *et al.*, 2002). ADAMs resemble MMPs in their possession of an N-terminal prodomain and Zn^{2+}-binding active domain, but they also have a disintegrin domain involved in cell-cell adhesion, and a cysteine-rich and an EGF repeat domain thought to be involved in substrate recognition (Moss *et al.*, 2001). ADAM9 is expressed on the cell surface and, outside the lens, has been shown to have collagenase activity as well as cleaving EGF binding proteins (Moss *et al.*, 2001)

The existence of another proteolytic pathway in the lens is suggested by the presence of tissue plasminogen activator (t-PA) in both the lens epithelial cells

(Tripathi *et al.*, 1988) and the aqueous humour (Giedrojc *et al.*, 1996). Also, plasmin itself is likely to be present in the aqueous humour as injection of plasmin activator in the eye successfully resolves postoperative fibrinous reactions in cataract patients (Muller-Jensen and Zimmerman, 1995). As the aqueous humour is in contact with the capsular bag during and after cataract surgery, any plasmin present in the aqueous could start degrading lens capsule components. Plasmin degrades laminin, fibronectin (Indyk *et al.*, 1999) and FGF-binding perlecan (Stetler-Stevenson and Yu, 2001) and promotes proliferation in retinal pericytes (Katsura *et al.*, 2000). Increase of t-PA expression by TGF-βs (Schacke *et al.*, 2002), together with therapeutic use of plasmin in the eye (Williams *et al.*, 2001) could exacerbate or maintain such matrix degradation. Lastly, the cysteine protease cathepsin B, which degrades collagen IV, laminin and fibronectin (Buck *et al.*, 1992) has been found in the lens epithelium (Wasselius *et al.*, 2003) and could participate in ECM degradation.

4.2. Aims:

The lens capsule is rich in growth factor binding proteins and the lens epithelial cells are rich in matrix proteases (see 4.1.2). In this Chapter, I wanted to investigate whether soluble extra-cellular factors were involved in the effects of the lens capsule on lens cell viability. First I examined whether the levels of FGF-2 and IGF-1,which are relevant to lens cell biology (see section 1.1.2 and section 1.1.3), could be correlated with cell viability. I then tested the hypothesis that the growth factors present in the lens cells medium were released by MMPs directly from the lens capsule.

4.3. Experimental procedure:

In this Chapter, the range of cell lines used was reduced compared to the previous two Chapters, due to the excessive cost of the enzyme-linked immunofluorescence substrate assay (ELISA) kits. H36LE2 was chosen as the only lens epithelial cell line used, as the presence of a αB-crystallin mutation would have complicated the interpretation of the results for the 9MaB cell line. MCF7 was chosen as the non-lens epithelial cell line to be compared to H36LE2, as previous work has been done on the effects of growth factor presence on MCF7 cells (Thompson and Kakar, 2005; Ciftci and Trovitch, 2003; Serrero and Lu, 2001; Wang *et al.*, 1998), which could therefore been called upon during the discussion of this section's results.

4.3.1. ELISA:

ELISAs were conducted to detect changes in medium levels of FGF-2, IGF-1 and TGFβ-2 with cell culture on the lens capsule and presence of staurosporine. The medium samples used for the ELISAs consisted of medium taken at the end of the viability assay described in 2.3.4, before the medium change and the addition of colorimetric reagent. 1ml of medium was taken for each well used in the viability assay, for a total of six samples per treatment for each experiment. The medium samples were then stored at -80^0C until later use.

IGF ELISAs were conducted using the OCTEA^R IGF-1 kit (ImmunoDiagnostic Systems Ltd., Bolden, UK) following the manufacturer's instructions. 25µl of calibrating or test sample were pipetted into Eppendorfs. 100µl of releasing agent were added to each tube, which were then vortexed before being left at room temperature for 10min. 1ml of sample diluent was added to each tube. After vortexing, duplicates of 50µl from each calibrator or test sample were pipetted into wells of the provided antibody-coated plate and, after addition of 200µl of enzyme conjugate to each well, incubated at room temperature for 2h. After incubation, wells were rinsed with wash solution by inverting and tapping the plate firmly onto tissue paper to empty the contents, followed by adding 250µl of wash solution to each well. That procedure was repeated twice for a total of three washes. 200µl of tetramethylbenzydine (TMB) substrate was then added to each well, followed by 30min incubation at room temperature. Finally, 100µl of stop solution (H_2SO_4, 2N) was added to all wells.

FGF-2 ELISAs were conducted using the Human FGF basic ELISA kit (Oncogen-Merck, Beeston, UK). 100µl of assay diluent was added to each well of an antibody-coated plate, followed by 100µl of each calibrator or test sample, after which the plate was left to incubate at room temperature for 2h. The plate was then rinsed four times by adding 250µl of washing solution to each well and by inverting and tapping the plate firmly onto tissue paper after each wash to insure complete removal of the washing solution. 200µl of conjugate was then added to each well and the plate was again left to incubate at room temperature for 2h, after which it was

again rinsed four times in washing solution. 200µl of substrate solution was then added to each well, followed by 30min incubation at room temperature. Finally, 50µl of stop solution (H_2SO_4, 2N) was added to all wells.

For all both ELISA kits, within 30 min of adding the stop solution, plates were read using an Anthos Lucy I microplate luminometer (Anthos Labtec Instruments, Salzburg, Austria) at 450nm and 550nm. Absorbance readings at 550nm were deducted from the readings at 450nm to remove the effects of the optical aberrations due to the microplate itself. The resulting test values were translated into the appropriate units of weight/volume using the calibrator values. The average test values were then plotted against the nature of the treatment, with standard deviation. As in Chapter 2, the significance of the differences between the averaged values was determined by independent sample t-test. The significance cut-point was set at P=0.05, with exact probability values being presented in Appendix 4.

For both H36LE2 and MCF7 cell lines, ELISA for the three growth factors examined was performed on three independent batches of medium samples. Each batch represented four treatments with three samples per treatment. The four treatments used were as described in 2.3.4, i.e. plastic substrate without staurosporine, plastic substrate with 500nM staurosporine, capsule substrate without staurosporine and capsule substrate with 500nM staurosporine. Three independent batches of medium from no-cell controls, with two treatments per batch and three samples per treatment, were also examined to determine the background levels of growth factors

in the culture medium for both substrates used, i.e. plastic and bovine lens capsule in the absence of staurosporine.

4.3.2. MMP detection:

The results of the ELISAs I conducted (see section 4.3.1) led me to formulate an hypothesis on the origin of the growth factors present in the lens cells culture medium. To test this hypothesis, I first determined the presence of the gelatinases MMP-2 and MMP-9 in the lens cells culture medium. To avoid false-positive detection of MMPs due to FCS presence in the culture medium, all following experiments were conducted using 0.1% FCS-supplemented DMEM. The persistence of H36LE2 cell viability on the lens capsule in 0.1%FCS-supplemented DMEM was checked beforehand by repeating the cell viability assay for this cell line (as described in 2.3.4). The results of this preliminary experiment are presented in Figure 4.1 and show that, when cells were seeded at a density of 5.10^4 cells/well (as set in section 2.3.3), viable cells were detected on the lens capsule, but not on the plastic substrate. This result is in accordance with previous studies on lens epithelial cells survival in low serum conditions (Ishizaki *et al.*, 1993).

4.3.2.1. Sample collection:

Medium samples were taken as described in 4.3.1, i.e. at the end of the viability assay described in 2.3.4, before the medium change and the addition of

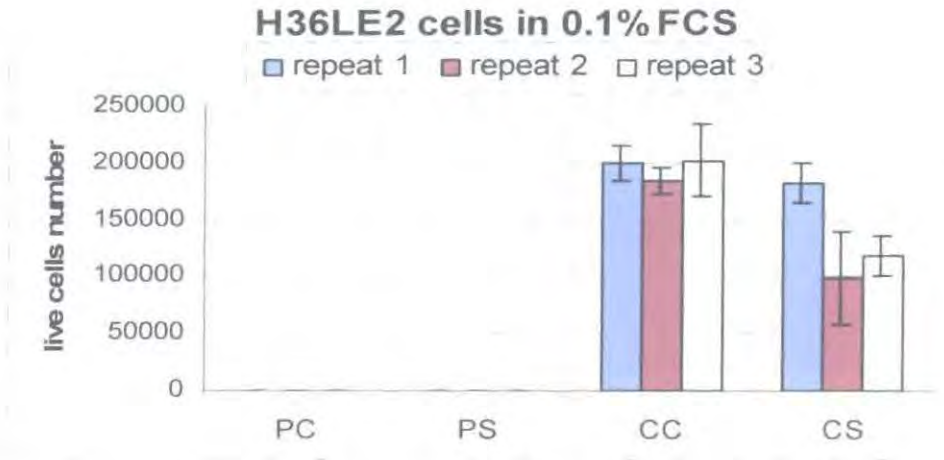

Figure 4.1 Culture on the bovine lens capsule allows lens cells' viability in low-serum culture medium.

H36LE2 cells were seeded onto either plastic or bovine lens capsule substrates and cultured in 0.1% FCS-supplemented cell culture medium. After a 48h recovery period, half of each substrate group was exposed to staurosporine at a concentration of 500nM while the other half was left undisturbed as a control. After a further 48h, live cells numbers were determined using the Aqueous 96 non-radioactive cell viability assay (Promega, Madison, USA) as described in Chapter 2 (2.3.4). The experiment was conducted independently three times. Bars represent the average of six samples with standard deviation for each independent repeat. Note that, although no viable H36LE2 cells were detected on the plastic substrate at this low serum concentration, culture on the lens capsule allows cell viability both in the absence and the presence of staurosporine.

Treatment legend:

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurossporine CS: capsule substrate, 500nM staurosporine

colorimetric reagent. 1ml of medium was taken for each well used in the viability assay, for a total of six samples per treatment for each experiment. The medium samples were then stored at -80^0C until later use. The only, but important, difference with the samples used in the ELISAs was that the samples used for MMP detection were taken from 0.1%FCS-supplemented DMEM cell cultures. Protein concentration in the cell culture medium samples was quantified using the BCA protein assay kit (Perbio-Pierce, Tattenhall, UK) as described in section in 4.3.2.1 (see Figure 4.2 and Table 4.1).

4.3.2.2. Gelatine zymography:

Gelatine zymography was used as a convenient method of detecting the presence and activation state of the gelatinases examined (Passi *et al.*, 1999; Esteve *et al.*, 1998). An equal amount of medium sample and medium sample buffer were mixed and left at room temperature for 10min, after which samples were run at 200V for 30min on an SDS-polyacrylamide gel, with a 4% (w/v) stacking layer and an 8% (w/v) separating layer supplemented with 0.1% gelatine (w/v). The gel was then washed in washing buffer for 30min on the room temperature shaker and rinsed three times in distilled water. This step ensures that SDS originally present in the gel is washed off with the buffer, thus allowing renaturation of the proteins in the gel. The gel was then left overnight in incubating buffer on the 37^0C shaker at 50 rpm. This step allows

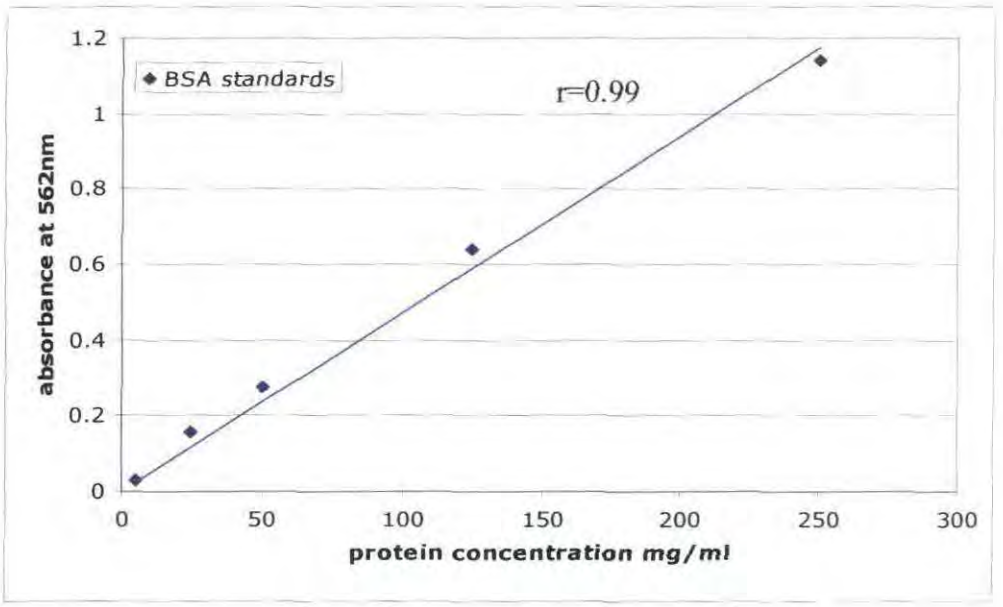

Figure 4.2 Example of a BSA standard curve used for the quantification of *H36LE2 cell culture medium* samples.

Sample name	Absorbance at 562 nm	Concentration in µg/ml	Equivalent volume in µl for 20µg of protein
H36LE2 PC	0.361	4778	4
H36LE2 PS	0.706	9357	2
H36LE2 CC	0.852	11294	1.8
H36LE2 CS	1.104	14625	1.4
H36LE2 PC<0	1.086	14384	1.4
H36LE2 PS<0	0.950	12590	1.6
H36LE2 CC<0	0.929	12316	1.6
H36LE2 CS<0	0.852	11285	1.8

Table 4.1 Quantification of H36LE2 cell culture medium samples with indication of volumes used for subsequent gelatin zymography.

degradation of the gelatine contained in the gel by the gelatinases. The next day, the gel was stained for 30 min in staining buffer and destained in distilled water for up to three days. The typical gel pattern obtained at that stage is of a dark background, containing intact gelatine, with white bands due to gelatine degradation and therefore indicating the presence of gelatinase. The gel was then photographed using the 'digitising' setting on a Fujifilm Intelligent Dark Box II. The image was visualised by the software IR-LAS1000. The composition of the solutions used during gelatine zymography is detailed in Table 4.2. Positive controls were provided by partially purified MMP-2 and MMP-9 from human serum (BioMol, Exeter, UK) and a molecular weight marker. Negative controls were provided by medium samples from cells exposed to the MMP inhibitor 1,10-orthophenathroline used at the concentration of 10nM (Sigma-Aldrich, Poole, UK) (Dawson *et al.*, 1986; Herman and Heumann, 1995; Mandal *et al.*, 2003).

4.3.2.3. Antibody detection:

MMP detection by monoclonal antibody was conducted to provide additional evidence for the presence of MMP-2 and MMP-9 in H36LE2-conditionned media on the lens capsule. The characteristics of the antibodies used are presented in Table 4.3. with the optimisation protocol being presented in Figure 4.3.

Sample Buffer	10% SDS (g/ml), 4% sucrose (g/ml), 1% bromophenol blue (g/ml), 0.25M Tris (pH=6.8).
Washing Buffer	2.5% (ml/ml) Triton X-100
Incubating Buffer	5mM $CaCl_2$, 50mM Tris (pH=8.0)
Staining Buffer	0.5 (g/ml) commasie blue R-250 in a 1:3:6 volumetric ratio of acetic acid: isopropanol: ddH_20 (respectively).

Table 4.2: List and composition of all solutions used during gelatin zymography.

Target	Name	Origin	Source	Concentration used
MMP-2	sc-13594	monoclonal	Santa Cruz	1/50
MMP-9	sc-21733	monoclonal	Santa Cruz	1/50
All primary monoclonals	Goat anti-mouse HRP	N/A	DAKO	1/1000

Table 4.3: Characteristics of all antibodies used for immunoblotting.

Commercial antibodies were obtained from Santa Cruz Biotechnology Inc., Mile Elm Calne, UK and from DakoCytomation, Olestrup, Denmark.

My MMP blot

I had the bands, there, right in front of me,/I had even shown they were activated, using zymography

But Roy said 'no, this can't be all'/'to really be sure, use a monoclonal'.

So back to my bench I went again/And for my first gel I loaded 2μl per lane.

But there was no bands on my gel, as far as I could see,/So I did another one and this time I loaded 30.

But it wasn't any better, I have to admit,/So I sat down and thought about it.

I looked on the net for MMP articles/And found that most people concentrated their samples.

So I concentrated mine, /and hoped that it would work, this time.

Again I tried to make my blot a success,/And, then it worked, well, more or less.

MMP-2 was too dark and MMP-9 too bright,/And they also were at the wrong height.

'They're complexes' in vain I cry,/'No they are not' ,the cruel reply.

In my samples I had BSA/ Maybe that was where my problems lay.

So much of it was in the gel, /MMPs could not run down well.

My next samples were serum-free/but still the bands eluded me.

I re-concentrated my last samples' lot/ and without much hope, ran a dot blot.

This one did work, could it be true? Another western I had to go through.

At last, o joy, after such a long wait,/there were the bands of the right weight.

Twenty-eight westerns I blotted/ to get that one figure sorted

Now you can see it at its best,/ Please just be kind and look impressed.

Figure 4.3: Optimisation protocol for both MMPs antibodies used and conditioned cell culture medium samples prior to immunoblotting.

4.3.2.3.1. Sample preparation:

Medium samples collected as described in section 4.3.3.1 were concentrated 25 folds using Amicon^{R} Ultra-4 centrifuge filters with a 10K nominal molecular weight limit (Millipore, Molsheim, France) to increase protein content of the samples, as has been described elsewhere (Fuchshofer *et al.*, 2003). Protein concentration was then quantified using the BCA protein assay kit (Perbio-Pierce, Tattenhall, UK) as described in section in 4.3.3.1. Samples were then precipitated with 10% (v/v) tri-chloro-acetic acid (TCA) and re-diluted in 2x sample buffer, to a final protein concentration of 50µg/µl (see Table 4.4).

4.3.2.3.2. Dot blotting:

Dot blotting was conducted before Western blotting as a less time-consuming way to optimise antibody concentration. Indeed, as I was the first person in my laboratory to investigate MMPs presence in the lens capsular bag model, the best antibody concentration to use against my samples was unknown. Dot blotting was conducting by applying either 50µg or 250µg of protein extract for each treatment unto nitro-cellulose paper. A positive control was provided in the form of partially-purified MMP-2 and MMP-9 from human serum (BioMol, Exeter, UK) and a negative control was provided in the form of medium samples from cells exposed to

Sample name	Absorbance at 562nm after sample centrifugation	Concentration (μg/ml)	Volume in μl for 750 μg	Volume to be re-suspended in after TCA precipitation for a final concentration of 50 μg/μl
H36LE2 PC	0.690	7845	97.5	15 μl
H36LE2 PS	0.913	11123	67.5	15 μl
H36LE2 CC	1.024	12934	60	15 μl
H36LE2 CS	0.809	9540	75	15 μl
H36LE2 CB	1.1005	14575	51.5	15 μl

Table 4.4 Quantification of H36LE2 concentrated cell culture medium samples with indication of volumes used for subsequent TCA precipitation.

the MMP inhibitor orthophenathroline used at the concentration of 10nM. Exposure to antibodies was as described in section 3.3.2.2.

4.3.2.3.3. Western Blotting:

Western Blotting was performed as described in Chapter 3 (section 3.3.2.2), with the following modifications:

- 250µg of protein was loaded per lane for all samples.
- positive control was provided by partially purified MMP-2 and MMP-9 from human serum (BioMol, Exeter, UK), although the sHSP marker was still used.

4.3.3. MMP inhibition studies:

To investigate the involvement of gelatinases in the release of growth factors and cell viability on the lens capsule, more viability assays and ELISAs were conducted.

The viability assays and subsequent data analysis were conducted as described in 2.3.4, with the following modifications :

- 0.1% FCS-supplemented DMEM instead of 10% FCS-supplemented DMEM was used as the cell culture medium.

- MMP-2-specific (Calbiochem-Merck, Beeston, UK) (Aye *et al.*, 2004; Emonard *et al.*, 1999) and MMP-9-specific (Calbiochem-Merck, Beeston, UK) (Pikul *et al.*, 1998) inhibitors were used. According to the manufacturer's instructions, the MMP-2-specific inhibitor was used at a concentration of 100μM and the MMP-9-specific inhibitor was used at a concentration of 5nM.
- Either inhibitor was added at the time of cell seeding. This was to prevent the cells from releasing the corresponding MMP for the whole duration of their culture on the capsule, i.e. 96h.
- No cells were cultured on plastic.

ELISAs were conducted as described in section 4.3.1, with medium samples collected from the viability assays described in the previous paragraph. Data analysis was as described in section 4.3.1, with exact probability values being presented in Appendix 4.

4.4. Results:

4.4.1. Growth factor release on the lens capsule:

In this Chapter, I chose to investigate the potential involvement of soluble factors in cell viability on the lens capsule. As FGF-2 and IGF-1 are well known diffusible growth factors in the lens (see sections 1.1.2 and 1.4.2.3), their medium levels were compared between H36LE2 and MCF7 cells, to determine whether

growth factor medium levels could be correlated with cell viability on the lens capsule.

As shown in Figure 4.4, there was no significant increase in FGF-2 medium levels with either culture on the bovine lens capsule or addition of staurosporine, and this for both H36LE2 and MCF7. As shown in Figure 4.5, there was a small, but not significant increase in IGF-1 medium levels for H36LE2 cells with culture on the lens capsule, but not with staurosporine addition. There was a decrease in IGF-1 medium levels in MCF7 cells with culture on the lens capsule, although it was significant for only two out of the three independent repeats shown. As for H36LE2 cells, addition of staurosporine to MCF7 cells had no consistently significant effect on IGF-1 medium levels.

As shown in Figure 4.6, when medium samples taken from cell-free capsules were examined, with the original aim of providing background growth factor levels for Figure 4.4 and 4.5, it was noticed that presence of the lens capsule resulted in an increase in growth factor medium levels. For FGF-2 this increase was not significant, but it was significant for IGF-1.

Both growth factors examined are known to bind, either directly or in complexed forms, to extracellular matrix components. FGF-2 is known to bind lens capsule's HSPGs (Shulz *et al.*, 1997). IGF-1 participates in IGF-1/IGFBP-3/fibronectin complex (Gui and Murphy, 2001) and therefore could be present in the fibronectin-rich layers of the lens capsule. This led me to investigate whether the

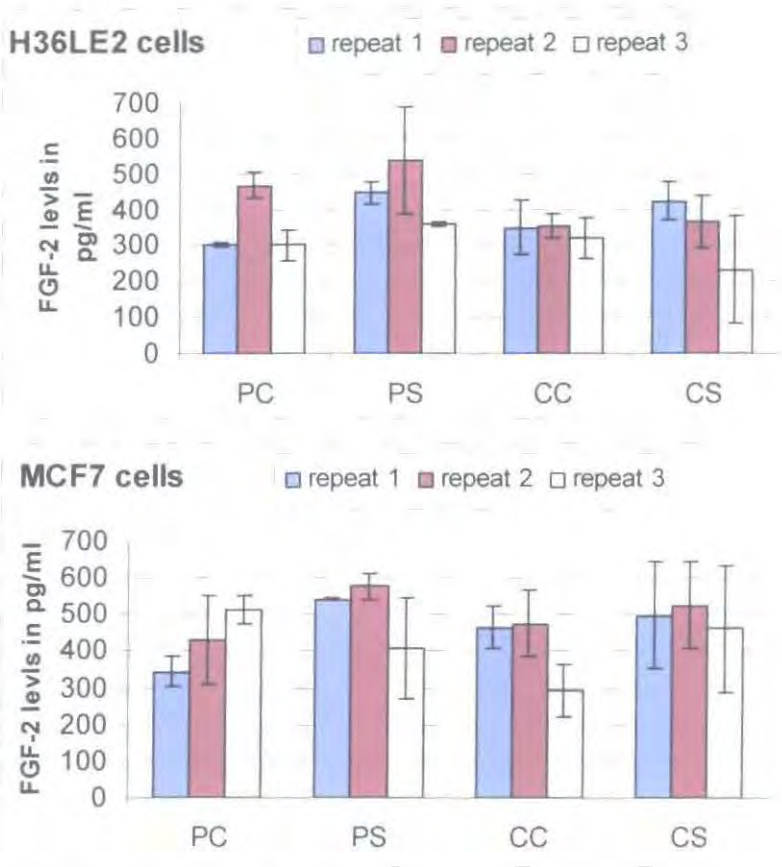

Figure 4.4 FGF-2 medium levels.

H36LE2 cells and MCF7 cells were cultured either on plastic or on bovine lens capsules for 48h in 10% FCS-supplemented cell culture medium, after which half of each group was exposed to staurosporine at a concentration of 500nM for a further 48h. Culture medium samples were then collected and total FGF-2 levels were assayed by ELISA as described in 4.3.1.

Note that, for both cell lines, there is no significant difference in FGF-2 levels between cells cultured on plastic and cells cultured on bovine lens capsules ($P>0.05$ for PC vs CC and PS vs CS for all three repeats for both cell lines). There is also no significant difference in FGF-2 levels between cells cultured without staurosporine and cells cultured with staurosporine ($P>0.05$ for PC vs PS and CC vs CS for all three repeats for both cell lines).

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend:

PC: plastic substrate, no staurosporine
PS: plastic substrate, 500nM staurosporine
CC: capsule substrate, no staurosporine
CS: capsule substrate, 500nM staurosporine

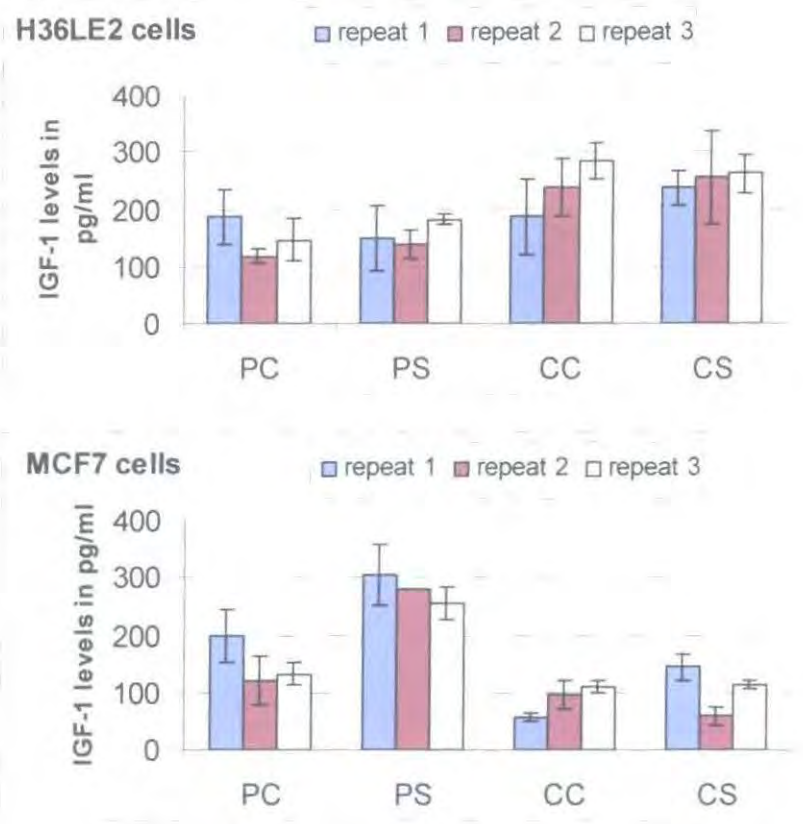

Figure 4.5 IGF-1 medium levels.

H36LE2 cells and MCF7 cells were cultured either on plastic or on bovine lens capsules for 48h in 10% FCS-supplemented cell culture medium, after which half of each group was exposed to staurosporine at a concentration of 500nM for a further 48h. Culture medium samples were then collected and total IGF-1 levels were assayed by ELISA as described in 4.3.1.

For H36LE2 cells, culture on the lens capsule resulted in a small, non-significant increase in FGF-2 medium levels compared to culture on plastic. The addition of staurosporine did not affect the FGF-2 medium levels on either substrate. For MCF7 cells, culture on the lens capsule resulted in a decrease in FGF-2 medium levels compared to culture on plastic. However, this decrease was not consistently significant across the three repeats shown. The addition of staurosporine did not affect the FGF-2 medium levels on either substrate.

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend:

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurosporine CS: capsule substrate, 500nM staurosporine

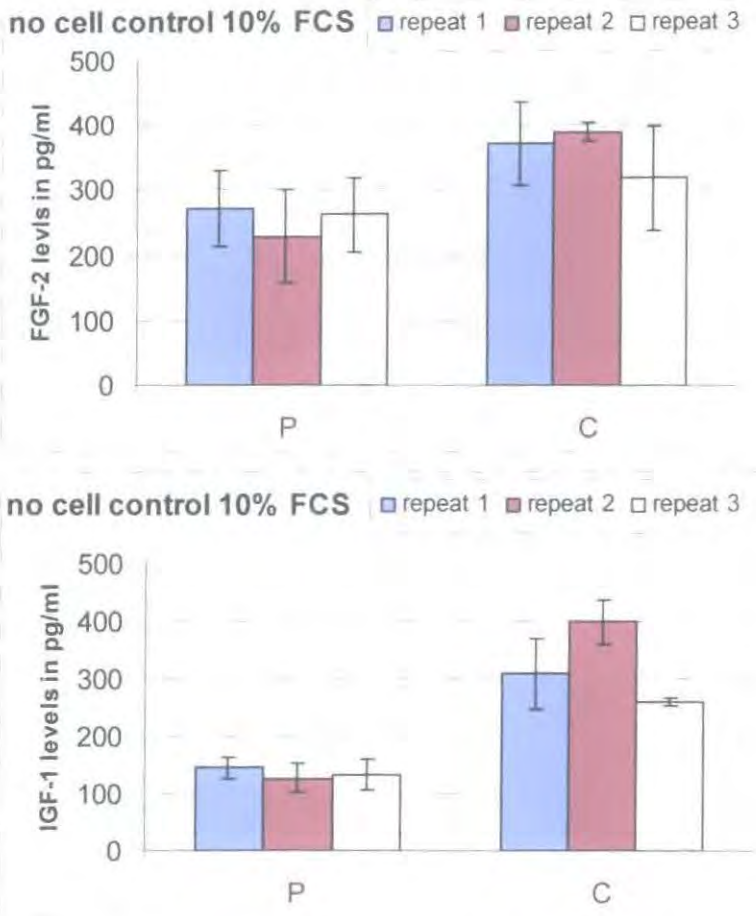

Figure 4.6 FGF-2 and IGF-1 medium levels in the absence of cells.

As a no-cell control for Figure 4.3 and Figure 4.4, cell-free plastic and bovine lens capsules were kept for 96h in 10% FCS-supplemented cell culture medium. Culture medium samples were then collected and total FGF-2 levels and IGF-1 levels were assayed by ELISA as described in 4.3.1.

Note that, for both growth factors, there is an increase in medium level on the bovine lens capsule compared to the plastic. For FGF-2, this increase is small and not significant ($P>0.05$ for all three repeats). For IGF-1 however, this increase is significant ($P<0.05$ for all three repeats).

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend: P: plastic substrate C: capsule substrate

growth factors present when cells are grown on the lens capsule originate from lens capsule release rather than increased cell secretion.

To investigate the possibility that the growth factors are being released from the lens capsule, I examined the expression patterns of the gelatinases MMP-2 and MMP-9 in my model. MMP-2 and MMP-9 appeared to be good potential growth factors-releasing agents as they are known to be present in the capsular bag (Kawashima *et al.*, 2000; Tamiya *et al.*, 2000) and can release all three growth factors examined from their binding proteins (Fowlkes and Winkler, 2002; Fukuda *et al.*, 2004).

4.4.2. MMPs are present and activated in staurosporine-resistant cells:

To first determine whether MMP-2 and MMP-9 were present in cells grown on the bovine lens capsule, conditioned medium samples from H36LE2 cells were run by gelatine zymography, as detailed in section 4.3.2.2. As seen in Figure 4.7, gelatinolytic activity at a size compatible with active MMP-2 was found in medium samples from both the plastic and capsule substrate, while gelatinolytic activity at a size compatible with active MMP-9 was found in medium samples from the capsule substrate. The absence of such activity in the negative control lanes suggests that these bands do correspond to active MMP-2 and MMP-9.

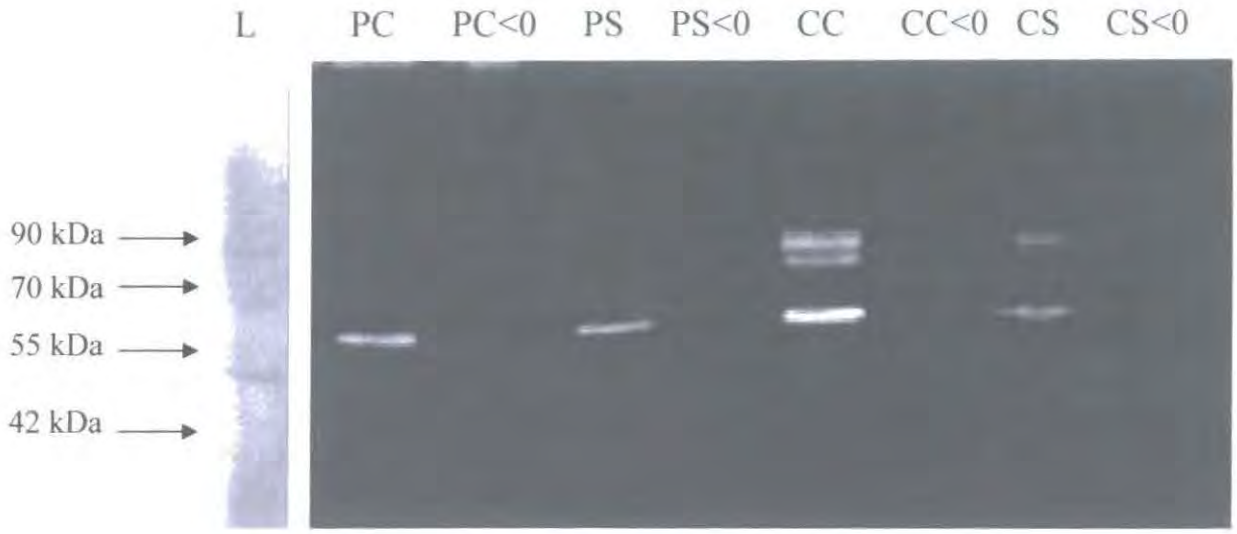

Figure 4.7 Gelatine zymogram of H36LE2 cells-conditioned medium.

Cell culture medium samples were taken from H36LE2 cells *in vitro* cultures and examined by gelatin zymography as described in 4.3.2.

Note that a single band was present in all samples, with a higher doublet being present in samples from the lens capsule treatments. Although the positive control used (partially purified MMP-2 and MMP-9 mix) was not concentrated enough to give a strong signal, the molecular weight used allowed the approximate sizing of the gelatinolytic bands present in the test samples. The lower single band was between 70kDa and 55kDa and the doublet between 90kDa and 70kDa.

The molecular weight marker presented was originally run on the same gel as the samples, although its colour pattern (dark blue bands against a blue background) made it hard to see, hence the increased exposure being used especially for the marker in this Figure.

Treatment legend:

L: molecular weight ladder

0<: negative control (1,10-orthophenanthroline 10nM)

PC: plastic substrate, no staurosporine

PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurossporine

CS: capsule substrate, 500nM staurosporine

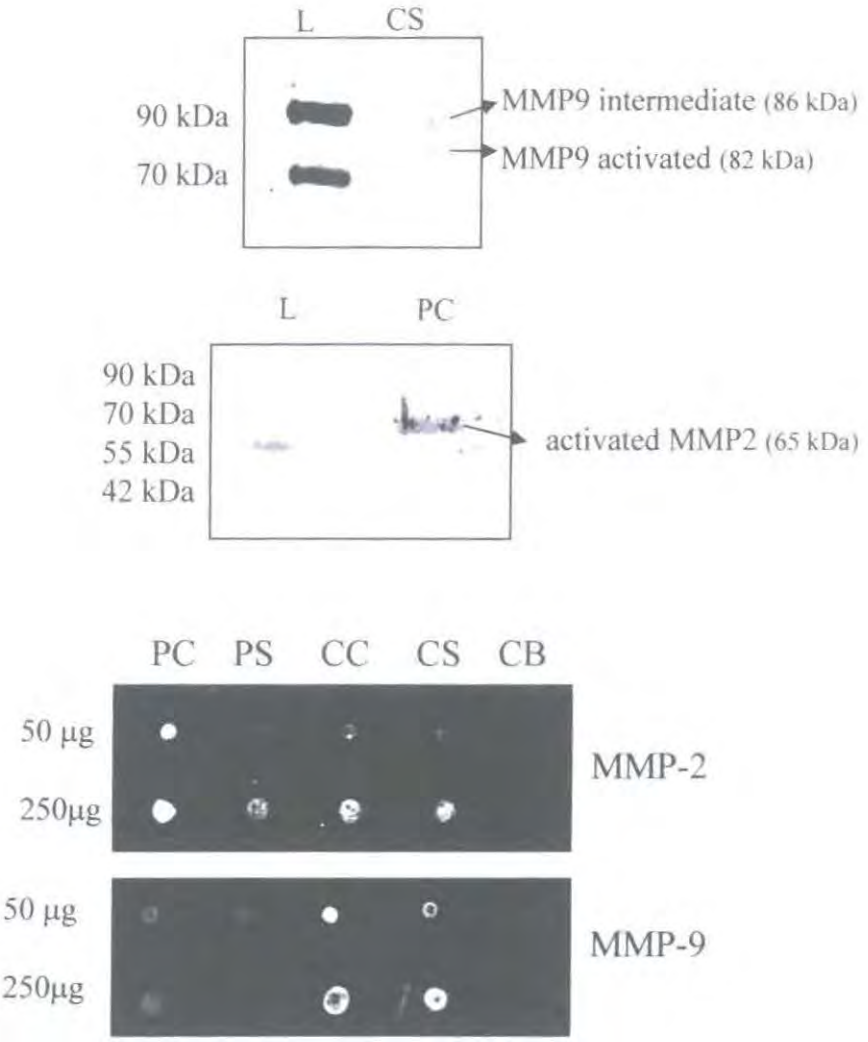

Figure 4.8 MMP-2 and MMP-9 are present in H36LE2 cells on the lens capsule.

Concentrated conditioned cell culture media samples were run against MMP-2 and MMP-9 monoclonal antibodies as described in 2.3.3. Specificity of the antibodies were checked by immunoblotting selected H36LE2 samples against a weight ladder (top two blots). A positive control was available but could not be concentrated enough to give a strong signal. The MMP antibodies were then run against the original range of H36LE2 cells samples by dot blotting (two bottom blots). Note that MMP-2 is expressed more weakly than MMP-9, but in all treatments, while MMP-9 is expressed preferentially on the capsule (bottom two blots).

Treatment legend:

L: molecular weight ladder

PC: plastic substrate, no staurosporine PS: plastic substrate, 500nM staurosporine

CC: capsule substrate, no staurossporine CS: capsule substrate, 500nM staurosporine

CB: capsule substrate, BB94 10nM (general MMP inhibitor (Botos *et al.*, 1996)): negative control

results, presented in Figure 4.8, show that the antibodies used recognised mainly the active forms of both MMPs. When they were run against H36LE2 samples on a dot blot, MMP-2 was detected on both plastic and lens capsule substrates in the absence and the presence of staurosporine, although expression was strongest on the plastic in the absence of staurosporine. MMP-9 was also detected, but mainly on the lens capsule, both in absence and presence of staurosporine, with much lower levels being detected on the plastic substrate.

4.4.3. MMPs inhibition's effect on growth factor levels on the lens capsule:

To then determine whether MMP-2 and MMP-9 were able to release growth factors from the bovine lens capsule, two separate experiments were conducted, the results of which are presented in Figure 4.9 and Figure 4.10. In the first experiment (see Figure 4.9), cell-free capsules were exposed to a mix of MMP-2 and MMP-9 partially purified from human serum. After 48h, the presence of MMPs resulted in a significant increase in FGF-2 medium levels while IGF-1 medium levels were unaffected.

To test whether these effects would be conserved in the presence of lens epithelial cells, a second experiment was conducted (see Figure 4.10). MMP-2-specific and MMP-9-specific inhibitors were added separately to H36LE2 cells grown on the bovine lens capsule and, after 96h, FGF-2 and IGF-1 medium levels were assayed by ELISA. Both MMP-2-specific and MMP-9-specific inhibitors resulted in a

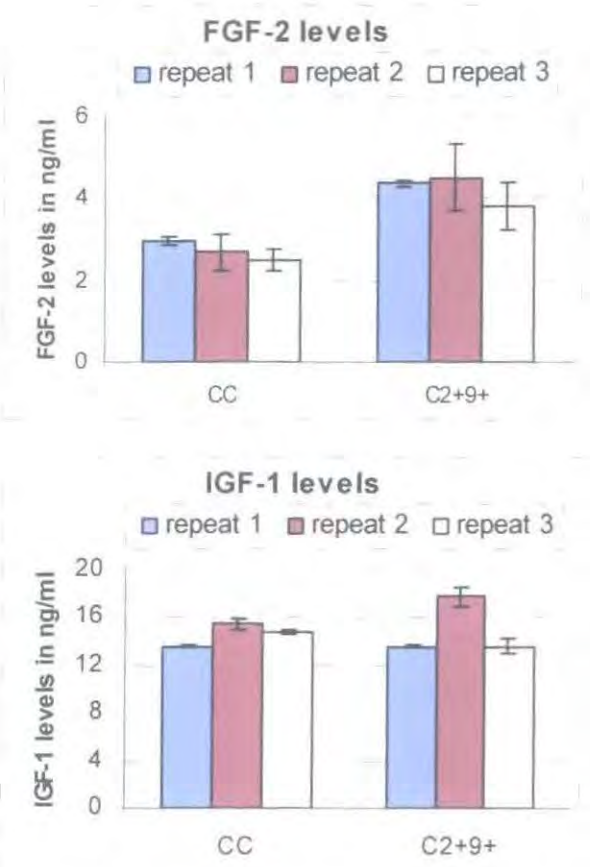

Figure 4.9 FGF-2 and IGF-1 medium levels in the presence of gelatinases.

Cell-free bovine lens capsules were kept for 96h in PBS at 37^{0}C. During that time, half the capsules were exposed to a partially-purified MMP-2 and MMP-9 mix, the other half being left undisturbed as a control. Culture medium samples were then collected and total FGF-2 levels and IGF-1 levels were assayed by ELISA as described in 4.3.1.

Note that, for FGF-2, there was a significant increase in medium level in the presence of the gelatinases mix ($P<0.05$ for all three repeats). For IGF-1, no consistent significant increase was noticed. ($P>0.05$ for two out of three repeats).

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend:

CC: cell-free capsule substrate, control

C2+9+: cell-free capsule substrate, MMP-2/MMP-9 mix added

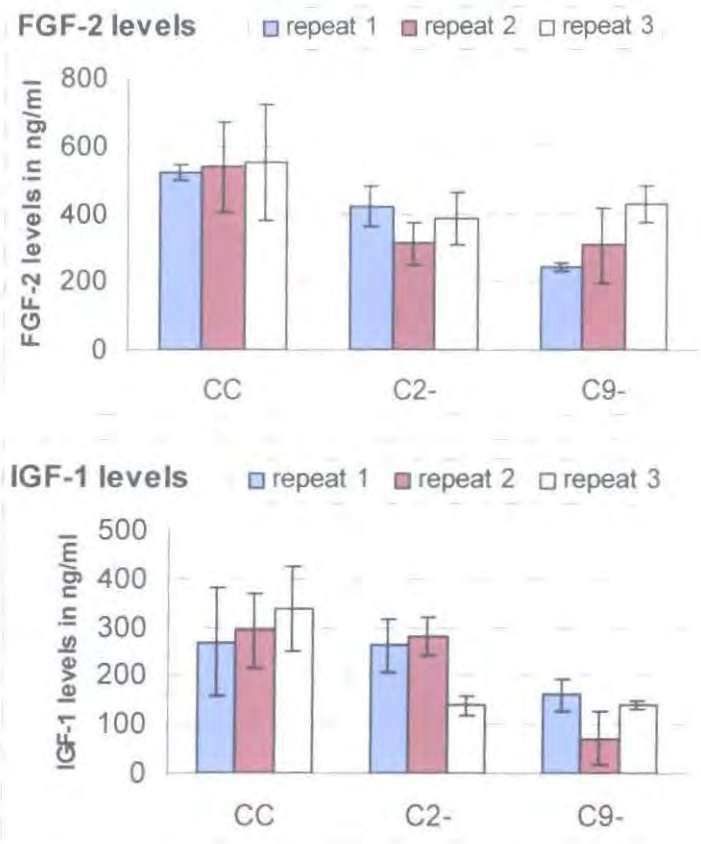

Figure 4.10: The effect of MMP inhibition on growth factors levels on the lens capsule.

H36LE2 cells were seeded onto bovine lens capsules in 0.1% FCS-supplemented cell culture medium and exposed to either an MMP-2-specific or an MMP-9 specific inhibitor for 96h, as described in section 4.3.3. Cell culture medium samples were then collected and FGF-2 and IGF-1 medium levels were assessed by ELISA as described in section 4.3.1.

The addition of either MMP-2-specific or MMP-9-specific inhibitor resulted in a decreased in both FGF-2 and IGF-1 medium levels, although these decreases were not consistently significant for all repeats.

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend:

CC: capsule substrate, control

C2-: capsule substrate, MMP-2-specific inhibitor added

C9-: capsule substrate, MMP-9-specific inhibitor added

decrease in FGF-2 levels and IGF-1 levels, but neither was consistently significant across all three repeats.

4.4.4. MMPs affect cell viability on the lens capsule:

To test whether the MMPs were involved in cell viability on the lens capsule, H36LE2 cells were grown on the lens capsule in the presence of MMP inhibitors for 96h, after which the viable cell numbers were assayed. As seen in Figure 4.11, in the absence of staurosporine, separate MMP-2 and MMP-9 inhibitions had different effects. The inhibition of MMP-2, but not MMP-9, resulted in a significant decrease in viable cell number in H36LE2 cells ($P<0.05$ for all three repeats for MMP-2-specific inhibitor), indicating a role for MMP-2 in cell viability on the bovine lens capsule. In the presence of staurosporine, however, and as shown in Figure 4.12, neither MMP-2-specific nor MMP-9-specific inhibitor significantly reduced cell viability ($P>0.05$, for all three repeats for both inhibitors).

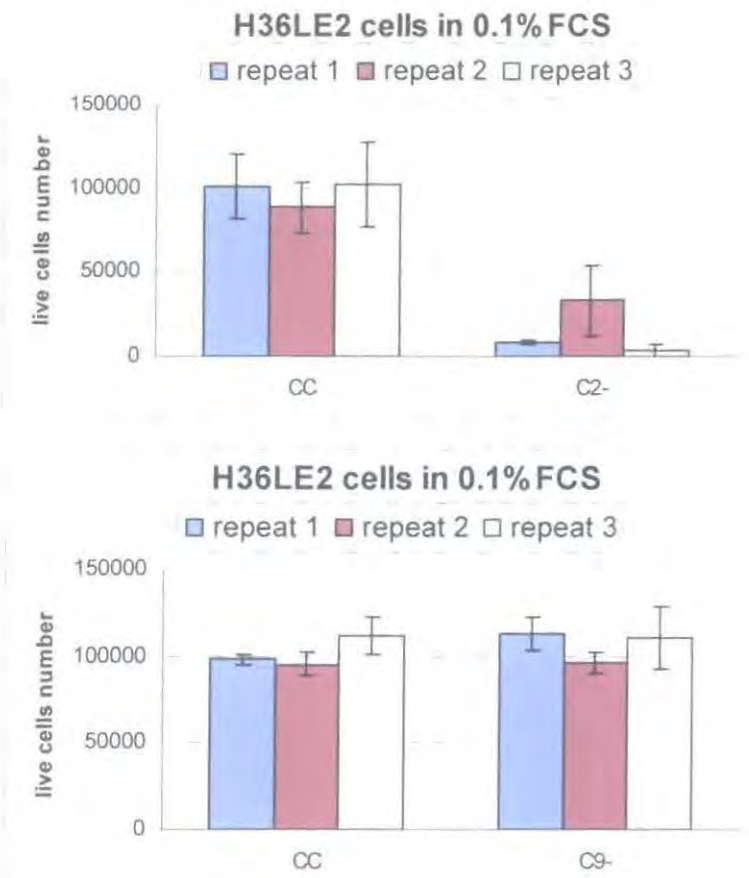

Figure 4.11: MMP-2 inhibition in lens epithelial cells affects cell viability on the lens capsule.

H36LE2 cells were seeded onto bovine lens capsules in 0.1% FCS-supplemented cell culture medium and exposed to either an MMP-2-specific or an MMP-9 specific inhibitor for 96h, as described in section 4.3.3. Viable cells numbers were then determined using the the Aqueous 96 non-radioactive cell viability assay (Promega, Madison, USA) as described in Chapter 2 (2.3.4).

Note that, in the presence of MMP-2-specific, but not MMP-9-specific inhibitor, H36LE2 cell viability is significantly decreased on the bovine lens capsule ($P<0.05$ for all three repeats for CC,C2-).

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend:

CC: capsule substrate, no staurosporine

C2-: capsule substrate, MMP-2 inhibitor, no staurosporine

C9-: capsule substrate, MMP-9 inhibitor, no staurosporine

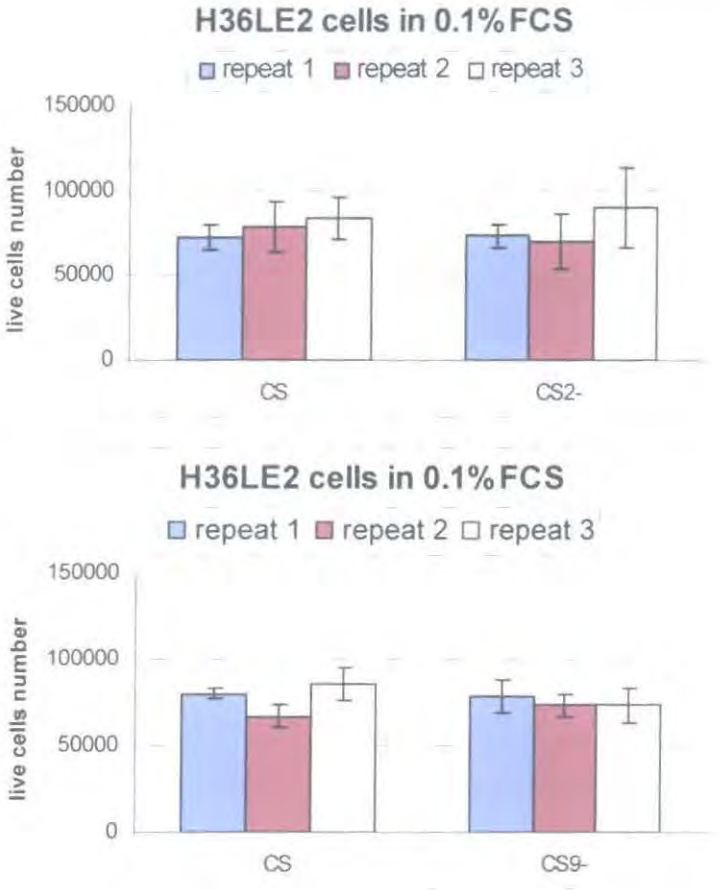

Figure 4.12: MMP-2 and MMP-9 inhibition do not decrease H36LE2 cells viability on the lens capsule in the presence of staurosporine.

H36LE2 cells were seeded onto bovine lens capsules in 0.1% FCS-supplemented cell culture medium and exposed to either an MMP-2-specific or an MMP-9 specific inhibitor as described in section 4.3.3. After 48h, staurosporine 500nM was added to all samples. Aqfter a further 48h, viable cells numbers were determined using the the Aqueous 96 non-radioactive cell viability assay (Promega, Madison, USA) as described in section 2.3.4.

Note that, in the presence of either MMP-2-specificor MMP-9-specific inhibitor, H36LE2 cell viability is not significantly decreased on the bovine lens capsule.

Bars represent the average of three samples with standard deviation. Three independent repeats are shown. Probabilities are as calculated by two-tailed, independent sample t-test within each repeat. Exact probability values are shown in Appendix 4.

Treatment legend:

CS: capsule substrate, staurosporine 500nM

CS2-: capsule substrate, MMP-2 inhibitor, staurosporine 500nM

CS9-: capsule substrate, MMP-9 inhibitor, staurosporine 500nM

4.5. Discussion

4.5.1. Involvement of FGF-2 and IGF-1 in cell viability on the lens capsule:

No clear differences could be observed in FGF-2 levels between staurosporine-resistant and staurosporine-sensitive cell lines when cultured on the bovine lens capsule. However, the effects of FGF-2 on cell proliferation are not purely concentration-dependent, but also cell-specific. While it promotes cell proliferation or apoptosis resistance in human lens epithelial cells (Ibaraki *et al.*, 1995) it also promotes apoptosis in MCF7 cells (Wang *et al.*, 1998) *in vitro*. Therefore, FGF-2 might still play a role in cell-specific proliferation and survival on the lens capsule despite the absence of clear quantitative differences between cell lines.

In the case of IGF-1, however, increased medium levels were present on the lens capsule in H36LE2 cells, which remain viable on the lens capsule, compared to MCF7 cells, which viability is reduced when cultured on the lens capsule (see Figure 2.6). IGF-1 signalling is well known in the lens to promote cell proliferation and differentiation, either on its own or in a coordinated manner with FGF-2 signalling (Chandrasekher and Sailaja, 2003; Ibaraki *et al.*, 1995). The possibility of IGF-1 contributing to lens cells viability in the capsular bag is important as the increase in fibronectin deposition in the lens capsule after cataract surgery (Tanaka *et al.*, 2002; Joo *et al.*, 1999) may increase IGF-1 capture from the aqueous humor (Arnold *et al.*, 1993) onto the lens capsule via IGFBP3 binding (Martin *et al.*, 2002; Gui and

Murphy, 2001). This would intensify IGF-1 signalling in lens epithelial cells following IGF-1 release from IGFBP-3 by the gelatinases (Manes *et al.*, 1999; Fowlkes *et al.*, 1995), with consequences on their viability.

An unexpected result, however, was the increased levels of FGF-2 and especially IGF-1 detected on the lens capsule in the cell-free controls. The thorough cleaning procedure used during capsule preparation (see section 2.3.3 and Figure 2.1) suggests that this increase cannot be due to remaining primary bovine cells. A better explanation for this increase in FGF-2 and IGF-1, can be found in the addition of 10% foetal calf serum to our cell culture medium. As the capsule is known to contain heparin-bound FGF-2 (Schulz *et al.*, 1997) and fibronectin (see section 2.1.3, which is itself capable of binding IGFBP-3-IGF-1 complexes (Martin *et al.*, 2002; Gui and Murphy, 2001), the presence of a serum factor with protease activity could generate an increase in free FGF-2 and IGF-1 without the need for cells to be present. It is true that, when MMP-2 and MMP-9 were added to cell-free capsules in a serum-free medium, there was no significant release of IGF-1. However, this difference between the effects of FCS and MMPs on IGF-1 release from the lens capsule could be explained by the presence in FCS of proteases other than MMP-2 and MMP-9, which would be directly responsible for IGF-1 release into the cell culture medium. The decrease in IGF-1 levels in the presence of MMP-2 and MMP-9 inhibitor when cells are cultured on the lens capsule could be explained by the indirect involvement of gelatinases in capsule-bound IGF-1 release. MMP-2 and MMP-9 might release an unidentified factor from the lens capsule, which then diffuses to the cells and induces

the release of a non-gelatinase protease that can in turn directly release IGF-1 from the capsule. Sadly, I did not have the opportunity to test this hypothesis in my study.

It is this observation that growth factors could be released from cell-free capsuled which first suggested the role of the lens capsule as a growth factor store, which would be accessible to cells expressing the right protease factors.

It is to test this hypothesis that I looked for MMP-2 and MMP-9 presence in my model, and evidence of their presence in their active form were indeed found in H36LE2 cells grown on the bovine lens capsule (see Figure 4.7 and Figure 4.8). The gelatinases were also found to contribute to FGF-2 release directly from the lens capsule (see Figure 4.9), in quantities which have been shown to promote lens epithelial cells' proliferation in vitro (Ibaraki *et al.*, 1995). As FGF-2 also promotes MMP-9 expression in a number of cell lines (Liu *et al.*, 2002), this result also suggests the presence of a positive feedback loop between MMP-9 and FGF-2, which would result in large quantities of FGF-2 being released from the capsule's stores.

4.5.2. Correlation of MMPs expression with cell viability on the bovine lens capsule:

The MMP-mediated release of growth factors from their capsule stores would represent an obvious advantage to cells growing on the capsule. Indeed, in other systems, gelatinase expression has been linked to increased cell proliferation. MMP-2 induces bronchial fibroblasts' proliferation *in vitro* (Xu *et al.*, 2002) and correlates

with hepatic fibroblasts' proliferation *in vivo* (Lichtinghagen *et al.*, 2001), while MMP-9 mediates mammary epithelial cells' proliferation *in vitro* (Lee *et al.*, 2000).

In my model, MMP-2, rather than MMP-9, was shown to have a significant effect in promoting cell viability on the lens capsule.

In the presence of staurosporine, however, neither MMP-2 nor MMP-9 inhibition had significant effects on cell viability. Other proteases, such as MMP-3, which is found in the lens capsular bag after cataract surgery (Kawashima *et al.*, 2000) could still be investigated as potential agents of cell viability on the lens capsule. MMP-3's substrates includes lens' capsule components and TGFβ and IGF binding proteins (Fowlkes and Winkler, 2002) and would therefore be able to release growth factors independently of the gelatinases.

4.6. Chapter's conclusions:

The aim of this chapter was to test whether soluble factors were involved in cell viability on the lens capsule with special emphasis on FGF-2, IGF-1 and the gelatinases MMP-2 and MMP-9. By the end of this chapter, my main finding has been that MMP-2 is indeed involved in cell viability on the lens capsule, as its inhibition significantly decreases cell viability (see Figure 4.11). Also, I have shown that FGF-2 and IGF-1 can be released from the lens capsule by MMP-2, MMP-9 and most likely other proteases (see Figure 4.6 and Figure 4.9), thus increasing the medium levels of growth factors relevant to lens epithelial cell viability.

5. Conclusion:

5.1. Summary of this study's main findings (also see Figure 5.1):

In Chapter 2 of this study I have provided evidence that:

- The viability of lens epithelial cells in the presence of staurosporine is greatly improved when the cells are cultured on their *in vivo* basement membrane, the lens capsule, compared to a plastic substrate (section 2.4.1 and section 2.5.2).

- When cultured in the absence of staurosporine, the viability of the non-lens MCF7 and U373-MG cells is greatly decreased if the cells are in direct contact with the bovine lens capsule, rather than with plastic (section 2.4.1 and section 2.5.3). This decrease in cell viability is also observed when the cells are co-cultured with the lens capsule (section 2.4.3).

- These results suggest that the lens capsule has a cell-specific effect on cell viability, with the likely involvement of soluble factors.

In Chapter 3 I have shown that:

- Both αA-crystallin and αB-crystallin are present in lens epithelial cells on the lens capsule, while only αB-crystallin could be detected in U373-MG cells and neither crystallins in MCF7 cells (section 3.4.2 and section 3.5.2).

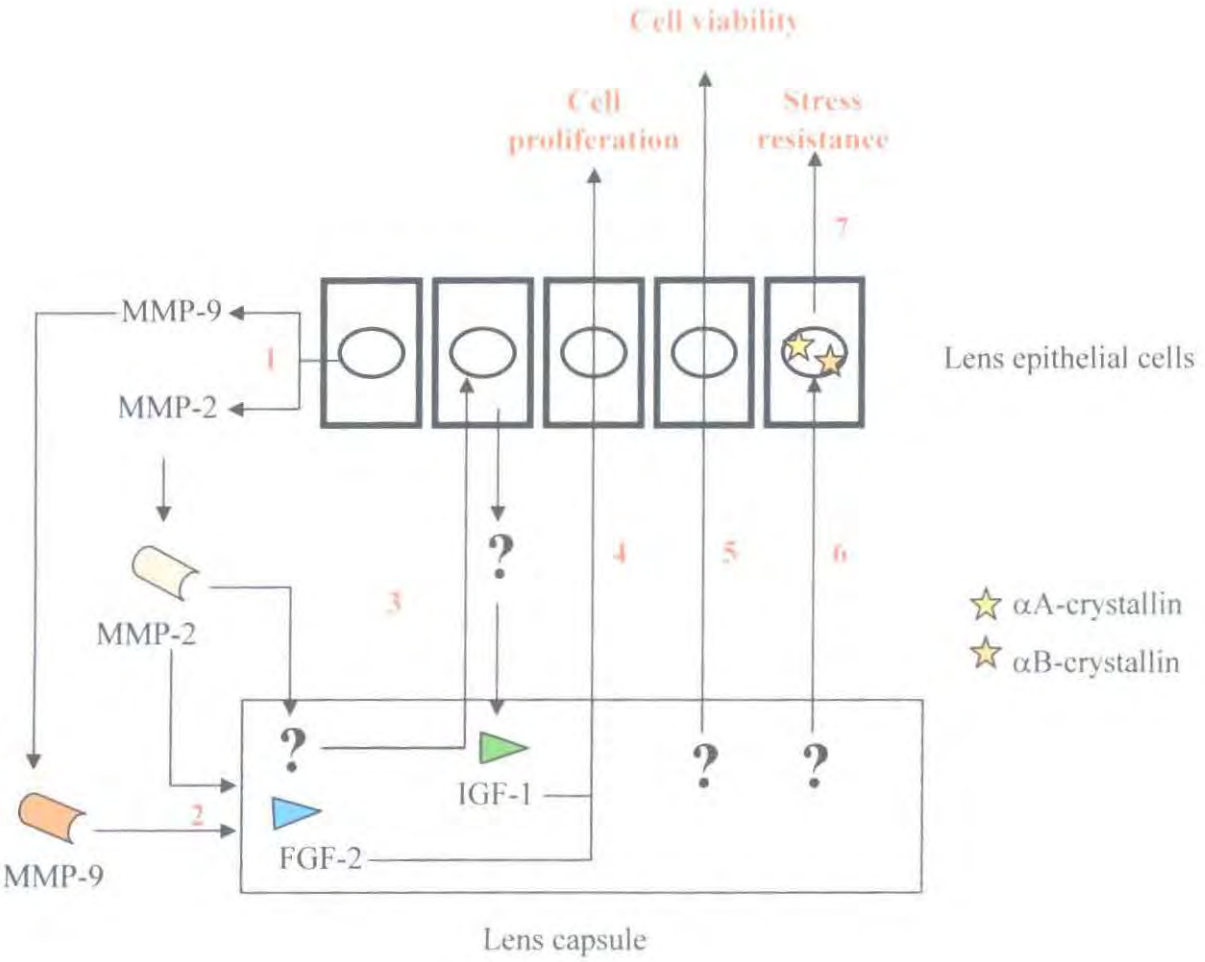

Figure 5.1: Summary diagram of this thesis' mains findings

1 MMP-2 and MMP-9 are released by lens epithelial cells (section 4.4.2).

2 MMP-2 and MMP-9 lead to the direct release of FGF-2 from the lens capsule (section 4.4.3).

3 hypothetical mechanism to explain the following findings:

IGF-1 can be directly released from the lens capsule by soluble factors (section 4.4.1).

IGF-1 cannot be directly released from the lens capsule by MMP-2 and MMP-9 (section 4.4.3).

IGF-1 soluble medium levels are decreased by inhibition of either MMP-2 or MMP-9 (section 4.4.3).

4 FGF-2 and IGF-1 are promoter of lens cell proliferation (section 4.5.1).

5 Culture of lens epithelial cells on the lens capsule promotes cell viability (section 2.4.1).

6 Culture of lens epithelial cells on the lens capsule promotes α-crystallin expression (section 3.4.2).

7 α-crystallins promote lens epithelial cells stress resistance (section 1.4.2.3.3)..

- The addition of staurosporine to cells cultured on the lens capsule did not result in a clear up-regulation of either crystallin (section 3.4.2).

- These results allowed me to formulate the hypothesis that the presence of both αA-crystallin and αB-crystallin is necessary for cell viability on the lens capsule, although I could not test this hypothesis due to lack of time.

In Chapter 4, I have shown evidence that:

- FGF-2 and IGF-1 can be released directly from the lens capsule (section 4.4.1 and 4.4.3).

- MMP-2 and MMP-9 are present in my in vitro model and their activity leads to the release of FGF-2 and IGF-1 from the lens capsule (section 4.4.2 and section 4.4.3).

- The inhibition of MMP-2, but not MMP-9 activity, leads to a decrease in lens epithelial cell viability on the lens capsule (section 4.4.4).

- These results demonstrate the importance of MMP-2, and potentially growth factors such as FGF-2 and IGF-1, in the ability of the lens capsule to provide lens epithelial cell viability.

5.2. Implications for cell-matrix interaction research:

The results of this study indicate that the lens capsule, which is the native basement membrane of the lens epithelial cells, is a promoter of cell viability and protein expression, as well as a store of growth factors. These notions correlate well with what is already known of extra-cellular matrices in other systems, but also add some knowledge to the field of ECM research.

The proliferative and anti-apoptotic roles of basement membranes are well known in both normal and disease processes (Raines, 2000; Rintoul and Sethi, 2002). and are heavily linked to integrin functions (see section 5.1.1).The actin cytoskeleton is also important in mediating these effects, by its participation, with the integrins, in focal adhesions (Wozniak *et al.*, 2004). Here, we show that another cytoskeletal protein, vimentin, does correlate with cell viability on the lens capsule in SW13 cell lines, which suggests that hemi-desmosomes, as well as focal adhesions, can be important for cell viability.

The role of native extracellular matrix as a maintainer of organ-specific protein expression has also been observed in other systems, such as for β-casein expression in the breast and osteocalcin expression in bone (Roskelley *et al.*, 1994; Franceschi, 1999). Our protein expression data shows that this induction also occurs in the lens for α-crystallins. This is a strong argument in favour of using native matrices in *in vitro* culture (Bissell and Barcellos-Hoff, 1987). Also, the use of native rather than artificial matrices is important, as only the native matrix contains all the

elements that influence cell behaviour *in vivo*. This point is exemplified by U373-MG and MCF7 cells, which do grow on artificial mix of laminin, fibronectin and collagen IV, but fail to do so on the lens capsule, possibly due to the presence of sulfated proteoglycans (see section 3.3.3).

I also presented evidence for the role of MMP-2 in cell viability on the lens capsule. MMP-2 was already known to promote cell proliferation outside the lens and cell migration in the lens *in vitro*, and, independently, to count growth factor-binding proteins amongst its substrates (see section 5.1.2). The novelty of our results is that we showed that release of capsule-bound FGF-2 and IGF-1 by MMP-2, or MMP-2-activated MMP-9, does occur in the lens, offering a mechanism for MMP-2-mediated cell proliferation. This also suggests that TGFβ-2, which is traditionally associated with cell differentiation (Plisov *et al.*, 2000; Schofield and Wolpert, 1990) rather than proliferation (Jennings et al., 1994; Jennings *et al.*, 1988), could play a role in cell proliferation in the lens, via a positive feedback loop on MMP-2 secretion that would amplify the release of other growth factors.

5.3. Implications for after-cataract research:

The results of this study show that the lens capsule, via the release of sequestered growth factors, is an important actor in the viability of lens epithelial cells. These interactions between lens cells and lens capsule should be taken into account when devising new strategies to delay or prevent the development of posterior capsular opacification.

5.3.1. Decreasing cells-capsule compatibililty:

As shown in Chapter 2, the promotion of viability by the lens capsule is not available to all cells. Cells from non-lens origin, and even lens cells in long-term *in vitro* cell culture, showed poor viability on the lens capsule. Therefore, the question arises of whether decreasing the compatibility of the *in vivo* lens cells with their substrate might hinder their ability to be viable on it.

The lens cells can be manipulated to make them unsuitable to their original substrate. Blocking integrins activity, by means of specific antibodies or exogenous disintegrins, has proved a successful way of preventing cell attachment of both retinal and corneal cells (Li and Sakagushi, 2004; Doane *et al.*, 2002). In this study, I have suggested the role of α6β4 integrin in vimentin-mediated viability on the lens capsule (see Chapter 2). Blocking α6β4 integrin expression or activity might therefore decrease lens cells attachment to the laminin-rich capsule or their resistance against therapeutic apoptogens. However, α6β4 is also present at the leading edge of migrating cells in wounded corneas (Gipson *et al.*, 1993) and any perturbation of this activity, by non-lens-specific disintegrins, could impair corneal wound healing after cataract surgery.

A different, lens-specific protein in the eye is αA-crystallin. With a good lens-sepcificity and correlation to viability on the lens capsule (see Chapter 3), it remains a strong candidate for intra-cellular mediation of apoptosis resistance on the lens

capsule and its down-regulation, by techniques such as sRNAi, could allow greater sensitivity to apoptotic agents. The simultaneous addition of αA-crystallin RNAi and apoptogen at the time of surgery might result in decreased lens epithelial cell viability and delay the full colonisation of the posterior capsule by the remaining lens epithelial cells.

Decreasing the suitability of the lens capsule itself is also conceivable. Spraying the capsule with a synthetic, adhesion-refractory and transparent material could be a potential solution. However, and as found for materials used in IOL design, any remaining lens epithelial cells could eventually colonise the lens capsule by first laying down new matrix onto this artificial covering. Still, covering the lens capsule with a lens-incompatible material would present the advantage of denying the lens epithelial cells access to capsule-bound growth factors.

5.3.2. *Preventing growth factors availability:*

As seen in Chapter 4, the lens capsule acts as a store for growth-promoting factors that are releasable by lens epithelial cells via MMP-2 and MMP-9 secretion. Therefore, another potential way to reduce lens cells growth on the lens capsule might be by preventing gelatinase activity.

MMP-2 activity in the anterior segment is a feature of pathological conditions (Sakimoto *et al.*, 2004), and its inhibition should therefore not negatively affect other cell populations. Indeed, Endogenous TIMP-1 and TIMP-2 are readily found in the

aqueous humour after lens injury and effectively inhibit MMP-2 activity, although their levels returns to negligible values after a few days (Ni *et al.*, 2004). For more sustained effects, the heparin-binding TIMP3 could be used (Arris *et al.*, 2003). TIMP-3 injection during surgery should allow its binding to the lens capsule. When released by active MMP-2 cleavage of HSPG, TIMP-3 would inhibit MMP-2 by a negative feedback loop.

Apart from MMP-2 and MMP-9, which were the only matrix proteases investigated in my study, lens epithelial cells express other matrix proteases such as cathepsin B and ADAM9, which also degrade ECM components and are likely to release growth-promoting factors. Indeed, my results suggest that MMP-2 is not the only factor able to release growth factors from the lens capsule (section 4.4.1). To fully prevent lens cell viability, any additional factors need to be characterised and inhibited.

Another matter is whether the growth factors that are released from the lens capsule are also accessible to the lens cells from other sources. During and after primary cataract surgery, the aqueous humour is in direct contact with the lens epithelial cells and exposes them to growth factors such as EGF, KGF, HGF, PDGF, FGF, NGF and Gas6, several of which promote epithelial cells' proliferation (Klenker and Sheardown, 2004; Valverde *et al.*, 2004). If left exposed to the growth factors in the aqueous humour, lens epithelial cells should still proliferate, even after preventing them from releasing growth factors bound to the lens capsule. The inhibition of the aqueous humour factors themselves is not a desirable option, as they are necessary for

the maintenance of the corneal cells. Rather, to inhibit cell viability, the access of the lens epithelial cells to the aqueous humour must be restricted, which should be feasible by re-sealing the capsulorhexis flap during primary cataract surgery.

In the same way, after Nd:YAG laser treatment, cells growing onto the posterior capsule are exposed to the FGF-rich vitreous humour, which presents the advantage of antagonising the TGFβ-induced matrix wrinkling by decreasing α-smooth muscle actin expression (Cavallaro *et al.*, 2001), but also the disadvantage of promoting proliferation or fibre differentiation. This last aspect is especially relevant for the Soemmering's ring and Elshnig's pearl forms of after-cataract, which already represent abnormal fibre differentiation and might be exacerbated by the increased exposure to FGF-2 after Nd:YAG laser treatment.

5.4. Future experiments:

Several questions remained unanswered by my study and could be addressed by further experiments.

Factors involved in the promotion of lens-associated protein expression by the lens capsule were not investigated. Factors capable of such an induction include the transcription factors FoxE3 and Pax6, as well as the growth factor FGF-2. A close examination of their expression pattern, together with knock-out or knock-down studies, might reveal a correlation between their presence and lens-specific protein expression on the lens capsule.

The hypothesis I formulated about the role of αA-crystallin, αB-crystallin and HSP27 in cell viability on the bovine lens capsule could not be tested. The completion of RNAi experiments for lens epithelial cells on the bovine lens capsule is needed to ascertain their involvement.

The identity of both attachment factors and non-gelatinases factors in the promotion of cell growth and survival was not investigated. Potential candidates include α6β4 integrin, which is up-regulated by TGF-β2 and participate in hemidesmosomes with vimentin, and MMP-3 or MMP-7, which are present in the eye after cataract surgery and are inhibited by the resistance-reducing BB94.

Lastly, an aspect that was not investigated by our study was the effect of the lens capsule on lens epithelial cells' migration. Reducing migration of lens epithelial cells would be an effective way of delaying the obstruction of the visual axis, and therefore the need for laser treatment. Factors expressed by lens epithelial cells or present in their environment after cataract surgery, such as MMP-2, α-smooth actin and HGF, stimulate migration (Wong *et al.*, 2004; Nagamoto *et al.*, 2000; Wormstone *et al.*, 2000), as do components of the lens capsule itself (Oharazawa *et al.*, 1999). Here again, interactions between ECM components and MMPs have proved important for cell migration. Exposure to the α3 form of collagen IV can inhibit MMP-2 activation *in vitro* (Pasco *et al.*, 2000), and MMP-2 inhibition is independently known to decrease lens epithelial cell migration on the lens capsule

(Wong *et al.*, 2004). α3 collagen IV is already present in the capsule (Kelley *et al.*, 2002) and its exposure by selective proteolysis or addition at the time of cataract surgery might be an additional way of inhibiting MMP-2 activation and the resulting lens epithelial cells proliferation and migration on the lens capsule.

6. APPENDIX 1: Excerpts from 'Biostatistics: Experimental Design and Statistical Inference' (Zolman, 1993)

The following paragraphs are presented to support my choice in the use of standard deviation over standard error of the mean and of Pearson's correlation coefficient (r) over its square (r^2) throughout the thesis.

5.2 STANDARD DEVIATION AND STANDARD ERROR OF THE MEAN

The *standard deviation* (SD) and the *standard error of the mean* (SEM) are often confused in descriptive statistics (5.1). Most biologists summarize their data with the *standard error of the mean* because it is always smaller than the standard deviation; consequently, the data plotted look better (less variable). The standard deviation quantifies the variability in the sample data such that about 68 percent of the data obtained (assuming a normally distributed population, 5.1) will fall within one standard deviation from the mean, and about 95 percent of the data obtained within two standard deviations from the mean. Reviewers are usually interested in evaluating the data obtained in the experiment, and the best way to convey this information is to present the mean and its standard deviation for each treatment condition.

The *standard error of the mean* (SEM) quantifies the precision with which the sample mean *estimates* the population mean (5.1). The SEM is, therefore, the standard deviation of the sampling distribution of the mean and is calculated as:

$$\text{SEM} = \frac{\text{SD}}{\sqrt{n}}$$

If the standard error of the mean is confused with the standard deviation, the range of the sample values would be incorrectly assumed to be very narrow. Therefore, if the SEM is given in a report the number of animals in the sample *must* also be given so that the SD can be calculated to determine the variability in the sample data.

The Pearson correlation coefficient r is a measure of the linear relation between two random variables. The coefficient may vary between -1.00 and $+1.00$ where 0 signifies the absence of a relation and either -1.00 or $+1.00$ indicates a perfect relationship: A *positive correlation* implies a direct relation between the variables, as X increases Y increases, and a *negative correlation* implies an inverse relation, as X increases Y decreases. In summary, the correlation coefficient r is a measure of the strength of the linear association between two variables, X and Y, and the closer r comes to either $+$ 1.00 or -1.00, the stronger is the relation and the more nearly it approximates a straight line. A scatter diagram (also called a scatterplot or scattergram) allows you to view the relationship between the two variables. The predictor variable traditionally is represented on the abscissa, or X axis, the criterion variable on the ordinate, or Y axis.

7. APPENDIX 2: Probability values for Chapter 2

Legend to the treatments abbreviations used in the following probabilities tables:

stau: staurosporine

PC: cells grown on plastic without staurosporine

PS: cells grown on plastic with staurosporine

CC: cells grown on the lens capsule without staurosporine

CS: cells grown on the lens capsule with staurosporine

Ant C: cells grown on the anterior lens capsule without staurosporine

Ant S: cells grown on the anterior lens capsule with staurosporine

Post C: cells grown on the posterior lens capsule without staurosporine

Post S: cells grown on the posterior lens capsule without staurosporine

EC: early passage cells grown on the lens capsule without staurosporine

ES: early passage cells grown on the lens capsule with staurosporine

LC: late passage cells grown on the lens capsule with staurosporine

LS: late passage cells grown on the lens capsule without staurosporine

PI: MCF7 or U373-MG cells grown on the insert with cell-free palstic substrate

CI: MCF7 or U373-MG cells grown on the insert with cell-free capusle substrate

NP: cell-free plastic substrate used during co-culture experiment

NC: cell-free capsule substrate used during co-cultue experiment

S+PC: SW13vim+ cells grown on plastic without staurosporine

S+CC: SW13vim+ cells grown on the lens capsule without staurosporine

S-PC: SW13vim- cells grown on plastic without staurosporine

S-CC: SW13vim- cells grown on the lens capsule without staurosporine

Table A2.1: t-test P values for Figure 2.1 Effectiveness of the lens capsule cleaning process.

P values for No cell control	Plastic vs Capsule
Repeat 1	0.27
Repeat 2	0.34
Repeat 3	0.10

Table A2.2: ANOVA P values for Figure 2.1 Effectiveness of the lens capsule cleaning process.

P values for No cell control	Repeat 1 vs Repeat 2 vs Repeat 3
Plastic	0.06
Capsule	0.62

Table A2.3: t-test P values for Figure 2.2 Quantification of cell numbers in confluent cultures.

P values for Pearson's coefficient	Repeat 1	Repeat 2	Repeat 3
H36LE2	0.99	0.99	0.99
9MaB	0.99	0.99	0.99
MCF7	0.99	0.99	0.99
U373-MG	0.98	0.97	0.99
SW13vim+	0.99	0.99	0.98
SW13vim-	0.98	0.98	0.98

Table A2.4: ANOVA P values for Figure 2.2 Quantification of cell numbers in confluent cultures.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3				
	10 µl	50 µl	100 µl	200 µl	500 µl
H36LE2	0.48	0.64	0.53	0.66	0.72
9MaB	0.74	0.055	0.055	0.93	0.14
MCF7	0.23	0.81	0.92	0.24	0.79
U373-MG	0.47	0.11	0.23	0.16	0.13
SW13vim+	0.07	0.06	0.10	0.19	0.93
SW13vim-	0.18	0.76	0.14	0.45	0.14

Table A2.5: t-test P values for Figure 2.3 Determination of staurosporine concentration to be used in cell viability assays.

P values for t-test		0 vs 50	0 vs 125	0 vs 250	0 vs 500	0 vs 1000
H36 LE2	Repeat 1	<0.001	<0.001	<0.001	<0.001	<0.001
	Repeat 2	<0.001	<0.001	<0.001	<0.001	<0.001
	Repeat 3	<0.001	<0.001	<0.001	<0.001	<0.001
9MaB	Repeat 1	<0.001	<0.001	<0.001	<0.001	<0.001
	Repeat 2	<0.001	<0.001	<0.001	<0.001	<0.001
	Repeat 3	<0.001	<0.001	<0.001	<0.001	<0.001
MCF7	Repeat 1	<0.001	<0.001	<0.001	<0.001	<0.001
	Repeat 2	0.002	0.003	0.001	0.001	0.001
	Repeat 3	<0.001	<0.001	<0.001	<0.001	<0.001
U373-MG	Repeat 1	0.34	0.12	<0.001	<0.001	<0.001
	Repeat 2	0.84	0.25	<0.001	<0.001	<0.001
	Repeat 3	0.49	0.09	<0.001	<0.001	<0.001

Table A2.6: ANOVA P values for Figure 2.3 Determination of staurosporine concentration to be used in cell viability assays.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3					
	no stau	50nM	125nM	250nM	500nM	1000nM
H36LE2	0.21	0.26	0.05	0.02	0.31	0.11
9MaB	0.81	0.17	0.02	0.01	0.48	<0.001
MCF7	0.59	0.07	0.55	0.31	0.26	0.08
U373-MG	0.82	0.62	0.43	0.18	0.66	0.38

Table A2.7: ANOVA P values for Figure 2.4 Standard curves for conversion of absorbance values into viable cell numbers.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3					
	no cells	10^4 cells	5.10^4 cells	10^5 cells	5.10^5 cells	10^6 cells
H36LE2	0.25	0.08	0.22	0.14	0.055	<0.001
9MaB	0.01	0.08	0.15	0.14	0.01	0.16
MCF7	0.003	<0.001	<0.001	<0.001	<0.001	0.27
U373-MG	0.50	<0.001	<0.001	<0.001	0.44	0.15
SW13vim+	<0.001	<0.001	0.004	<0.001	<0.001	0.01
SW13vim-	<0.001	<0.001	0.66	0.85	0.97	<0.001

Table A2.8: t-test P values for Figure 2.6 Culture on the bovine lens capsule significantly reduces the staurosporine-induced decrease in cell numbers in H36LE2 and 9MaB cells, but not in MCF7 and U373-MG cells.

P values for t-test		PC vs PS	CC vs CS	PC vs CC	PS vs CS
H36 LE2	Repeat 1	<0.001	0.002	0.65	<0.001
	Repeat 2	0.03	0.73	0.15	0.002
	Repeat 3	<0.001	<0.001	<0.001	<0.001
9MaB	Repeat 1	<0.001	0.02	<0.001	<0.001
	Repeat 2	0.001	0.01	0.29	0.007
	Repeat 3	<0.001	0.014	0.08	0.008
MCF7	Repeat 1	<0.001	<0.001	<0.001	0.004
	Repeat 2	<0.001	0.048	<0.001	0.12
	Repeat 3	0.003	<0.001	<0.001	0.056
U373-MG	Repeat 1	<0.001	<0.001	<0.001	<0.001
	Repeat 2	0.008	0.01	<0.001	0.001
	Repeat 3	<0.001	0.06	<0.001	0.16

Table A2.9: ANOVA P values for Figure 2.6 Culture on the bovine lens capsule significantly reduces the staurosporine-induced decrease in cell numbers in H36LE2 and 9MaB cells, but not in MCF7 and U373-MG cells.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
	PC	PS	CC	CS
H36LE2	0.33	0.21	0.17	<0.001
9MaB	0.079	0.59	0.23	0.003
MCF7	0.13	<0.001	0.39	0.001
U373-MG	0.21	<0.001	0.004	<0.001

Table A2.10: t-test P values for Figure 2.7 Increased length of *in vitro* culture decreases cell viability on the lens capsule for both H36LE2 and 9MaB cells.

P values for t-test		EC vs E S	L C vs L S	EC vs LC	E S vs E S
H36 LE2	Repeat 1	<0.001	<0.001	<0.001	<0.001
	Repeat 2	0.002	<0.001	<0.001	<0.001
	Repeat 3	<0.001	<0.001	0.06	<0.001
9MaB	Repeat 1	<0.001	<0.001	<0.001	<0.001
	Repeat 2	0.85	0.068	<0.001	<0.001
	Repeat 3	0.83	<0.001	<0.001	<0.001

Table A2.11: ANOVA P values for Figure 2.7 Increased length of *in vitro* culture decreases cell viability on the lens capsule for both H36LE2 and 9MaB cells.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
H36LE2	E C	E S	L C	L S
	0.28	0.06	<0.001	<0.001
9MaB	E C	E S	L C	L S
	0.12	0.19	0.07	0.03

Table A2.12: t-test P values for Figure 2.8 Co-culture of MCF7 and U373-MG cells with the lens capsule.

P values for t-test		PC vs CC	PI vs CI
MCF7	Repeat 1	0.03	<0.001
	Repeat 2	<0.001	<0.001
	Repeat 3	<0.001	<0.001
U373	Repeat 1	<0.001	<0.001
	Repeat 2	<0.001	<0.001
	Repeat 3	<0.001	0.016

Table A2.13: ANOVA P values for Figure 2.8 Co-culture of MCF7 and U373-MG cells with the lens capsule.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3					
	NP	PC	PI	NC	CC	CI
MCF7	0.38	0.95	<0.001	0.53	0.74	0.16
U373	0.14	0.20	0.19	0.91	0.053	0.26

Table A2.14: t-test P values for Figure 2.9 Vimentin-expressing SW13vim+ cells have an increased viability on the lens capsule compared to the vimentin-lacking SW13vim-cells.

P values for t-test		S+PC vs S+CC	S-PC vs S-CC	S+PC vs S-PC	S+CC vs S-CC
SW13	Repeat 1	0.62	<0.001	0.07	<0.001
	Repeat 2	0.09	0.001	0.35	<0.001
	Repeat 3	0.0504	<0.001	0.20	0.028

Table A2.15: ANOVA P values for Figure 2.9 Vimentin-expressing SW13vim+ cells have an increased viability on the lens capsule compared to the vimentin-lacking SW13vim-cells.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
SW13	S+PC	S+CC	S-PC	S-CC
	0.98	0.02	0.065	0.27

8. APPENDIX 3: DNA sequences for Chapter 3

DNA sequence of the genes whose mRNA expression was investigated. Note that , whenever possible, the position of the primers allows for differences between the PCR product sizes amplified from DNA or cDNA templates.

Black: DNA sequence

Blue: cDNA sequence

Red: PCR primer sequence

Green: sequence picked up by BLAST during PCR product identification

Figure A3.1 LEDGF (sequence number AF199339):

Blue: LEDGF/p75 cDNA

Underlined: p52 cDNA

ggaaacatga ctcgcgattt caaacctgga gacctcatct tcgccaagat

gaaaggttat ccccattggc cagctcgagt aagtgatttg tagccctcgc

gcttagcagt gctaccctcc tctccagagg cctgctgccc accgtgtctc

tttaaaaaaa cttctgtcct ttctctccct cgctttagtc tcatttgtaa

cctgaccttt cattttagtt ttttgcccaa taatctcccg tgtaattatg

aaaatccaga cgtttctaca tacctgtgta tttccctatg gtttattggc

agacttggac agattttctt cctaggaaat atttaaatgc gttgctagtt

aatattccat tgtaggtgaa ataatgaaga gtgaattttt gtttaaaaat

tagtgaaaca ttgacatttc catagttttg gatcaagtca aaatatcaat

ggaatttgtg cagattacac acaaacactt ggatgtcaga gaagtgcgta

tgtgattaaa ttggatttac agggttttta aaaaaaaatc cttgtacttg

atgaggcctt ctggatttgc ttgcttaata cagattcagg actggatatt

cttgaaagta ttacagtagt ttagtaccga ggaagggcca gaaaattcat

ttctcctttt ttatttgaga tgtttatgac cttgaagaag ttgttttacc

tttttgtttc caataggcaa aaaggaggtg tggaccagat tcctcagttc

acatctagtg gtacagagaa atgattaagg acccaggatg ttttgtgaca

ttgaggaatt gagactgttt aaagatctac gctccatttt tagaaaaagg

gttctttttg tttatggtgg tcccaggctc tcaaacagag atagatagga

cctaagatat gttgcaatca attagattag gtaaactcaa gatcctttaa

aaatatactt acatggggtt ctgaagtttt aaaaaaccaa gccatttatt

ttcgactaat ttactgcctt tgaaaatgta tttcttaaaa ataattaaat

tctgtagttt tagttatgtc agcaggggaa tggtggaaaa cacccattag

ataaacggag tcctgtctag gagctgagag tgccatccaa gtgtaaaacg

atcactgttc cttgactttc ttccatctag ctgctctacg taaactcctg

ttcacaagtt tccttccaaa gctttttttt ccttgcctat aaagtctgcc

ttttcggtgc agtcttactt gaacaatcct tagtcataat ccttgtctgt

atattttaca ttctaatcta ttcctttcga gataatcttt tgttttaatt

cttaactctg tgtatgcttt gacctccaaa tatattgtga gaccgagggc

aacaacctta aatggaattt gtttataccc tccatagtcc gtagtatata

tgatgcacct ataaaacaca attcacgttc aaatggagcc tagggaaact

gatcaggttt aaggcccctc tgatatactt tttcatggtc ttcgaggtat

ccttgtttat tttgaggtgt ttgaaaatat gaggttgtct aatagatcta

tattccgatt tgcatgccac agtgaagtta aaagccaaag gaaatgttta

aaaaatctcc caaatcaccc tccccacgct catatgttaa gtgactaaga

gtgctactga aaataaaata gggtagcctt tggaagacca gagtgttttc

cttatttaac aagttctaca tttaacaagt tcatttggat tggaagggaa

gtgtttgtct tttccctttt ccccatcatt ttttctgaga aacacctcca

tttagtgttg gtgtattctg tgtccaacag taacattcct ttttctgtct

tcttcattct taaaaatttg aaatcttgct ggtagagttt agagtccata

tttaaagtnt gagccgtaaa ggtgacacct cttgttgcaa tttttgcttt

aacatttctg tctacatgtc ccaaacttca ttaattttta agtcttaaac

attcatgtac ataatcgttt accagtaata attatttaaa aaatcaactt

aaattttccc ggcatttgct aaaggctata ggtgctaggt atttgcataa

actctttaag ctgtttgcta gcagaaagag gcatatgaac tgggttaaca

gaaatcatct cttaagggat taaggatttt tgcaattatc gggacattcc

cacaaagttg agataacagg tgtgatcttc atcatataag tggggaaata

ggtccctaga gatattgggt ggtaatgatt ctgttggtgt gtgaaagcct

ttagacttcc ctcacaaatc agttcagtca tcctgatggt cttcttctgc

aacaccataa tgcttacaga ggatagtagg tcctctgatg gtcaaccccc

ttcttaacaa agtcagctga ttttcaaatt gaagtgtttt tgaaattatg

aaaattatga catttaaaat agatatgttg ttaaattctt ttttttgtt

ttttttaaag acaggatctc actctgtcac ccaggcttga gtgcagtggc

atgatctcgg ctcactgcaa cctctgttgc ccaggttcaa gtgattcgcc

tctggagtgg ctgagattac aggcacctga caccgtgccc tgctaatttt

tgtattttta gtagagaccg ggtttcacca tattggtcag gctggtcttg

aactcctgac ctcatgatcc atccaccttg ccctcccaga gtgctgggat

tacaggtgtg agccaccgcg cctggccgac atgttgttaa attctagcta

gatagtactg ctttagtcct gaggtctgct tttcctttta ttgatgaggg

aaagtaagaa gtattatgga tatattagac gtgacagcac ctgatgattt

cttccttaca taatttaaaa ggttgaaaga gcagtgaaaa aggaacaggt

agcctcatgt gattcaggca catcagtgaa ggatcttttc atagtaagtt

tttcagactt tgggaggtac tgatttaaac ttttacatta gtctgttatt

tgtatagatt tcctgcagaa ctaacattta gggttgtcaa ataggttcag

actccttacc tgaaatgata tgattaacaa ttcagaattc tgggatttta

ggaaggcagg gagagtacat atgccatata ttttgctgct ggtgcggtct

ggaatagcac ataataatat gtctgcaaag aaacaacagt ggaaacaggt

ggataaaaga aatgactgat accctcaatg tcagtccagg ttttgccacc

aaatgagttt tgacagaaat tttggttttc agagcttctt gggtttcaga

tttggggata ggagattatg ggcctgcaga actggcccat tttataacag

aacccttttg gatgttgtgc tcttgtttat tcaaataaat gttgaggtgt

gtatgattta aaatatttta atttttctca cttttctttt aggtagacga

agttcctgat ggagctgtaa agccacccac aaacaaacta cccattttct

tttttggaac tcatgagacg taagtcctgt tagctttaaa ggcaaatcag

agctaattta acagggttat taggcagtag acattaattc aaggggaaa

tcgtaacgta ataggaaact ttaaaatctt tgaactttgg aaagagtctt

taagtctcta tttttaaggt agcgtttaca gatgctatag gtttatttt

tactggaatt gatatttcct taaccaaaaa ggaaagtaga aattctctga

gcaataagaa tgattgatgg atgaaaatac ataaggaaag ttttaataag

tgatctgata ttcctataca gtaaaataag tgctttttat aaggactagt

tggtacctaa tttttggttt tatgatatca gatatgcaaa ctgtgtatat

tgtggagaag gtgagaagag gaagtctagc tagttccctc catgtgttta

tatggtccct gaagctctgc tgcctctggt gggtatgtgg gcagagaact

gatgtgaagc aaatttttca aaaattgcag ctgaatgttg acgtatgtta

ctgccaagtg catgggatca ctcaggaggc taccgttaat ttcccgaaga

atgttcctat gtacctataa tttaatggcc ttagaaatac tatataatct

ggttgatttt ggacttgaag tttggtgtct gtcataattt tggtagtctt

gtttacctgg gatgaggaga aacaaaacta ttgctattct gattagaatt

atgtattcct aagagtggct gcaatggcaa tatttggttt aaagttgctt

agctactgta taaactttca cacaatgtct ttaatagctc tattgagatg

gaatttacat acaaagaagt tcacctgctt aaagtgattt tcaatagatt

ttagtatatt tagtagattt tagtatattg actatcataa ccttttgatg

atcactttcc cctctccaac cgtaaacaac cactagtcta ctgtctgtat

agacttacca gttctggaca ttttatttga atggtgtcat acaatatttc

ataacaagtt tttaaaaaaa tacaactttg gatccagaat tggtttggcc

ttcatcataa ttagagttag gttcaagtgt attctatata taacaatata

atttttaaga agtgataagt ttctgattcc atgggatact ttaaagaaac

tcttcagtaa atttaataga ggacaaaagt atcttttaat ggaaaatggt

gcagtgtata ttagagtagg gcttttcaaa gtgtgttgtc aggtctagca

ccattagcat ctcttgcgaa ctttaattaa aatgtaagtt cttggctggg

tgtggtggct gacacctgta atcccaacac tttgggaggc caaggcggga

gggtcacttg agttcaggag ttggagacca gcctgcaaca tggtgaaact

gtgtctctac aaaaaataca aaaattagcc gtttgtggtg gcacacacat

gtggtcccag ctactccaga ggctgaggtg gaaccgtcac ctgagccggg

gaggcaaagg ttgcagtgag ccaagatctc accactgcag acctcatctc

aaaaaaaaaa aaaaaaaaaa agttcttagg ccttattcta gacctactga

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aactttcaga ctgctcacag aaggaatatg ctgaaaggcc aacatgagaa

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acttacttag atttattcta gttaagccat aagttcaacg tgtaaacttg

ttttcattaa agaatttttc tatcaaactc tgctagtaaa

Figure A3.2 DNA sequence retrieved from sequencing the 56.3^{0}C LEDGF PCR product:

Green: sequence used for BLAST

ttatnaacaaaaatttaagcnnntnancccnntaactcgggtgagaaacnggn
cacaacacannngctagngtaangcncnanccggggcanngactcnaantggn
ggancngangctccagatggagaatcagccncccatctcgngncagagcaatg
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ncagagagagactganatanctcngaaggnntnnacnctagnnanctnggtng
ncntncctgnnanatngagnacaccttgagaagtttgtaatggcnttacnntt
ncnatnnactgcggaaagnttcnnatnntnatnngnanagnnttnangttgaa
aacttgtnttgaggnnggaatcccctaaanaa

Figure A3.3 Vimentin (sequence number AL133415):

Black: DNA sequence
Blue: cDNA sequence
Red: primer sequence

acatcatgcg cctccgggag aagtaaggct gcgcccatgc aagtagctgg
gcctcgggag ggggctggag ggagagggga acgccccccc ggcccccgcg
agagctgcca cgcccttggg gatgtggccg gggggaggcc tgccagggag
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tgaactgata cagtggcaag tgaaaaagac atgcctttac aggatgagtc

aaaggagttt tagaagaaaa atccaccaga gaaagccaag caaatacagt

tcagagttac atttcttttc catttttcc tgaactgaat ctttggcatg

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gataatatct aaacaattct ctacttggtc

9. APPENDIX 4: Probability values for Chapter 4

Legend to the treatments abbreviations used in the following probabilities tables:

PC: cells grown on plastic without staurosporine (except for Table A4.7, Table A4.8, Table A4.9 and Table A4.10: cell-free plastic without stauropsorine)

PS: cells grown on plastic with staurosporine

CC: cells grown on the lens capsule without staurosporine (except for Table A4.7, Table A4.8, Table A4.9 and Table A4.10: cell-free lens capsule without stauropsorine)

CS: cells grown on the lens capsule with staurosporine

C2+9+: cell-free capsule with partially purified MMP-2/MMP-9 mix

C2-: cells grown on the lens capsule with MMP-2 inhibitor without staurosporine

C9-: cells grown on the lens capsule with MMP-9 inhibitor without staurosporine

CS2-: cells grown on the lens capsule with MMP-2 inhibitor with staurosporine

CS9-: cells grown on the lens capsule with MMP-9 inhibitor with staurosporine

Table A4.1: t-test P values for Figure 4.1 Culture on the bovine lens capsule allows lens cells' viability in low-serum culture medium.

P values for t-test		PC vs PS	CC vs CS	PC vs CC	PS vs CS
H36LE2	Repeat 1	N/A	0.02	<0.001	<0.001
	Repeat 2	N/A	<0.001	0.03	0.002
	Repeat 3	N/A	<0.001	<0.001	<0.001

Table A4.2: ANOVA P values for Figure 4.1 Culture on the bovine lens capsule allows lens cells' viability in low-serum culture medium.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
H36LE2	PC	PS	CC	CS
	N/A	N/A	0.60	0.19

N/A: non-applicable. Probabilities could not be calculated due to the absence of detectable viable cells in the corresponding treatment.

Table A4.3: t-test P values for Figure 4.4 FGF-2 medium levels.

P values for t-test		PC vs PS	CC vs CS	PC vs CC	PS vs CS
H36LE2	Repeat 1	0.90	0.97	0.96	0.92
	Repeat 2	0.66	0.91	0.29	0.36
	Repeat 3	0.68	0.91	0.29	0.36
MCF7	Repeat 1	0.08	0.79	0.15	0.75
	Repeat 2	0.32	0.69	0.71	0.64
	Repeat 3	0.70	0.64	0.27	0.89

Table A4.4: ANOVA P values for Figure 4.4 FGF-2 medium levels.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
H36LE2	PC	PS	CC	CS
	0.42	0.26	0.84	0.31
MCF7	PC	PS	CC	CS
	0.25	0.63	0.31	0.96

Table A4.5: t-test P values for Figure 4.5 IGF-1 medium levels.

P values for t-test		PC vs PS	CC vs CS	PC vs CC	PS vs CS
H36LE2	Repeat 1	0.42	0.55	0.30	0.25
	Repeat 2	0.46	0.45	0.17	0.81
	Repeat 3	0.25	0.45	0.03	<0.001
MCF7	Repeat 1	0.48	0.08	0.28	0.34
	Repeat 2	0.12	0.15	0.58	0.01
	Repeat 3	0.02	0.20	0.09	0.02

Table A4.6: ANOVA P values for Figure 4.5 IGF-1 medium levels.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
H36LE2	PC	PS	CC	CS
	0.003	0.51	0.43	0.86
MCF7	PC	PS	CC	CS
	0.43	0.24	0.04	0.01

Table A4.7: t-test P values for Figure 4.6 FGF-2 and IGF-1 medium levels in the absence of cells.

P values for t-test		PC vs CC
FGF-2	Repeat 1	0.11
	Repeat 2	0.06
	Repeat 3	0.14
IGF-1	Repeat 1	0.03
	Repeat 2	0.01
	Repeat 3	0.01

Table A4.8: ANOVA P values for Figure 4.6 FGF-2 and IGF-1 medium levels in the absence of cells.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3	
FGF-2	PC	CC
	0.70	0.12
IGF-1	PC	CC
	0.66	0.13

Table A4.9: t-test P values for Figure 4.9 FGF-2 and IGF-1 medium levels in the presence of gelatinases.

P values for t-test		CC vs C2+9+
No cell control FGF-2	Repeat 1	<0.001
	Repeat 2	<0.001
	Repeat 3	<0.001
No cell control IGF-1	Repeat 1	0.90
	Repeat 2	0.40
	Repeat 3	0.04

Table A4.10: ANOVA P values for Figure 4.9 FGF-2 and IGF-1 medium levels in the presence of gelatinases.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3	
No cell control FGF-2	CC	C2+9+
	0.80	0.18
No cell control IGF-1	CC	C2+9+
	0.07	0.05

Table A4.11: t-test P values for Figure 4.10: The effect of MMP inhibition on growth factors levels on the lens capsule.

P values for t-test		CC vs C2-	CC vs C9-	C2- vs C9-
H36LE2 FGF-2	Repeat 1	0.17	0.01	0.04
	Repeat 2	0.37	0.36	0.97
	Repeat 3	0.21	0.34	0.90
H36LE2 IGF-1	Repeat 1	0.93	0.38	0.18
	Repeat 2	0.85	0.04	0.03
	Repeat 3	0.04	0.04	0.97

Table A4.12: ANOVA P values for Figure 4.10: The effect of MMP inhibition on growth factors levels on the lens capsule.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3		
	CC	C2-	C9-
H36LE2 FGF-2	0.61	0.06	0.59
H36LE2 IGF-1	0.38	0.08	0.17

Table A4.13: t-test P values for Figure 4.11: MMP-2 inhibition in lens epithelial cells affects cell viability on the lens capsule in the absence of staurosporine.

P values for t-test		CC vs C2-	CC vs C9-
H36LE2	Repeat 1	0.02	0.57
	Repeat 2	0.006	0.79
	Repeat 3	<0.001	0.53

Table A4.14: ANOVA P values for Figure 4.11: MMP-2 inhibition in lens epithelial cells affects cell viability on the lens capsule in the absence of staurosporine.

P values for ANOVA	Repeat 1 vs Repeat 2 vs Repeat 3			
H36LE2	CC (A)	C2-	CC (B)	C9-
	0.61	0.36	0.79	0.06

Table A4.15: t-test P values for Figure 4.12: MMP-2 and MMP-9 inhibition do not decrease H36LE2 cells viability on the lens capsule in the presence of staurosporine.

P values for t-test		CS vs CS2-	CS vs CS9-
H36LE2	Repeat 1	0.79	0.84
	Repeat 2	0.18	0.39
	Repeat 3	0.61	0.07

Table A4.16: ANOVA P values for Figure 4.12: MMP-2 and MMP-9 inhibition do not decrease H36LE2 cells viability on the lens capsule in the presence of staurosporine.

P values	Repeat 1 vs Repeat 2 vs Repeat 3			
H36LE2 : between repeats ANOVA	CS (A)	CS2-	CS (B)	CS9-
	0.14	0.55	0.60	0.22

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