The Expression of Tumour Necrosis Factors during Chick Lens Development

Thesis submitted to Cardiff University for the degree of Doctor of Philosophy

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Abstract

During development of the lens, epithelial cells at the lens equator begin a differentiation process to become secondary fibre cells. The differentiating cells elongate and migrate towards the centre of the lens where they envelop the older, central fibre cells. Differentiation into fibre cells is accompanied by the breakdown of all organelles, such as the mitochondria. All organelle degradation is completed and denucleation occurs at the border of the organelle free zone (OFZ) which contains the central, terminally differentiated, fibre cells. The differentiation pathway is not well characterised, though it is believed to have similarities to an attenuated form of apoptosis supported by the identification of apoptosis related genes, such as TNF, in the lens. This study continues the search for and characterisation of apoptosis related genes expressed during lens development, focusing on TNFs and their extended family.

Reverse Transcriptase- (RT-) PCR was carried out, identifying a number of TNF and extended family member genes in the chick lens, expression studies established novel, statistically significant differential expression for TRAF2 and TRAF3. TRAF2 protein expression from western blotting, similar to RT-PCR expression was found to decline as the lens developed. TRAF2 localisation studies showed limited expression in the equatorial region but there was extensive signalling found in the developing iris, a region in the corneal-scleral boundary and some staining was also detected in the ciliary body.

TRAF3 protein and RT-PCR expression were similar, with increasing expression as the lens developed. Western blotting identified two bands and subcellular fractionation confirmed different localisation for the two isoforms. Immunofluorescence identified increasing TRAF3 staining in the cortical fibre cells, this staining was found to be similar to proteins that were reported to be involved in lens fibre cell remodelling and maintenance, suggesting a possibly similar role for TRAF3.

Following interest in TRAIL as a gene therapy for Posterior Capsule Opacification (PCO) its expression was examined using RT-PCR and Western blotting which showed low, similar levels of expression throughout the stages of lens development studied. Peroxidase staining showed interesting staining in the equatorial epithelial cells and those just beginning to differentiate at the transition zone. Novel nuclear staining was identified at all time points in both epithelial and fibre cells containing nuclei.

Characterisation of whole lens culture was undertaken to discover the optimum culture system for the whole chick lens. Of the published research using whole chick lens culture none stated the basic morphology of the developing lens in organ culture, though each lab had their preferred methodology. The characterisation resulted in the preference of E10 chick lenses being grown with vitreous attached in medium containing glucose. Understanding the morphology of lenses in culture will be invaluable when undertaking the functional studies required to clarify the roles in the lens of the newly identified genes, specifically TRAF2 and TRAF3.

List of Abbreviation

αSMA – alpha Smooth Muscle Actin
ADP – Adenosine Diphosphate
AP1 – Activator Protein -1
Apaf – Apoptotic protease activating factor 1
ATP – Adenosine Triphosphate
Bad – Bcl2 family protein - proapoptotic
Bax – Bcl2 family protein – proapoptotic
BCA assay – Bicinchoninic acid assay
BLAST – Basic local alignment search tool
BMP – Bone Morphogenetic Protein
BrdU – Bromodeoxyuridine
CARD – Caspase Recruitment Domain
CART1– C-rich motif associated with RING and TRAF domains 1
CD30L – CD30 ligand; Tumour Necrosis Factor ligand superfamily member8
CD40 – Tumour Necrosis Factor ligand superfamily member5
cDNA – complementary Deoxyribonucleic Acid
Cds – Coding Sequence (NCBI website)
CHOP – C/EBP homologous protein, a leucine zipper transcription factor
CP49 – phakinin
CRD – Cysteine Rich Domain
CRYG – Gamma Crystallin
crmA – Cytokine response modifier A
Cx – Connexin
DAPI – 4',6-Diamidino-2-phenylindol
DcR – Death Decoy Receptor
DD – Death Domain
DEVDase – Cleaves the cpp32 protein recognition site asp-glu-val-asp and is indicative of caspase-3 activity
DFF – DNA fragmentation factor
DISC – Death-Inducing Signalling Complex
DLAD – DNase II like DNase
DNA – Deoxyribonucleic Acid
DR(1-5) – Death Receptor (1-5)
DTT – Dithiothreitol
E – Embryonic Day
ER – Endoplasmic Reticulum
ERK – Extracellular-receptor kinases
Eya1 – Eyes absent 1
FCS – Fetal Calf Serum
FGF – Fibroblast Growth Factors
FADD – Fas-Associated Death Domain
Fas –TNF receptor superfamily, member 6
FoxE3 – a lens-specific member of the large forkhead transcription factor family
GAPDH – Gylceraldehyde-3-phosphate dehydrogenase

GJA1 – Connexin 43
GSK – Glycogen Synthase Kinase-3
H &E – Haematoxylin and Eosin
hLEC – Human Lens Epithelial Cells
HSP – Heat Shock Protein
IAP1 – Inhibitor of Apoptosis Protein1
IGg – Immunoglobin G
IκB – Inhibitor of NF-κB
IKK – IκB Kinase
IMS – Industrial Methylated Spirit
IOL – Intraocular lens
IRE1 – Intracellular Transmembrane ER receptor
Jag1 – Notch ligand Jagged 1
JNK – Jun N-terminal kinase
L-Maf – lens-specific Maf
LMP – Latent Membrane Protein
LTβ– Lymphotoxin- beta
15-LOX – 15- Lipoxygenase
MAPK –Mitogen-activated protein kinase
MIP-T3 – TRAF3 interacting protein-1
MOPS – 4-Morpholinepropanesulfonic acid
Nd:YAG – Neodymium: laser capsulotomy
NF-κB – Nuclear factor-kappa B
NIK – NF-κB-inducing kinase
OFZ – Organelle Free Zone
OPG – Osteoprotegerin
Otx2 – Orthodenticle homeobox 2
Ox40 – Tumor necrosis factor receptor superfamily 4
p38 – p38 MAP kinase
p53 – tumour protein 53
p60 – tumour protein 60
p62 – nucleoporin 62kDa
PARP – poly (ADP-ribose) polymerase
Pax6 – Paired box gene 6
PBS – Phosphate Buffered Saline
PCO – Posterior Capsule Opacification
PCR – Polymerase Chain Reaction
PI3K – Phosphatidylinositol 3-kinase
Pitx – Paired-like homeodomain transcription factor
PLE – Presumptive lens ectoderm
Prox – Prospero-related homeobox gene
RAIDD – Receptor-interacting protein (RIP)-associated ICH-1/CED-3 homologous protein with a death domain
RANKL–TNF-related activation-induced cytokine/ receptor activator of NF-κB ligand
RING – Really Interesting New Gene, a RING-finger is a specialised type of Zinc finger of 40 to 60 residues
RIPA – Radio-Immunoprecipitation Assay buffer
RIP – receptor-interacting protein

RNA – Ribonucleic Acid
RPE – Retinal Pigment Epithelium
Rx – Retinal homeobox gene 3
SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Six3 – sine oculis-related homeobox gene
Sox2 – SRY-box containing gene 2
TAE – Tris Acetate Buffer
TBS – Tris-buffered saline
TE buffer –Tris EDTA buffer
THD – TNF Homology Domain
TGF – Transforming Growth Factors
TLR2 – Toll-like receptor 2
TNFs – Tumour Necrosis Factors
TNFα – Tumour Necrosis Factor α
TNFR – Tumour Necrosis Factor Receptor
TRADD – TNF Receptor Associated Death Domain
TRAF – TNF Receptor-Associated factor
TRAIL – TNF-Related Apoptosis Inducing Ligand
UPP – Ubiquitin- Proteasome Pathway
UVB – Ultraviolet A/ Black light
VEIDase – Caspase -6-like proteolytic activity
Wnt – name derived from a mixture of wingless and INT (genes), pathway known in embryogenesis and cancer

Table of Contents

Declaration i
Acknowledgements ii
Abstract iii
List of Abbreviation iv
Table of Contents vii
List of Figures xi
List of Tables xiii
Chapter 1: Introduction 1
1.1 General Introduction 2
1.2 Aims of this PhD 3
1.3 The use of model organisms 3
1.4 Ocular Structure 4
1.5 Ocular Development 6
1.6 The Lens 7
1.6.1 Lens Development 9
1.6.2 Induction of the lens 10
1.6.3 The Lens Vesicle 11
1.6.4 Lens Fibre Cell Differentiation 12
1.6.5 Secondary Fibre Cell Differentiation 13
1.6.6 Lens Organelle degradation 14
1.7 Molecular signals in lens development 17
1.7.1 Crystallins 18
1.7.2 Pax6 19
1.7.3 Bone morphogenetic protein (BMP) 20
1.7.4 Notch Signalling 20
1.7.5 Fibroblast Growth Factors (FGF) 21
1.7.6 Wnt signals 21
1.7.7 Caspases 22
1.7.8 Connexins 23
1.8 Cytoskeleton remodelling during differentiation 23
1.9 Cataract 25
1.9.1 Congenital Cataract 25
1.9.2 Age-Related Cataract 26
1.9.3 Posterior Capsular Opacification 27
1.10 Apoptosis 27
1.11 Tumour Necrosis Factor Ligands and Receptors 29
1.11.1 Tumour Necrosis Factor Ligands (TNFs) 30
1.11.2 Tumour Necrosis Factor Receptors (TNFR's) 31
1.11.3 The Tumour Necrosis Factors in the lens 31
1.11.4 Selected Tumour Necrosis Factors and their adaptors 32
1.11.5 TNF Related Apoptosis Inducing Ligand (TRAIL) 32

1.12 TNF receptor-associated factors (TRAFs) 33
1.12.0.1 NF-κB 35
1.12.0.2 MAPK pathways 36
1.12.1 TNF receptor-associated factor 1 (TRAF1) 37
1.12.2 TNF receptor-associated factor 2 (TRAF2) 38
1.12.3 TNF receptor-associated factor 3 (TRAF3) 39
1.12.4 TNF receptor-associated factor 4 (TRAF4) 40
1.12.5 TNF receptor-associated factor 5 (TRAF5) 41
1.12.6 TNF receptor-associated factor 6 (TRAF6) 41
1.12.7 TNF receptor-associated factor 7 (TRAF7) 42
1.13 Summary of Introduction 43
1.13.1 Objectives: 43
Chapter 2: Materials and Methods 45
2.1 Tissue Collection 46
2.1.1 Egg collection and incubation 46
2.1.2 Tissue collection 47
2.2 Polymerase Chain Reaction (PCR) 49
2.2.1 Preparation of RNA (Ribonucleic Acid) from tissue 49
2.2.2 TRIzol Preparation 50
2.2.3 Quantification and Integrity of RNA 51
2.2.4 DNase digestion 52
2.2.5 Reverse Transcriptase (RT) Reaction 52
2.2.6 Primer design 53
2.2.7 Optimisation of Primers 55
2.2.8 Polymerase Chain Reaction (PCR) 55
2.2.9 PCR Visualisation 56
2.2.10 Normalisation of GAPDH 57
2.2.11 Analysis of Genes 57
2.3 Western Blotting 58
2.3.1 Protein Isolation 58
2.3.2 Subcellular Fractionation 58
2.3.3 Protein Quantification 60
2.3.4 SDS-Page 61
2.3.5 Preparation of protein sample sets 62
2.3.6 Protein transfer 62
2.3.7 Membrane blocking 63
2.3.8 Primary antibody incubation 63
2.3.9 Secondary antibody incubation 63
2.3.10 Developing films from the membranes 64
2.3.11 Stripping membrane 64
2.3.12 Re-incubation with Actin antibody 64
2.3.13 Western Blotting Analysis 65
2.4 Histology 65
2.4.1 Preparing tissue for histology 65
2.4.2 Embedding tissue 66
2.4.3 Sectioning tissue 66
2.4.4 Selecting sections/ slides for staining 67
2.4.5 Haematoxylin and Eosin Staining 67

2.4.6 Peroxidase staining 68
2.4.7 Immunofluorescence 69
2.5 Organ Culture 70
2.5.1 Preparing Medium 70
2.5.2 Preparing Tissue 70
2.5.3 Assessment of tissue quality 71
2.5.4 Tissue collection 71
2.5.5 Analysis of cultured lens opacities 71
2.5.6 Fixing cultures 71
2.5.7 Haematoxylin and Eosin Staining 72
Chapter 3: TNF family member expression in developing Chick Lens 73
3.1 Introduction 74
3.2 Experimental design 75
3.3 Integrity of RNA samples 75
3.4 PCR primer optimisation 76
3.5 Semi-quantitative RT(reverse transcriptase)-PCR 78
3.6 Analysis of Semi-quantitative RT-PCR 78
3.7 Statistical analysis of RT-PCR 80
3.8 Discussion 81
Chapter 4: TNF-related factors in the Developing Chick Lens: Spatio-temporally Regulated Expression of TRAF2, TRAF3 and TRAIL proteins. 83
4.1 Introduction 84
4.2 Experimental Design 85
4.2.1 Western Blotting 86
4.2.2 Subcellular Fractionation 86
4.2.3 Histology 87
4.3 Results 89
4.3.1 Western Blotting 89
4.3.1.2 TRAF3 90
4.3.1.4 TRAIL 93
4.3.2 Histology 93
4.3.2.1 Haematoxylin and Eosin staining 94
4.3.2.2 TRAF2 97
4.3.2.3 TRAF3 102
4.3.2.4 TRAIL 105
4.4 Discussion 106
4.4.1 TRAF2 107
4.4.2 TRAF3 108
4.4.3 TRAIL 110
4.4.5 Summary 111
Chapter 5: Optimisation of whole chick lens culture 112
5.1 Introduction 113
5.2 Experimental Design 114
5.3 Results 116
5.3.1 Darkfield visualisation of cultured whole lenses. 116
5.3.2 Haematoxylin and Eosin staining. 118
5.4 Discussion 129

Chapter 6: Discussion **135**
6.1 Discussion 136
Bibliography **144**
Appendices **166**
Appendix 1- Solution concentrations 166
Appendix 2- PCRs, primers, densitometry and statistics 169
Densitometry Results 170
Statistical Analysis 171
TRAIL 171
TRAF1 173
TRAF2 175
TRAF3 177
TRAF4 179
TRAF7 181
Appendix 3- Western Blotting analysis 184
TRAIL western blotting analysis 184
TRAF3 (higher band) western blotting analysis 186
TRAF3 (lower band) western blotting analysis 188
TRAF2 western blotting analysis 190
Appendix 4- Lens culture 193
Lens opacity following whole lens culture 193

List of Figures

Figure 1.1 Diagram showing the structure of the eye. 5
Figure 1.2 Basic stages of vertebrate eye development 7
Figure 1.3 Diagrammatic view of the various regions found in the lens 8
Figure 1.4 A diagram of lens development 9
Figure 1.5 Proposed structures of the known TRAFs 34
Figure 2.1 Octagon 100 incubator containing eggs. 46
Figure 2.2 Stages in obtaining embryos 48
Figure 2.3 Graph showing GAPDH standard curve with corresponding PCRs. 55
Figure 2.4 Flow diagram explaining the stages undertaken during subcellular fractionation. 59
Figure 2.5 Diagram of the orientation of the tissue within a wax block 66
Figure 3.1 Image of RNA denaturing gel. 76
Figure 3.2 Shows PCR standard curves for selected genes. 77
Figure 3.3 Semi-quantitative RT-PCRs with no RT controls are representative of the three replicates of PCRs gained form each set of primers. 78
Figure 3.4 Graphical representation of RT-PCR results obtained. 79
Figure 4.1 Representative western blot of TRAF2 protein expression with actin as a sample loading control. 89
Figure 4.2 Graphical representation of densitometry results of TRAF2 protein expression. 89
Figure 4.3 Western blotting of TRAF3 at all developmental time points with actin as a loading control 90
Figure 4.4 Graphical representation of densitometry results from TRAF3 protein expression 91
Figure 4.5 Subcellular fractionation of TRAF3 at E16 92
Figure 4.6 Western blot of TRAIL in the developing chicken embryo lens 93
Figure 4.7 Graphical representation of densitometry results from TRAIL protein expression 93
Figure 4.8 H and E staining at E6, E8 and E10 94
Figure 4.9 H and E staining at E12, 14 and E16 95
Figure 4.10 TRAF2 staining at E6, E8 and E10 97
Figure 4.11 TRAF2 staining at E12, E14 and E16 98
Figure 4.12 TRAF2 cytosolic staining at E16 99
Figure 4.13 TRAF2 staining of the iris and ciliary body from E10 to E16 100
Figure 4.14 Immunofluorescent staining of TRAF3 at E6, 8 and 10 with a Rabbit IGg control of an E10 section 102
Figure 4.15 Immunofluorescent staining of TRAF3 at E12, 14 and 16 with a Rabbit IGg control of an E16 section 103
Figure 4.16 Immunofluorescent staining of TRAF3 at E14 104
Figure 4.17 TRAIL staining from E6 to E16 105
Figure 4.18 TRAIL immunocytochemical staining at E14 106

Figure 5.1 Dark field images of E10 lenses cultured for 2, 4, 6 and 8 days (as shown on left of table) with various methods (at the top of table), scale bar 0.5mm. 116
Figure 5.2 Diagram showing the measurements of the lenses used in this experiment 119
Figure 5.3 E10 lens cultured for 4 hours 120
Figure 5.4 E10 lens cultured for 2 days 121
Figure 5.5 E10 lens cultured for 4 days 122
Figure 5.6 E10 lens cultured for 6 days 123
Figure 5.7 E10 lens cultured for 8 days 124

List of Tables

Table 2.1 Hamburger & Hamilton stages selected for study of lens development..... 47
Table 2.2 Grid showing amount of tissue and solution values used for TRIzol® methodology explained below. 49
Table 2.3 Amounts used for GO Taq system..... 55
Table 2.4 Number of embryos required for each time point..... 58
Table 2.5 Volumes for BSA standards. 60
Table 3.1 TRAF1 results of Tukeys post hoc test..... 80
Table 3.2 TRAF2 results of Tukeys post hoc test..... 80
Table 3.3 TRAF3 results of Tukeys post hoc test..... 80
Table 4.1 TRAF2 p values obtained from Tukey's post hoc test. 90
Table 4.2 TRAF3 p values obtained from Tukey's post hoc test 91
Table 5.1 Showing the amount of opacification in whole lens cultures 116
Table 5.2 Mean measurements (in μm) of E10 lenses cultured for 4 hours 125
Table 5.3 Mean measurements (in μm) of E10 lenses cultured for 2 days..... 125
Table 5.4 Mean measurements (in μm) of E10 lenses cultured for 4 days..... 125
Table 5.5 Mean measurements (in μm) of E10 lenses cultured for 6 days..... 126
Table 5.6 Mean measurements (in μm) of E10 lenses cultured for 8 days..... 126
Table A.2.1 Primers designed showing primer sequences, band size, optimised temperature and cycle number 169
Table A.2.2 Densitometry results of gene expression found via semi-quantitative PCR 170
Table A.2.3 TRAIL Descriptives..... 171
Table A.2.4 TRAIL ANOVA 171
Table A.2.5 TRAIL Multiple Comparisons..... 172
Table A.2.6 TRAF1 Descriptives 173
Table A.2.7 TRAF1 ANOVA..... 173
Table A.2.8 TRAF1 Multiple Comparisons 174
Table A.2.9 TRAF2 Descriptives 175
Table A.2.10 TRAF2 ANOVA..... 175
Table A.2.11 TRAF2 Multiple Comparisons 176
Table A.2.12 TRAF3 Descriptives 177
Table A.2.13 TRAF3 ANOVA..... 177
Table A.2.14 TRAF3 Multiple Comparisons 178
Table A.2.15 TRAF4 Descriptives 179
Table A.2.16 TRAF4 Test of Homogeneity of Variances 179
Table A.2.17 TRAF4 ANOVA..... 179
Table A.2.18 TRAF4 Multiple Comparisons 180
Table A.2.19 TRAF7 Descriptives 181
Table A.2.20 TRAF7 Test of Homogeneity of Variances 181
Table A.2.21 TRAF7 ANOVA..... 181
Table A.2.22 TRAF7 Multiple Comparisons 182
Table A.2.23 Genes shown to be expressed at E12, without further studies undertaken 183

Table A3.1.1 TRAIL densitometry results 184
Table A3.1.2 TRAIL Descriptives from SPSS analysis 184
Table A3.1.3 Test of Homogeneity of Variances 184
Table A3.1.4 TRAIL ANOVA 184
Table A3.1.5 TRAIL Multiple Comparisons using Tukey's post hoc test 185
Table A3.1.6 TRAIL Tukey's Post Hoc 185
Table A3.2.1 TRAF3 (higher band) densitometry results 186
Table A3.2.2 TRAF3 (higher band) Descriptives from SPSS analysis 186
Table A3.2.3 Test of Homogeneity of Variances 186
Table A3.2.4 TRAF3 (higher band) ANOVA 186
Table A3.2.5 TRAF3 (higher band) Multiple Comparisons using Tukey's post hoc test 187
Table A3.2.6 TRAF3 (higher band) Tukey's Post Hoc 188
Table A3.3.1 TRAF3 (lower band) densitometry results 188
Table A3.3.2 TRAF3 (lower band) Descriptives from SPSS analysis 188
Table A3.3.3 Test of Homogeneity of Variances 188
Table A3.3.4 TRAF3 (lower band) ANOVA 189
Table A3.3.5 TRAF3 (lower band) Multiple Comparisons using Tukey's post hoc test 189
Table A3.3.6 TRAF3 (lower band) Tukey's Post Hoc 190
Table A3.4.1 TRAF2 densitometry results 190
Table A3.4.2 TRAF2 Descriptives from SPSS analysis 190
Table A3.4.3 Test of Homogeneity of Variances 190
Table A3.4.4 TRAF2 ANOVA 191
Table A3.4.5 TRAF2 Multiple Comparisons using Tukey's post hoc test 191
Table A3.4.6 TRAF2 Tukey's Post Hoc 192
Table A4.1 Densitometry results of opacities in cultured lens 193

Chapter 1:
Introduction

1.1 General Introduction

The lens, an organ found behind the pupil of the eye, refracts light onto the retina providing the ability to focus. At a cellular level, it is known that once the lens vesicle forms, cells at the posterior of the lens elongate into the lumen of the lens vesicle and differentiate into primary fibre cells. At the anterior of the lens is a layer of epithelial cells which, after initial lens development, continue to proliferate and migrate, along the basement membrane, towards the equatorial region. At the equatorial region, cells then differentiate into secondary fibre cells and wrap around the primary fibre cells found at the centre of the lens. Differentiation into fibre cells involves the cells elongating and losing all of their intracellular organelles; once this occurs the cell becomes a part of the organelle free zone (OFZ), which is essential for transparency of the lens.

The development of the lens, at a molecular level, has been studied and hypothesised upon for a number of decades and research is still ongoing. The degradation of intracellular organelles, mentioned above, was once described as an attenuated form of cell death or apoptosis (Dahm, 1999). As research continued, many complex signalling pathways have been identified in the lens to investigate this process. Recent research has identified a number of apoptosis genes that are differentially expressed at stages of lens development where a substantial amount of organelle degradation occurs (Wride *et al.*, 2003; Mansergh *et al.*, 2004; Geatrell, 2007a). Prior to this a number of Tumour Necrosis Factors (TNFs), which have many roles including inflammation, cell proliferation and apoptosis (MacEwan, 2002), were identified during chick lens development (Wride & Sanders, 1998). Since this time there has been no attempt to study the expression and function TNF-related signalling pathways during lens development in any species.

In this chapter the, structure and development of the lens will be described before background on our current understanding on TNF-related genes, and their protein products studied during this research, will be provided.

1.2 Aims of this PhD

In 1998 (Wride & Sanders) identified a number of TNFs expressed in the lens during development, which they had previously termed TNF-cross reactive proteins (Wride & Sanders, 1998). It was hypothesised that TNFs may have a number of roles in embryonic development, based on their known roles in inflammation and in the immune system, including apoptosis (Wride & Sanders, 1995). It was then proposed that differentiation of lens fibre cells was an attenuated form of apoptosis (Dahm, 1999). This hypothesis has been born out by various studies, including research using microarrays investigating gene expression profiles in mouse lenses of various ages, which revealed that a number of TNFs and apoptosis-related genes such as Lymphotoxin B and Caspase6 were up-regulated during stages of development associated with lens fibre cell differentiation (Wride *et al.*, 2003). Further research into the expression of TNFs and their related family members in lens development is required to determine their possible roles during lens development and the overall objective of this thesis is to provide new insights into the roles of these factors in chick embryo lens development (shown in figure 1.4). The overarching hypothesis and specific objectives are provided at the end of this chapter.

1.3 The use of model organisms

There are many species that are used in scientific research and selecting the organism to be used has to take into account many variables that affect the decision. Each investigator will have their preference dependant on factors such as their own experience, available knowledge of the species, the species used in previous related research, availability, financial and time constraints.

The source of tissue in this investigation was *Gallus gallus*, though there were many other options e.g. mouse, rat, bovine and zebrafish, each with benefits and disadvantages for use of that particular organism for research (Norton, 1999). As research continues the similarities and variations between organisms are becoming clearer. An example of this is a recent study that highlights similarities and differences between lens development in zebrafish and humans. Though there are likenesses,

zebrafish lenses have been found to develop in markedly different ways to mammalian and avian lenses; i.e. as the ingression of a single mass of tissue rather then the invagination of a section of ectoderm to form a lens vesicle (Dahm *et al.*, 2007b).

Embryonic chicks were used here for their ease of purchase, quantity of tissue available, relatively low costs, production time and because the original paper identifying TNFs in the lens used the chicken (Wride & Sanders, 1998).

The Chicken can live for up to eleven years depending on breed and the incubation time for eggs is 21 days. The eggs will only begin to develop when incubated, allowing a large number of eggs to be collected and incubated at the same time. This means that a larger volume of the required tissue can be obtained in less time and with less expense, when compared to other model systems such as the mouse or rat. Increased tissue volume collection is also aided by the fact that the lens in *Gallus gallus* is relatively large, for example in comparison to the mouse. Though there are morphological differences between lenses in different species; e.g. umbilical sutures in avian lenses and the complex star architecture of adult primate sutures (Kuszak *et al.*, 2006), the molecular pathways that are of interest in this thesis are thought to be similar between mammalian and avian species (Dahm *et al.*, 2007a).

1.4 Ocular Structure

The eye is one of the key sensory organs of the body. Figure 1.1., shows the main structures of the eye. The eye receives images via refracted light. Light is refracted from the cornea and through the pupil opening to the lens which then focuses light onto the retina.

The light passes through the cornea, which is transparent and coated by a tear film, which keeps the cornea moist. The cornea and the sclera together form the outer surface of the eyeball. The sclera is where the extra-ocular muscles attach externally. The optic, sensory and motor nerves and blood vessels enter the eye through the sclera at the posterior of the eye (Batterbury & Bowling, 2001).

The sclera surrounds the choroid, a vascular layer, which in turn surrounds the retina. The retina is found on the interior of the eye wall and covers the posterior of the eye. The inner retina is clear, so the focused light can pass through to the

photoreceptors, which convert the light into electrical signals that are passed on by axons to the optic nerve. The optic nerve then carries these signals to the visual processing centres in the brain (Batterbury & Bowling, 2001).

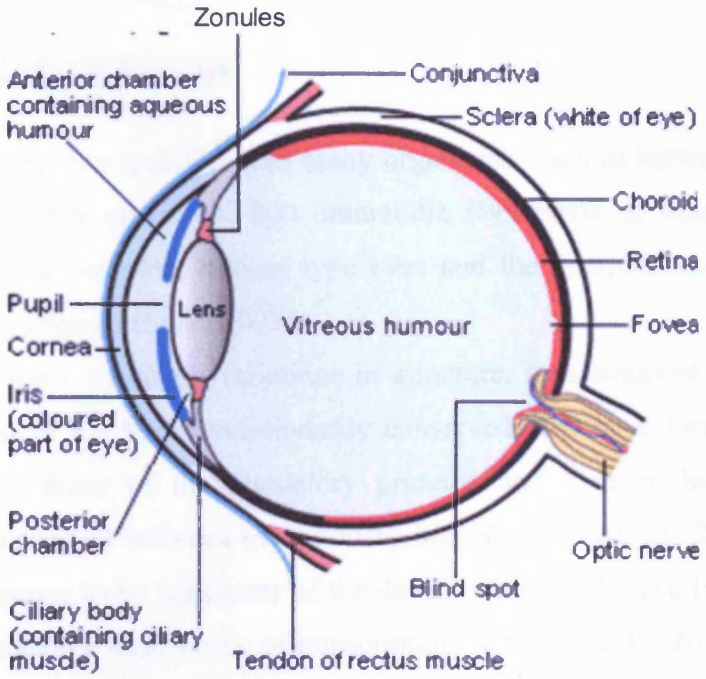


Figure 1.1 Diagram showing the structure of the eye. Adapted from www.mydr.com.

At the anterior of the eye, behind the cornea, is the iris, which is a coloured diaphragm that expands and contracts as light intensities increase and decrease respectively. The pupil defines the area found in the centre of the iris and once light passes through the pupil it hits the lens, which changes shape so that the image projected onto the retina is in focus, this is known as accommodation (Beebe, 2003).

The anterior chamber, found between the cornea and the lens, is filled with a clear fluid called the aqueous humour which maintains the intraocular pressure of the eye; it also supplies the cornea and lens with nutrients, and removes waste, as both these structures are avascular (Ofri, 2002). The aqueous humour is produced by the ciliary body, which is found at the most anterior point of the retina and behind the iris.

The ciliary body also has fibres called zonules, which attach to the lens and anchor it in place. Furthermore, the zonules help alter the shape of the lens. When the ciliary muscles contract, the zonules relax allowing the lens to thicken, sharpening near focus. If focusing in the distance the ciliary muscles relax and the zonules contract

exerting pressure that makes the lens flatten its shape to focus the light on the retina (Batterbury & Bowling, 2001).

1.5 Ocular Development

The structure of the eye is different in many organisms, such as between *Drosophila*, compound eyes made up of 700-800 ommatidia (Wawersik & Maas, 2000); *Mus musculus*, a pair of complex, camera type eyes and the *Platyhelminthes* (flatworm) with a pinhole eye design (Land, 2005).

Though there are many variations in structure, it is believed that embryonic ocular development has been evolutionarily conserved (Oliver & Gruss, 1997). It is astonishing how many of the regulatory proteins and their molecular signalling pathways are conserved between evolutionary branches (Treisman, 2004). There are several genes known to be regulators of the development of the eye (Oliver & Gruss, 1997). These include a large group of transcription factors (e.g. Pax6) and growth and differentiation factors (e.g. transforming growth factors (TGFs) and Fibroblast Growth Factors (FGF)). These factors are found in various locations throughout the embryo and are not only involved in eye development (Sanders & Wride, 1997). Though there has been a steady increase in our knowledge, the regulatory pathways that induce ocular development still have yet to be fully understood.

The induction of the eye begins when the diencephalic vesicle (presumptive optic vesicle) extends from the diencephalic neural plate towards the surface ectoderm of the embryo; this region is termed the optic placode (Creuzet *et al.*, 2005). As the optic vesicle nears the optic placode, the ectoderm thickens and the mesenchyme is displaced forming the lens placode, shown in figure 1.2, (Chow & Lang, 2001). The optic vesicle is important in the induction of the lens but other tissue interactions are also required as shown by numerous transplantation studies since the 1950's (Reviewed in Fisher & Grainger, 2004). As the lens placode forms, crystallins begin to be expressed and the invagination of the lens placode into the lens vesicle coincides with the development of the optic vesicle into the optic cup, the inner layer developing into the neural retina, the outer layer of the cup will form the RPE (retinal pigment epithelium) (Chow & Lang, 2001). The lens is internalised at this stage (Shimada *et*

al., 2003) and, as the lens continues to develop, it secretes factors that induce other ocular tissues to develop, such as the cornea (Coulombre & Coulombre, 1964). The cornea forms from development of the surface ectodermal, mesenchymal and neural crest cells (Graw, 2003). The cornea, like the lens, is clear, though the cornea is more a layer of sugars and proteins rather than the cellular tissue of which the lens is made (Dahm, 2004).

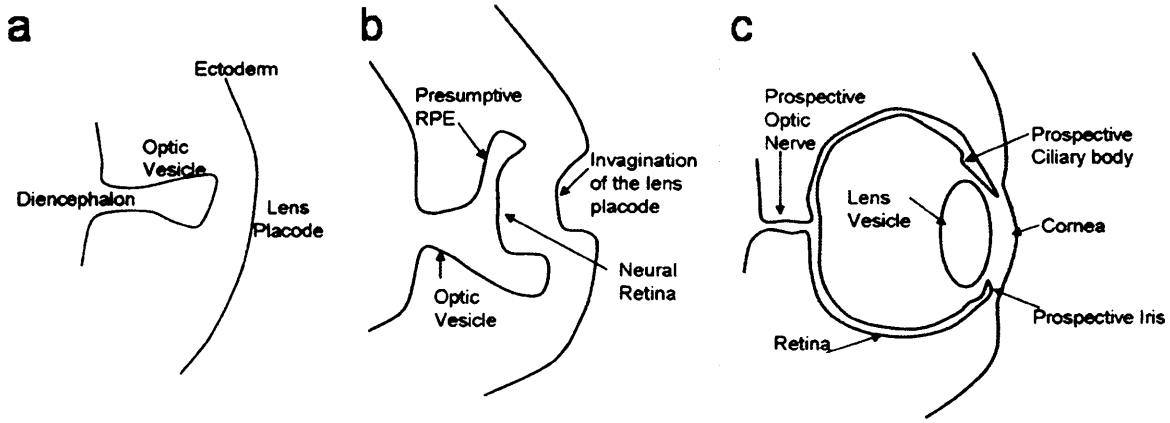


Figure 1.2 Basic stages of vertebrate eye development A: Diencephalon extends out towards the presumptive lens placode forming the optic vesicle. B: The lens placode invaginates forming the lens cup while the optic vesicle also invaginates to form the presumptive retina. C: the lens vesicle is internalised with the cornea forming from the surface epithelium and the retina becoming more specialized.

1.6 The Lens

The lens is a unique tissue with all stages of development in evidence throughout the life of the lens. At the anterior of the lens is a single epithelial layer. As the epithelial cells reach the equatorial region of the lens, the epithelial cells begin to differentiate into fibre cells. The fibre cells, once fully differentiated, do not contain any nuclei or organelles, but are maintained throughout the life of the organism at the centre of the lens (Modak & Perdue, 1970). Surrounding the lens is a capsule; within this capsule the age of the cells increases the closer to the centre they get. For an 80 year old organism, the fibre cells at the centre of the lens will have been *in situ* since the lens initially developed some 80 years previously. The presence of cells from initial development to the newly proliferated cells makes the lens an excellent model system, at both a cellular and molecular level, for the study of development, ageing and differentiation (Bloemendal, 1977; Piatigorsky, 1981a; Wride, 1996).

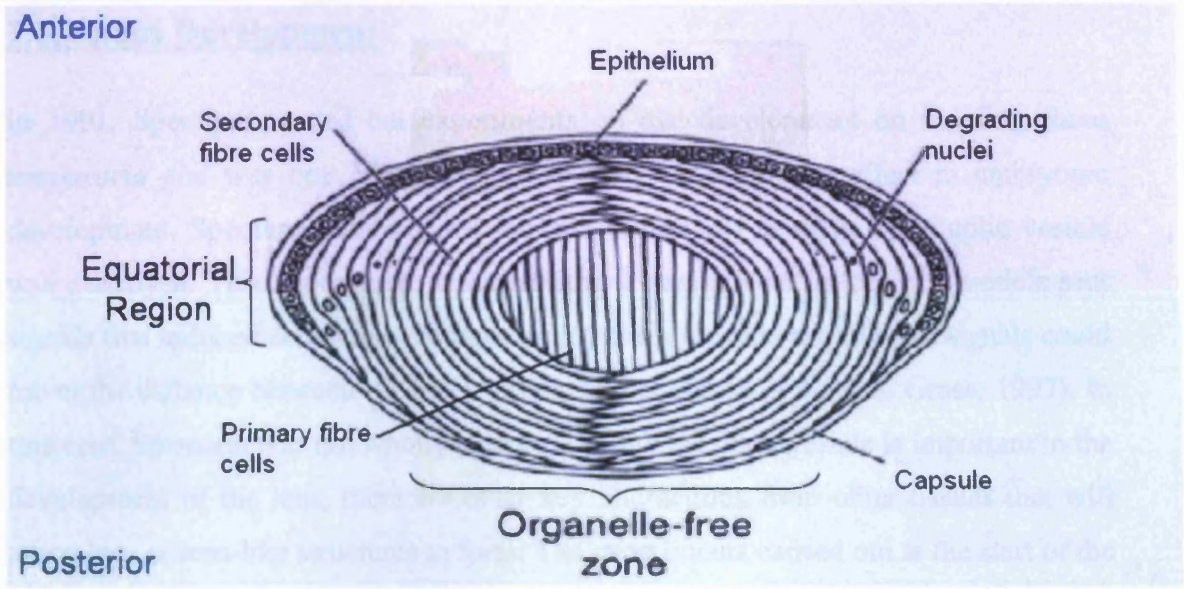


Figure 1.3 Diagrammatic view of the various regions found in the lens The fibre cells at the centre of the lens are the primary fibre cells. Surrounding these primary fibre cells are the secondary fibre cells which continue to differentiate from the epithelial cells as they pass the equatorial region in their migration towards the posterior of the lens (adapted from Bloemendal, 1977).

The function of the lens is the transmission of light, and protein products called crystallins are involved in lens cells' differentiation towards transparency (Gilbert, 2000). The lens is supported by the zonules and when the zonules relax the lens alters its shape and becomes more spherical. As the zonules become tense the lens stretches. This change in shape is how the lens focuses at different distances and is called accommodation. Once initial development of the lens is completed, the cellular structure is similar to figure 1.3, above. The epithelial cells are found at the front of the lens and, as cells proliferate; epithelial cells are pushed around the periphery of the lens towards the equatorial region. In this region, the cells elongate and differentiate into the secondary fibre cells, while migrating towards the centre of the lens. Differentiated fibre cells have a cytoskeletal framework, but the organelles usually contained within cells, such as mitochondria and nuclei, are degraded (Menko *et al.*, 1984). This is thought to resemble a type of programmed cell death or apoptosis (Wride & Sanders, 1998; Wride, 2000) and also involves activation of proteolytic pathways (Wride *et al.*, 2006). As the secondary fibre cells move into the centre of the lens, they spread over the primary fibre cells; this overlapping means that the lens is constantly growing and becoming more condensed throughout the life of the organism.

1.6.1 Lens Development

In 1901, Spemann carried out experiments on eye development on the frog *Rana temporaria* and was one of the first to identify the inductive effect in embryonic development. Spemann believed that the lens would not develop if the optic vesicle was destroyed. This experiment, amongst others, suggested that the optic vesicle sent signals that induced ectodermal competence to form the lens and that the signals could travel the distance between the optic vesicle and ectoderm (Oliver & Gruss, 1997). In this case, Spemann was not wholly correct. Though the optic vesicle is important in the development of the lens, there are other key interactions from other tissues that will allow lens or lens-like structures to form. The experiments carried out at the start of the 20th century gave rise to more and more in depth studies which have led us to a fairly detailed knowledge of the tissue interactions involved in lens development (Ogino & Yasuda, 2000). We are now on our way to identifying the regulatory factors that have a role in lens development and as with all questions have now led us to begin to examine how these factors are themselves induced and controlled (Lovicu & McAvoy, 2005).

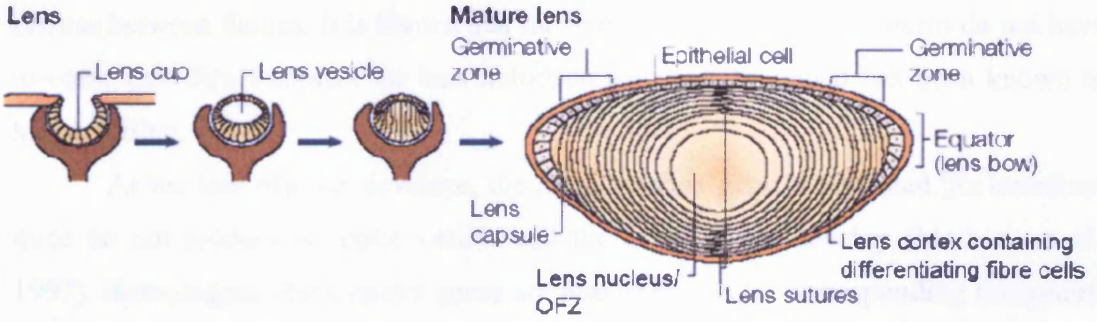


Figure 1.4 A diagram of lens development, adapted from (Graw, 2003).

In a review on the structural analysis of chick development, which was first printed in 1951 (Hamburger & Hamilton, 1992), it is stated that invagination of the optic vesicle occurs at roughly between 50-55 hours (Hamburger & Hamilton, 1992). In comparison, lens induction in the mouse has been stated to begin as early as 35 hours (Wride, 1996) with human lens development occurring at about the fourth week of gestation (Francis *et al.*, 1999). The gestation periods between species can be very different, but the stages in which the vertebrate lens develops are surprisingly similar and are described in the following sections.

1.6.2 Induction of the lens

In early chick development, Wnt (described in 1.7.6) signals in the neural border are known to influence Bone Morphogenetic Protein (BMP) activity in the ectoderm to signal either olfactory or lens placode character cells during development of the embryo ectoderm. The length of the period of BMP (described in 1.7.3) exposure is thought to influence which character is undertaken (Sjodal *et al.*, 2007; Patthey *et al.*, 2008). FGF (described in 1.7.5) is also thought to be required in placode development possibly in a proliferation or maintenance role (Litsiou *et al.*, 2005; Bailey *et al.*, 2006). Around this stage, *Pax6* (described in 1.7.2) is expressed in the head ectoderm (prior to diencephalon protrusion), which indicates that the area is competent for lens induction.

The frontal neural plate (diencephalon) protrudes and elongates towards the developing PLE (presumptive lens ectoderm) forming the optic vesicle (Kondoh, 1999; Ogino & Yasuda, 2000). Once the optic vesicle nears the PLE, the mesodermal layer is excluded allowing an extracellular matrix to form between the optic vesicle and the PLE (Wakely, 1977). The signalling proteins produced during lens development can diffuse between tissues; it is known that the optic vesicle and the ectoderm do not have to come into direct contact for lens induction to occur, though it has been known to happen (Bhat, 2001).

As the lens placode develops, the *Rx* homeobox gene is activated. *Rx* knockout mice do not produce an optic vesicle and the eyes do not develop (Mathers *et al.*, 1997). Homologous chick *rax/rx* genes are also expressed at corresponding timepoints and regions indicating a similarily key function in the chick (Ohuchi *et al.*, 1999). Downstream of *Rx, Pax6* is also found in the optic vesicle as it develops, but knockdown studies reveal that Pax6 is not necessary for development of the optic vesicle. Otx2 and Six3 have been identified to have a role during the evagination of the optic vesicle (Zhang *et al.*, 2000).

The evagination of the optic vesicle towards the surface tissue and expression of its molecular signals, such as BMP4 and downstream signals such as Sox2 (Furuta & Hogan, 1998), leads to ectodermal thickening of the PLE, through cellular elongation to form the lens placode (Ogino & Yasuda, 2000). Crystallins (described in 1.7.1) begin to be expressed during placode formation and are induced by the mutual

expression of Sox2 and L-Maf transcription factors; downstream of Pax6, they are both essential for lens placode formation and δ-crystallin induction (Kamachi *et al.*, 1998; Ogino & Yasuda, 1998; Reza *et al.*, 2002).

1.6.3 The Lens Vesicle

After the lens placode has fully thickened, the optic vesicle invaginates around the same time as the lens placode to form the optic cup and lens pit respectively, as shown in figure 1.2 (b). It has been demonstrated that the extracellular matrix formed between the two tissues during lens induction is likely to have a strong role in the synchronised invagination, but the molecular/ biochemical signals have not been fully characterised. It is known that retinoic acid signalling, important in forebrain and retina development and also known to be able to activate crystallin expression (Patek & Clayton, 1990; Tini *et al.*, 1993) is involved. There are retinoic acid receptors in the early PLE and experiments have shown that inhibition of these signals prevents or disrupts the lens placode invagination (Kastner *et al.*, 1995; Bavik *et al.*, 1996; Mic *et al.*, 2004). The activity of the receptors is believed to be regulated by Pax6 and downstream signals (Enwright & Grainger, 2000).

The formation of the lens pit is thought to be affected by cell proliferation, rearrangement of the cytoskeleton, contributed to by actin, and finally by the movement of adhesion molecules (Piatigorsky, 1981a).

As the lens placode sinks, forming the lens pit, the upper-most points of the lens pit come together and fuse forming the lens vesicle, which is separated and internalised from the surface ectoderm. The lens vesicle is held within the 'cup' that the optic vesicle forms (Bhat, 2001). The molecular pathways involved in the formation of the lens vesicle are not well understood, but some of the genes involved in the development in the lens placode, e.g. Pax6, also have roles here, such as in modulating the expression of cell adhesion molecules, such as integrin, Ndst, N-Cadherin and β-catenin, which are known to be involved in the formation of the mouse lens vesicle (reviewed in Cvekl & Duncan, 2007).

The lens capsule is produced when the lens vesicle invaginates, effectively turning the ectodermal cells upside down causing the basement membrane to be on the outside of the lens vesicle, therefore forming the lens capsule. The lens capsule is

primarily made of collagen that is strong and flexible allowing the accommodation of the lens via the zonules to which the capsule is attached (Krag & Andreassen, 2003).

1.6.4 Lens Fibre Cell Differentiation

The lens vesicle is made up of a single layer of epithelial cells and lens differentiation begins as soon as the vesicle is separated from the surface ectoderm. The anterior cells remain epithelial in nature, but the posterior cells exit the cell cycle and begin to differentiate, which is characterised by the cells elongating, accumulating crystallins and ultimately the degradation of subcellular organelles. The elongating posterior cells move into the lumen of the lens vesicle eventually filling up the whole space within the lens (figure 1.4).

In the 1960s Coulombre and Coulombre carried out a number of experiments that demonstrated that the surrounding tissues maintained the polarity of the lens and also showed that the surrounding tissues could influence the development of a new whole lens from the lens epithelium (Coulombre & Coulombre, 1969, 1971). The polarization of the lens means that the anterior cells remain as epithelial cells and the posterior cells are signalled to differentiate into lens fibre cells. It also means that once the posterior cells have produced the "primary" fibre cells, the continued polarity from the surrounding tissue signals the epithelial cells at the equator of the lens to differentiate into "secondary" fibre cells.

Since the 1960s the molecular signals to maintain the lens' polarity, provided by the surrounding tissue, and from within the lens, have begun to be elucidated. Pax6 expression is important in the anterior lens epithelial cells as it may have a role in maintaining epithelial polarity, it also has other roles in the activation of some of the crystallin genes (Francis *et al.*, 1999). The expression of crystallins is also influenced by fibroblast growth factors (FGFs) which are known to have a role in adjusting the regulation of homeobox genes as well as Pax6 (Plaza *et al.*, 1993). As Pax6 becomes down regulated in the ageing anterior epithelial cells, the cells move towards the back of the lens vesicle and differentiation into lens fibre cells occurs (Duncan *et al.*, 2004).

It is thought that Prox1 is important in the differentiation of the posterior cells in the lens vesicle as studies using the Prox1 knockout mouse show the posterior cells do not elongate and the lens of the lumen remains unfilled (Wigle *et al.*, 1999). It is

thought that expression and function of Prox1 in lens development is conserved between organisms namely mouse, rat, human and chicken (Duncan *et al.*, 2002). Though the molecular pathway is not fully understood, its expression is known to increase at the equatorial region where differentiation occurs (Tomarev *et al.*, 1996) and is thought to be involved as a transcriptional activator of β-crystallins as mentioned in 1.7.1 (Chen *et al.*, 2008a).

Notch signalling (described further in 1.7.4) has recently been suggested to be involved in mediating the differentiation of fibre cells. A larger than normal number of epithelial cells at the anterior segment of the lens vesicle will begin to differentiate in the absence of Notch signalling and this premature differentiation results in the development of a smaller than normal lens due to the reduction in the number of future epithelial cells available to undergo proliferation and eventually differentiate (Jia *et al.*, 2007).

Wnt signalling, described in greater detail in 1.7.6, is known to be transcriptionally activated through β-catenin and has an active role in the maintenance of the lens epithelium in an undifferentiated state and early differentiation into fibre cells (Stump *et al.*, 2003; Cain *et al.*, 2008). It has also recently been identified that interaction of Wnt signalling with frizzled receptors is important in cytoskeletal remodelling of the differentiating fibre cells (Chen *et al.*, 2004, 2006; Chen *et al.*, 2008b). Studies using the chick throughout the embryonic development of the lens have identified 11 Wnt proteins expressed in the anterior eye; with 2 expressed in the lens epithelium and 4 were specifically identified in the differentiating fibre cells. Staining was found throughout the epithelium and differentiating primary fibre cells which was then later restricted to the epithelium and transition zone where secondary fibre cell differentiation is initiated (Ang *et al.*, 2004; Fokina & Frolova, 2006).

1.6.5 Secondary Fibre Cell Differentiation

After the primary fibre cells have filled the lumen in the lens vesicle, the lens continues to grow via proliferation of germinal cells which are part of the epithelial layer at the lens anterior. As the epithelial cells divide they move around the periphery of the lens, towards the posterior, until they reach the equatorial region where the cells then begin to differentiate (Gilbert, 2000). The expression of numerous genes is involved in

secondary fibre cell differentiation, though there are also genes that are required to be inactivated for differentiation to occur normally. FoxE3 is expressed from the development of the lens placode and remains expressed in epithelial cells throughout the life of the lens. It is essential for both survival and proliferation in epithelial cells and also for the normal development of the lens vesicle (Blixt *et al.*, 2000). FoxE3 expression disappears when the epithelial cells reach the lens equator and normal differentiation occurs. If FoxE3 expression continues in the fibre cells, remodelling of the cytoskeleton and organelle degradation is impaired (Landgren *et al.*, 2008).

The differentiating secondary fibre cell undergoes similar stages as the primary fibre cells (i.e. exit from the cell cycle, cell elongation and crystallin, gap junction proteins and intermediate filament production). As these cells elongate, they migrate away from the equatorial region towards the centre of the lens. The tips of the fibre cells move along the epithelium at the anterior and the lens capsule at the posterior of the lens. As the tips of the cells reach their counterparts from the opposite equator, they no longer touch the epithelium or the capsule, but touch each other at the sutures and the cells gradually move into the lens nucleus as newer fibre cells overlap them (Bassnett *et al.*, 1999).

1.6.6 Lens Organelle degradation

As fibre cells enter the nucleus (centre) of the lens, differentiation concludes via degradation of the organelles and denucleation. In primary fibre cells, the nucleoplasm condenses and dense bodies form in both the nuclei and the cytoplasm. The bodies are then invaded by vesicles and lysosomal elements as they move towards the fibre membrane, which suggests that membrane-related proteases, such as calpains help to degrade the proteins within the bodies. The waste products are thought to be extruded to the extracellular space and the presence of these waste products at the posterior and anterior poles suggests that they are transported here though what occurs after this is unknown (Vrensen *et al.*, 1991). This may be similar to the process that occurs during development of erythrocytes, which also lose their nuclei during their development (Lockshin & Zakeri, 2004).

Secondary fibre cells undergo terminal differentiation through a different process. The mechanism that carries out this differentiation has been proposed to

utilise the apoptosis pathway. Some differences are that apoptosis in normal cells occurs in hours, whereas the breakdown in nuclei during terminal differentiation takes days (Bassnett & Mataic, 1997). Lens cells also have an undetermined signal that halts cell degeneration before the framework of the cell is also destroyed. A proposed gene that may be involved in this stage is Galectin-3, which, when it is down regulated, suggests some role in slowing degeneration (Dahm *et al.*, 2003).

In support of the apoptotic-like degradation hypothesis is the identification of a number of signalling and downstream cascade molecules identified in the lens. For example, it is believed that factors that have similarities to, and including, Tumour Necrosis Factor α (TNFα) help to signal the degeneration of DNA within the nucleus of lens fibre cells during initial developmental stages and differentiation (Wride & Sanders, 1998). It is also thought that caspases, such as caspase-1 through to caspase-4, and *Bcl-2* family members, like Bax and Bad, are involved in the breakdown of organelles required for complete fibre cell differentiation (Wride *et al.*, 1999). Further research over-expressing bcl-2 in the embryonic chick lens resulted in reduced activation of caspase-9 and the lack of chromatin condensation, which also suggests that apoptotic nuclear degradation and denucleation of fibre cells appear to use similar molecular pathways (Sanders & Parker, 2003). Downstream of the caspases, a number of substrates have been identified and are known to be cleaved in the region of organelle degradation. These substrates include laminB and poly (ADP-ribose) polymerase (PARP), both known to be cleaved by caspase-3 (Ishizaki *et al.*, 1998; Slee *et al.*, 2001). Recent research has challenged the proposal of involvement of an apoptosis-like pathway in lens fibre cell denucleation, through studies involving knockout mice for the executioner caspases -3, -6 and -7. The single caspase knockout mice had a relatively normal phenotype with organelle degradation indistinguishable from wild-type mice controls. A further experiment using caspase-3 and caspase-6 double knockouts, to test whether functional redundancy was involved, showed that even without both caspase-3 and -6, organelle degradation was relatively normal. These results indicate that the executioner caspases are not vital in organelle degradation (Zandy *et al.*, 2005).

Nuclei in lens fibre cells are lost via a series of steps including the clumping of chromatin, which is then cleaved into sections through activity similar to that of DNase I, which has been identified in bovine lens fibres (De Maria & Arruti, 2004). This is

then followed by the use of kinases, where phosphatidylinositol 3-kinase (PI3K) has a role in actin cytoskeleton reorganisation in the early stages of differentiation, which is sufficient to induce differentiation, while later on PI3K inactivates glycogen synthase kinase-3 (GSK3) signalling which protects against apoptosis (Weber & Menko, 2006).

The lysosomal nuclease DNase II-like acid DNase (DLAD) has also been identified in the lens and deficiency in it resulted in partial nuclei persisting in the normal OFZ (Nishimoto *et al.*, 2003). The recognition of expression of DLAD and other lysosomal enzymes/ nucleases involved in nuclear degradation in the lens fibres has increased the uncertainty about the involvement of apoptosis-like pathways during terminal fibre differentiation (Counis *et al.*, 1998; Zandy *et al.*, 2005; Nakahara *et al.*, 2007).

15-Lipoxygenase (15-LOX) expression has also been identified in the lens and is restricted to the region where organelle degradation occurs (van Leyen, 1998). 15-LOX had previously been identified in the precursors to erythrocytes immediately prior to organelle degradation, which was shown to allow the access of proteases to organelles and release of organelle proteins by permeabilising the membranes of organelles, while leaving the plasma membrane intact. Further studies identified that 15-LOX formed pores in membranes of organelles, which allowed further degradation via cytosolic proteases, perhaps such as those involved in the ubiquitin/ proteasome pathway (Grullich *et al.*, 2001).

The Ubiquitin-proteasome pathway (UPP) has been suggested as an alternative to the apoptosis-like pathway proposed above and its interaction with 15-LOX increases the validity of this suggestion. The UPP is a key proteolytic pathway found in the cytosol. First, poly-ubiquitinated conjugates are formed via the ligation of ubiquitin to the substrate protein, the marked protein is then degraded by the 26S proteasome (Hershko *et al.*, 2000). The UPP is required in many processes from transcription and cell cycle regulation to immune responses and DNA repair. The UPP was first identified in the lens, in epithelial cells (Jahngen *et al.*, 1986) and later studies showed that as fibre cell differentiation began there is an increase in ubiquitin conjugation (Shang *et al.*, 1999). In a following study, rat epithelial cell explants were used to carry out bFGF-induced proliferation and differentiation. The expression levels for a number of substrates and enzymes were monitored during proliferation and differentiation and a number of the components of the UPP were differentially regulating during both

processes (Guo *et al.*, 2004). Localisation studies of the UPP components have shown that they leave the cytoplasm as differentiation occurs and staining becomes specific to the fibre cell nuclei (Girao *et al.*, 2005). The most recent study again using rat epithelial cell explants, carried out bFGF-induced proliferation and differentiation with the use of a proteasome-specific inhibitor. The addition of the inhibitor delayed both proliferation and differentiation and cells exhibited both molecular and morphological changes, supporting the hypothesis that proteasome activity has a key role in lens development (Guo *et al.*, 2006).

The fibre cells, which no longer contain organelles, are not thought of as dead, though they cannot repair themselves like normal cells. These fibre cells are found in the OFZ and receive only nutrients for "survival" via passive diffusion from the surrounding cells. In the chick, the OFZ begins to form around embryonic day 12 (Modak & Perdue, 1970) and by hatching the OFZ is as large as the pupil opening therefore insuring light is not disrupted by organelles (Bassnett, 1992).

The border of the OFZ is where denucleation and degradation of organelles occurs (Bassnett & Beebe, 1992; Bassnett, 1995). It has been determined that DNA degradation occurs after the degradation of the mitochondria, ER and breakdown of the nuclear membrane (Bassnett & Mataic, 1997). The Golgi apparatus has been shown to be the first of the organelles to fragment and is only present at the earliest stages of lens differentiation (Bassnett, 1995). The last stage in organelle degradation occurs when fibre cell nuclei first change shape, followed by chromatin collapse, the nuclear envelope loses integrity and then finally the DNA fragments (Bassnett & Mataic, 1997). Physiologically, the stages in denucleation are similar to those undertaken in apoptosis. Mitochondrial molecules are known to be released during organelle degradation, which may be related to the effect of 15-LOX on its membrane (Vijayvergiya *et al.*, 2004).

1.7 Molecular signals in lens development

The maturation of the eye is strictly ordered during development via molecular signals. Mutations in the genes involved can cause numerous different disorders (Graw & Loster, 2003), some of which have or will be mentioned. There are many signals from

within the lens that help to maintain a functional lens but there have been a number of experiments that have clearly shown that there are key signals from the surrounding tissues. Examples of this include the Coulombre and Coulombre experiments in the 1960s briefly described above in section 1.6.4. Some of the key molecules that influence development and maintain the order of the lens are described below.

1.7.1 Crystallins

Crystallins are soluble proteins made up of broad "classes" segregated by their physical properties and immuno-chemical properties. α-crystallins are members of the small heat shock protein (HSP) family and are molecular chaperones important in the maintenance of cytoskeletal integrity and also have a role in the prevention of apoptosis (Andley *et al.*, 1998; Xi *et al.*, 2003). A lack of αA-crystallins has been shown, in knockout mice models, to be involved in early onset cataract and an increase in epithelial cell death (Xi *et al.*, 2003), while αB-crystallins in a knockout mouse model showed epithelial cell hyper-proliferation (Andley *et al.*, 2001). Double knockout mice of both αA and αB- crystallin produced the disintegration of fibre cells after organelle degradation (Boyle *et al.*, 2003). Further research on these double knockouts suggested that the α-crystallins were involved with the suppression of caspase activity, which in the normal lens would retain cytoskeletal integrity after apoptosis-like degradation of the intracellular organelles (Morozov & Wawrousek, 2005).

β-crystallins are the last crystallins to be expressed and found in the differentiated fibre cells. Pax6 represses its activity while L-Maf, c-Maf, MafB and Prox1 are thought to have a role in β-crystallin regulation (Duncan *et al.*, 1998; Cui *et al.*, 2004).

δ-crystallin is a key crystallin in the avian lens and is not found in mammalian lenses. These crystallins are early markers of lens differentiation and are the first crystallins to be expressed, during the formation of the lens placode, in the chick (Piatigorsky, 1981b). Pax6 and Sox2 form a complex that activates transcription of δ1-crystallin (Kamachi *et al.*, 2001).

It has also been shown that L-Maf, a *maf* gene family member, is required in regulating the expression of crystallins in the mouse, though no homologus proteins

have as yet been identified in humans (Reza & Yasuda, 2004). It is thought that L-Maf, along with Pax6 and Sox2, have important roles in the regulation of transcription, though the complex interaction of these proteins needs further study (Muta *et al.*, 2002; Reza *et al.*, 2002; Shimada *et al.*, 2003).

Crystallins have evolved through divergence from ancestral proteins of an alternate functional role and gene duplication. Beta and gamma crystallins are thought to "be related to a bacterial spore coat calcium binding protein" (Wistow & Piatigorsky, 1988). The rest have evolved from regular cellular enzymes and some still have enzyme activity. Crystallins, the major lens fibre structural proteins, comprise ~90% of the water soluble proteins in the lens. Their high concentration, along with metabolic enzymes, gives the lens transparency and refractive properties by a concentration gradient of crystallins through the layers of fibre cells (Cvekl & Piatigorsky, 1996).

1.7.2 Pax6

Pax6 is a gene encoding a key transcription factor in the ectoderm during lens development and as the lens develops it becomes segregated to the lens epithelium where it continues expression even after development has been completed. Upstream signals in the ectoderm had not been identified in 1993 though *Pax6* autoregulation had been identified; it is now known that BMP and FGF have a role in Pax6 regulation (Plaza *et al.*, 1993). *Pax6* gene expression becomes restricted to the presumptive lens placode where *Sox, Pitx3, Eya1* and *Otx* genes are also expressed. Studies in murine lenses have identified that up to 500 genes may be expressed downstream of Pax6 (Chauhan *et al.*, 2002a; Chauhan *et al.*, 2002b; Chauhan *et al.*, 2002c) Mutations in *Pax6* can result in aniridia (human), small eye (mouse) and eyeless (*Drosophila*) phenotypes (Gehring, 1996). *Sox3* ectopic expression produced ectopic lens in medaka (Koster *et al.*, 2000). Mice with the phenotype *aphakia* (a deletion in *Pitx3*) and *Drosophila* with the deletion of *Eya1* (the eyes absent gene) have both been identified to have homologues in humans. The mutations of these genes have been identified in congenital cataract and anterior segment anomalies. These last two genes, though expressed during lens induction, are thought to have an important role later on in lens development (Semina *et al.*, 1997; Azuma *et al.*, 2000; Semina *et al.*, 2000).

1.7.3 Bone morphogenetic protein (BMP)

Both BMP4 and BMP7 are known to be required in normal mouse lens placode formation. BMP7 is thought to have a role in maintaining the expression of Pax6 as without this signal Pax6 expression is lost in the lens placode area and lens induction ceases to occur (Wawersik *et al.*, 2000). BMP4 is believed to be necessary later on in placode formation as mutants still express Pax6 but the lens placode does not form (Furuta & Hogan, 1998). The BMPs have similar signalling cascades and in the chick it is thought that BMP4 and BMP7 signal different morphological processes to those found in the mouse; eg BMP4 is known to be the key BMP in lens placode development, while BMP7 is only found in the optic vesicle at the presumptive pigment epithelium during development (Trousse *et al.*, 2001). The receptors of BMPs seem to be similar between organisms, suggesting that downstream signalling remains the same (Dewulf *et al.*, 1995; Dudley & Robertson, 1997).

Once the chick lens vesicle forms, studies on BMPs and Pax6 confirm that many signals from a number of tissues are required for normal lens placode formation with interaction between each other being essential (Cvekl & Duncan, 2007). Later in development, BMPs have recently also been shown to have a role in the differentiation of fibre cells and blocking BMP signals delays the elongation of differentiating fibre cells and an increase in the normally very limited cell death in epithelial cells was identified (Belecky-Adams *et al.*, 2002).

1.7.4 Notch Signalling

Notch signalling, mentioned in 1.6.4 and involved in primary fibre cell differentiation, is also believed to be involved in secondary fibre differentiation. Research has suggested that Notch signalling maintains epithelial cells and, when the signal gradient decreases; the epithelial cells can undergo differentiation. Supporting this theory is the expression of *Jag1* by differentiating fibre cells. *Jag1* initiates feedback signals to nearby epithelial cells to activate Notch receptors, which helps maintain the epithelial cells from differentiation. Only when Notch signalling is suppressed or the differentiation signal is stronger will the next epithelial cells in the equatorial region begin to differentiate (Jia *et al.*, 2007).

1.7.5 Fibroblast Growth Factors (FGF)

FGFs are also strong candidates for the uncharacterised signals that induce epithelial cells to differentiate into fibre cells. The signal is thought to be emitted from the retina and produces a gradient from the posterior to the anterior, possibly determining the boundary of the epithelial cells (Lovicu & McAvoy, 2005). This was proposed when it was discovered that lens epithelial cells were induced to proliferate when low doses of FGF was present, but at high doses these cells began to differentiate (McAvoy & Chamberlain, 1989). It is also known that extracellular regulated kinase 1/2 (ERK1/2) (explained in 1.12.0.2) is required downstream of FGF to induce proliferation or differentiation. Though the morphological changes of differentiation are stopped when ERK signalling is blocked, the molecular mechanisms, such as β-crystallin production, are not inhibited. This suggests that multiple signalling pathways are involved in the normal maturation of the lens (Lovicu & McAvoy, 2001). As well as FGF's role in fibre differentiation it is also known that FGF8 has a role in the expression of L-Maf, a widely known lens induction marker (described in 1.7.2) in *Gallus gallus* (Vogel-Hopker *et al.*, 2000).

FGFs used to be thought of as key in signalling fibre cell differentiation for many years, but more recently it has been suggested that FGF works together with BMPs (described in 1.7.3) and Wnt signalling (described in 1.7.6, below) in the regulation of differentiation (Fokina & Frolova, 2006).

1.7.6 Wnt signals

As mentioned in 1.6.2 Wnt signals are involved in the earliest specification of the lens placode (Litsiou *et al.*, 2005), and their interaction with BMPs and FGFs are key in this role. A number of Wnts have been identified in the chick developing lens and two of those, *Wnt5b* and *Wnt7b,* are expressed in the lens epithelium just before elongation of the differentiating fibre cells. In addition to a role in primary fibre cell differentiation, *Wnt5b* is downregulated in the cortical fibre cells then becomes more highly expressed in the cortical fibres suggesting a role in the formation of the syncytium, a network of cell-cell interactions described in 1.8 (Shestopalov & Bassnett, 2000, 2002, 2003). *Wnt7a* is expressed later in development at the region of differentiation for the

secondary fibre cells. Wnt2 and Wnt2b have been suggested to have a role in the proliferation of epithelial cells; Wnt5a is involved in a different (non-canonical) pathway to the other Wnt's and is expressed in the equatorial region. It is suggested that it may have a role in epithelial cells' exit from the cell cycle and the start of differentiation of the secondary fibre cells (Fokina & Frolova, 2006).

1.7.7 Caspases

Caspases are apoptosis-associated molecules known to be involved in lens development, though their roles are still being elucidated; it has been shown that lens epithelial cells that undertake apoptosis also express caspase-3 (Ishizaki *et al.*, 1998; Yao *et al.*, 2003). Caspases are effector proteases that act in a cascade where the initiator caspases cleave the effector caspases found downstream in molecular pathways (Thornberry, 1997). The key executioner/ effector caspases then cleave a number of structural and regulatory proteins; for example, caspase-3, degrades death substrates, which then leads to DNA-fragmentation.

Research involving knockout mice of caspase-3, -6 and -7 found that both caspase-6 and -7 lenses exhibited normal morphology, while caspase-3 knockout mice had polar cataracts in about 75% of cases, only a few weeks after birth. The executioner caspases are not thought to be involved in organelle degradation but may have a role in lens transparency suggested by the development of cataract in caspase-3 knockouts (Zandy *et al.*, 2005).

From the above research, an interaction has been proposed between αA-crystallin and caspase-3 and -6. Further research discovered that αA- and αB-cystallin double knockout mice experienced fibre cell disintegration resulting from increased DEVDase and VEIDase activity which are indicative of caspase3 and-6 activity respectively. From these findings it is thought that αA-crystallin suppresses caspase activity, which in turn maintains the integrity of the terminally differentiated fibre cells (Morozov & Wawrousek, 2006).

Though knockout studies are still being carried out, it is known that it will be difficult to characterise the precise roles of the caspase family members, due to their close relationship, which results in a functional redundancy between members. This

means that the loss of one or even a number of caspases may have little effect on the end result (Zandy *et al.*, 2005).

1.7.8 Connexins

There are two connexin subunits that have been identified in the formation of intercellular channels called gap junctions in the lens fibres. These gap junctions transport water, ions and metabolites in the lens. Alpha3, otherwise known as connexin46 or Gja3, is vital for transparency of the lens (Gong *et al.*, 1997). Alpha8, known as connexin50 or Gja8, is important for both transparency and growth of the lens and can also be found in low concentrations in the lens epithelium (White *et al.*, 1998). The gap junctions formed from various subunits are thought to modulate different roles in either primary or secondary fibre cell differentiation as described in Xia *et al* (2006b). The research showed that alterations to which subunits are used, or alterations to the subunits themselves, resulted in various cataracts confirming that connexins and gap junctions are necessary for lens formation and transparency (Xia *et al.*, 2006a; Gong *et al.*, 2007)

1.8 Cytoskeleton remodelling during differentiation

Since early studies in the late 1970's, it has been confirmed that the lens is a living organ despite the initial failure of electrophysical studies to distinguish individual cells within the lens due to the low electrical resistance between cells (Benedetti *et al.*, 1976; Nonaka *et al.*, 1976). The circulating current, which transports the metabolites, ions and water throughout this avascular system, is of a uniform current throughout the lens and is the reason that distinct cells could not be identified within the lens nucleus during early studies (Mathias *et al.*, 1997). Along with the circulating current, the lens contains a huge number of gap junctions made up of connexins (described in 1.7.8) which are key in cell-cell communications.

It is now also suggested that there is a syncytium within the lens core that allows large molecules to diffuse around the lens nucleus (Shestopalov & Bassnett, 2003). Both the gap junctions and the permeable membrane, which forms the

syncytium, are formed during the remodelling of the cytoskeleton during differentiation.

Actin, a major cytoskeleton protein, has been well characterised in the lens and actin filament assembly and integrity is known to be important in fibre differentiation (Weber, 2004). Actin binds to myosin forming assemblies that have a role in the formation and stability of junctions and adhesions; mutation in myosinIIA (important in cell adhesion) is thought to be involved in the development of cataract (Rao & Maddala, 2006). Changes in the organisation of the actin cytoskeleton are associated with the differentiation of epithelial cells, which elongate as they migrate as described in section 1.6.4. As the differentiating fibre cells lose contact with the epithelium and the capsule, when they reach the suture, there is an increase in plasma membrane folding and interdigitations (Bassnett, 1995), the membranes then fuse prior to organelle degradation (Shestopalov & Bassnett, 2000), pores are present between adjacent cells and expression of a number of proteins increases. There are a number of proteins that have been identified in the fibre cells that act as cell-cell adhesion molecules: N-cadherin (a transmembrane protein that links and stabilises contact between neighbouring cells), vinculin (a regulator of cell-cell interactions, such as the complexes containing N-cadherin, with the actin cytoskeleton) and paxillin (expression has been identified around the time of fusing between cells and prior to organelle degradation) (Beebe *et al.*, 2001).

Microtubules have been identified along the long axis of the differentiating fibre cells, though their role has not been clarified. The presence of multiple motors e.g. kinesin and dynein suggests a role in transporting organelles and membrane proteins, such as aquaporin during elongation and differentiation (Lo *et al.*, 2003).
Two intermediate filaments have been identified (filensin and CP49), thought to be exclusively expressed in the lens fibre cells, and which make up the beaded filament. Mutations in both of these proteins have been linked with cataract and myopia respectively. Mouse knockout models for these proteins have shown that although the morphology of the lens remains normal there is increased light scatter with age and abnormalities in the fibre membrane architecture suggesting a role in the maintenance of the differentiated fibre cells (Alizadeh *et al.*, 2003; Sandilands *et al.*, 2003; Alizadeh *et al.*, 2004; Perng *et al.*, 2007).

Cell shape, integrity and polarisation are some of the cellular processes dependant on the cytoskeleton. The lens is a finely balanced system, which with only one protein mutation, as shown above, can cause cataract or myopia. Some of the possible types of cataract are described below.

1.9 Cataract

There has been a great deal of research into cataract formation with numerous studies focusing on the molecular networks that contribute to cataract. Recent work has shown that connexins, crystallins and other proteins are very important in lens development. The key to understanding the formation of cataract may be found in identifying the signalling pathways undertaken by these and other proteins. The leading reason for blindness throughout the world is cataract, which is the opacification of the lens (Vijaya *et al.*, 1997). Cataract can be cortical, nuclear, posterior, sub capsular or found throughout the lens as a combination of the stated types (Asbell *et al.*, 2005). Barring cataract formed by mechanical or chemical insult, cataract can be congenital or age-related (Reddy *et al.*, 2004). Other defects that can occur in the lens includes abnormalities in shape, size, position and complete absence of the lens (Graw, 1999). Changes in the structure of lens cells, or the aggregation of soluble proteins (e.g. crystallins), scatters the light that would normally be transmitted through the lens. Blockage of the transmission of the light is commonly known as cataract.

1.9.1 Congenital Cataract

Congenital cataract occurs less frequently than its age-related counterpart and though some of the causes of congenital cataract are intrauterine infection, trauma, ocular inflammatory disease or metabolic disorders, around half of these cataracts are attributed to genetic factors (Francis & Moore, 2004; Krishnamurthy & Vanderveen, 2008). There have been a number of mouse models of cataract that have helped to identify genetic mutations that relate to human congenital cataract. While many mutant models do not have a human homologue there a number of models which do. Examples of some of the known mutations that can cause cataract during each of the

stages of lens development in both humans and animal models are shown in the table below.

Table 1.1 A selection of genes from various stages of development whose mutations that can cause cataract

Stage of lens development	Gene/ Mutation	Reference
Lens Induction	Pax6	(Grindley *et al.*, 1995)
Lens Vesicle	Pitx3	(Semina *et al.*, 2000)
Primary fibre elongation	c-Maf	(Ring *et al.*, 2000)
Membrane stability	*Gja3*	(Jiang *et al.*, 2003; Gong *et al.*, 2007)
Fibre cell differentiation	CRYG	(Smith *et al.*, 2000; Graw *et al.*, 2002)
Gap junctions	Connexion, *Cx50*	(Shiels *et al.*, 1998; White *et al.*, 1998)

1.9.2 Age-Related Cataract

Without taking into account any other factors that cause cataract, genetic predisposition can count for over 50% of cortical cataract alone (Heiba *et al.*, 1995). Other studies on the different types of cataract have also found a statistically significant heritability (The Italian-American Cataract Study Group 1991). A genetic predisposition can result in age related cataract when taking into consideration other factors that can contribute to the development of cataract. Some of the major contributors to age-related cataract include excessive sunlight /UVB radiation (McCarty & Taylor, 1996), smoking (Flaye *et al.*, 1989), diet e.g. alcohol (Munoz *et al.*, 1993) and disease related cataract such as sugar cataract from diabetes (West & Valmadrid, 1995) and from steroid use for asthma (Robman & Taylor, 2005).

It has recently been confirmed that specific combinations of weak attraction and repulsion between dissimilar proteins produces a molecular formation that was transparent. If this fine balance of forces is disrupted, it causes the interactions to becomes unstable and the proteins can aggregate causing light entering the lens to scatter (Stradner *et al.*, 2007).

Along with this basic science above and the contributions of external factors, it is known that the aggregation of proteins, structural defects and nuclear remnants form opacifications in the lens and scatters the entering light.

1.9.3 Posterior Capsular Opacification

Currently there is only one effective treatment for cataract, which is surgery. Cataract surgery can vary, but phacoemulsification involves an opening being made into the anterior of the lens capsule and the lens being removed. An intraocular lens (IOL) is then substituted into the "capsular bag" created by the remaining lens capsule. Though this surgery is relatively simple and widely used, there is a common complication that occurs; this is known as Posterior Capsular Opacification (PCO). PCO occurs when lens epithelial cells, left behind following cataract surgery, proliferate. Only a few epithelial cells left behind can proliferate to form a monolayer between the IOL and the lens capsule. Though in some cases this does not go any further, there are many cases in which the epithelial cells begin to differentiate or wrinkling of the capsular bag occurs (Apple *et al.*, 1992). PCO is currently treated by Neodynium: Yttrium Aluminium Garnet laser treatment (Nd:YAG). Though cataract surgery and PCO treatment are effective, there are risks such as retinal detachment (Ranta *et al.*, 2004). The study of the biological pathways involved in cataract formation could give insight to novel treatments that may delay, stop or even reverse the development of cataract and/or PCO. Though this may seem a far-distant aim, recent research has found that the introduction of either procaspase-3 or bax into the capsular bag when the IOL is inserted has prevented PCO in rabbits (Malecaze *et al.*, 2006); this is now being followed by research involving other inhibitory molecules (to inhibit proliferation of epithelial cells) with the possible aim of inserting IOLs pre-treated with pro-apoptotic molecules, such as those mentioned above.

1.10 Apoptosis

Apoptosis was characterised in 1972 (Kerr & Searle, 1972), but was originally identified as shrinkage necrosis (Kerr, 1971). As more molecular mechanisms were identified in cell death, it became clear that the cell death system was becoming more complex with each discovery. Instead of increased understanding, research just increased confusion about cell death. Even into the 1990s, the differences between necrosis and apoptosis had not been clarified (Columbano, 1995). Though apoptosis

was first described in 1972 and has now been researched for a number of decades, there are still many questions still to be answered (Evan, 2003).

Apoptosis, otherwise known as programmed cell death, is critical during development as well as during adulthood. Apoptosis is used by organisms to cause cell death for many reasons, including if the cell is damaged, infected, cancerous, or no longer required and it is also important during development (Evan, 2003). Apoptosis occurs through a signal cascade, which is thought to include a number of possible signalling pathways that are either intra or extra-cellular (Dahm, 1999). The mechanism of apoptosis was initially studied using a nematode worm *Caenorhabditis elegans*. There are two apoptosis pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway.

Extracellular induction of apoptosis can occur from signals, such as reduction in survival factor concentrations or the interaction of pro-apoptotic factors with cell surface receptors. If induced, Tumour necrosis factor (TNF) ligands (e.g. Fas) bind to the death receptor (DR) e.g. Fas-associated protein with a death domain (FADD), which is extracellular and also a member of the TNFR family (Boatright & Salvesen, 2003). Proteins then bind to the intracellular death domain (DD) of the DR (e.g. FADD). These interactions form the death-inducing signalling complex (DISC). The DISC then recruits an adaptor protein and an apoptosis initiator such as pro-caspase-8. Proteolysis occurs and caspase-8 is activated and released intracellularly (LeBlanc & Ashkenazi, 2003). Caspase-8 then activates caspase-3 and caspase-7, which are found in the cytosol and are thought to be involved in the regulation of apoptotic executioner caspases, such as caspase-6 (Boatright & Salvesen, 2003).

Interactions are known to occur between caspases involved in both the intrinsic and extracellular apoptosis pathway and caspases from one pathway may serve as an amplifier to the apoptotic signal of the other pathway (Kidd *et al.*, 2000).

The intrinsic pathway, otherwise known as the mitochondrial pathway, can be triggered by a number of signals including mitochondrial damage or DNA fragmentation (Nagata, 2005).

Caspase-8 is necessary in apoptosis, though it depends on other protein signals such as caspase-3 to activate it. Once caspase-8 is activated via an intracellular signal, such as DISC activation, it can either go directly to the effector caspases to carry out apoptosis or can initiate the intrinsic pathway. In the intrinsic pathway, caspase-8 can

activate bid and bax (Bcl-2 family members), which move to the mitochondrial surface and signal the release of proteins such as cytochrome-c (a pro-apoptotic protein) (Kidd *et al.*, 2000; Harada & Grant, 2003). Cytochrome c is released into the cytosol and together with Apaf-1 and ATP activates caspase-9 (Kidd *et al.*, 2000).

Both apoptotic pathways initiate a protease cascade involving caspases. The initiator caspases, once activated, proteolytically cleave the effector (or executioner) caspases, which in turn cleave a number of protein substrates (Salvesen & Riedl, 2008). The substrates include poly (ADP-ribose) polymerase (PARP), which has been found in the differentiating lens fibre cells of rats (Ishizaki *et al.*, 1998), DNA fragmentation factor (DFF), identified in the developing chick lens (Wride *et al.*, 1999) and Dnase I (Counis & Torriglia, 2000) shown to be present in the bovine lens fibres (De Maria & Arruti, 2004). The activation via cleavage of these and other substrates leads to the morphological changes signifying apoptosis, including shrinkage of the cell, condensation and marginalisation of chromatin and DNA fragmentation, which has also been identified in differentiating lens cells (reviewed by Bassnett & Mataic, 1997; Kerr, 2002).

Necrosis occurs when cell membrane integrity is lost leading to the unregulated release of the cell contents causing an inflammatory response in the tissue. A key difference between apoptosis and necrosis is that apoptosis is tightly controlled by a complex network of signals that constantly regulate the molecular pathway with pro-survival signals, which may signal cell survival, which is itself regulated by another signalling cascade that can feedback and inhibit the anti- apoptotic signals, allowing apoptosis to conclude (Jin & El-Deiry, 2005). Apoptosis terminates with the formation of apoptotic bodies containing the cellular contents, which are either taken in by lysosomes of neighbouring cells and digested or phagocytosed by macrophages that recognise the bodies through phosphatidylserine signalling proteins found on the extracellular surface of the plasma membrane (Fadok *et al.*, 1998).

1.11 Tumour Necrosis Factor Ligands and Receptors

At the start of July 2008 entering a TNF search into the PubMed database resulted in little less than 70,000 articles. This immense amount of knowledge is being built upon

daily yet there is still much to learn. The TNF family is broken down into two super families; the ligands and receptors. Each of the members of these individual families have conserved structures and interact in similar fashions (Bodmer *et al.*, 2002).

The TNF ligands (cytokines) are known by their THD (TNF homology domain) and have well known roles in the immune system (Bodmer *et al.*, 2002). These TNFs undertake their roles through interaction with membrane bound receptors termed the TNF-R (Tumour Necrosis Factor Receptor) super family (Wajant *et al.*, 2003). TNF-Rs have cysteine rich domains (CRDs) to which the THD may bind (Bodmer *et al.*, 2002).

As with any large molecular family, there is a fine balance to be maintained, within the normal boundaries, TNF members carry out their usual functions, which include signalling for apoptosis, developmental processes (Zhang, 2004) and also has roles in the aggravation of, or protection from diseases/ injury such as malignancies or Crohns disease (Wajant *et al.*, 2003).

Whether TNFs are a help or a hindrance depends on the concentration and length of time that TNFs are involved. Even without disease or injury TNFs can have a serious effect; for example, if concentrations are low for a substantial length of time, cachexia, a wasting disease may occur. On the other hand, at high concentrations, TNFs may induce septic shock (Locksley *et al.*, 2001).

In 2002, Bodmer *et al.* reported that there were 19 ligands and 29 receptors identified in humans that were grouped in the TNF superfamilies, though these numbers have since increased. Originally Wride and Sanders proposed a hypothesis for the potential roles of TNFs in general development and they later confirmed the expression of TNFα, TNFR1 and TNFR2 was present in the differentiating lens fibre cells (Wride & Sanders, 1995, 1998).

1.11.1 Tumour Necrosis Factor Ligands (TNFs)

The TNF ligands are part of a large family of cytokines. Cytokines, when bound to a corresponding receptor, act as a signal in many mechanisms including cell growth, differentiation and death (Thain & Hickman, 2000) TNF ligands are type II transmembrane proteins with a complex structure. All TNF ligands exist as trimers and each has a 25-30% amino acid similarity with the other members in the family

(Locksley *et al.*, 2001). When these ligands bind their receptors they can either initiate a signalling cascade themselves or form a complex so that another ligand can bind; this more intricate complex, when bound, will increase the competence in the signalling cascade (Wajant *et al.*, 2003).

1.11.2 Tumour Necrosis Factor Receptors (TNFR's)

There are many more TNF receptors than ligands and some of these receptors may form complexes with adaptors before the ligand binds or the signal is sent (Dempsey *et al.*, 2003). There are also adaptor proteins that are involved in TNF signalling, which allows flexibility in the signalling pathways. Examples of this are TRAFs (explained in more detail in 1.12) and FADD (Fas-associated Death Domain protein). FADD, when bound to Fas, will recruit Caspase-8 or even Caspase-10 (Locksley *et al.*, 2001). Of the two apoptosis pathways only adaptors called CARD ("caspase recruitment domain") molecules are involved in the intrinsic pathway, but these are also involved in response to inflammation and not just apoptosis. Many members of the TNF family are involved in pathways other than those carrying out apoptosis, such as those involved in inflammatory responses or cell proliferation (Locksley *et al.*, 2001)

1.11.3 The Tumour Necrosis Factors in the lens

The TNFs have a standard role throughout the development of the eye, which relates to all parts of the body and that is to induce apoptosis in cells that are damaged, infected or no longer required, such as cells that make up the lens stalk when the lens vesicle invaginates from the lens placode (Ozeki *et al.*, 2001). In lens fibre cell differentiation, full apoptosis does not occur, but it has been suggested that TNFs or TNF-like molecules may induce a pathway with similarities to apoptosis (Wride & Sanders, 1998); this does not result in the cell being engulfed by macrophages, but rather the organelles within the cells are degraded leaving a "living skeleton" of the cell intact. Understanding TNFs as signalling factors and their affect on apoptosis pathways may be of use in slowing/ curing diseases and may even relate to problems within the lens. This has been tested briefly by the use of TRAIL and downstream apoptosis-related

molecules Bax, procaspase3 and p53 over expression to induce apoptosis in cells that remain in the lens capsule after phacoemulsification (Malecaze *et al.*, 2006).

1.11.4 Selected Tumour Necrosis Factors and their adaptors

Though selected TNFs were identified in the lens previously (Wride & Sanders, 1998) their role in the lens has not been studied any further. It is known that many processes are undertaken through TNF superfamily signalling and that while adaptors containing death domains activate signalling leading to apoptosis, TNF receptor associated factor (TRAF) protein signalling pathways can lead to survival, differentiation, immune and inflammatory responses (Dempsey *et al.*, 2003). Prior to the start of the research described in this PhD, only a select number of TNFs and TRAFs were identified in the *Gallus gallus*. When primers were first designed, the choice was limited, though many more became available throughout the study (e.g. TRAF7). The same issues were present in the selection of antibodies as none of those chosen were recommended/ identified for the detection of the *Gallus gallus* proteins. BLAST (www.ncbi.nih.gov/blast/cgi) analysis was undertaken to find antibodies against TNF-related proteins in other species with the closest homology to the *Gallus gallus* proteins. The design of primers and selection of antibodies is explained in section 2.26 and 4.2 respectively.

1.11.5 TNF Related Apoptosis Inducing Ligand (TRAIL)

TRAIL/Apo2L is a type II transmembrane protein that can initiate apoptosis through binding to two of its five death receptors (Kimberley & Screaton, 2004). The first receptor is soluble OPG, osteoprotegerin, (which also binds to RANKL) but this has low affinity at normal physiological temperatures in comparison to the other receptors (Truneh *et al.*, 2000). OPG is a decoy receptor along with two others that are not soluble; DcR1 and DcR2. These decoy recptors have homology to the active receptors, but DcR1 does not have the required cytosolic region so the apoptotic signal cannot be transmitted through the plasma membrane into the cytosol. DcR2 has the cytosolic

region, but it is truncated and therefore this induction signal also cannot be initiated into the cytosol.

TRAIL initiates apoptosis when it binds to its active, non-soluble death receptors DR4 or DR5. The two active receptors contain the full cytoplasmic death domains, which signal the induction of apoptosis on the intracellular side of the membrane. The death domains engage the cytoplasmic protein FADD (Fas associated death domain) and caspase-8, which then forms the DISC (Kamradt *et al.*, 2005). Caspase-8 proteolyses and then activates other caspases, which are termed effectors, such as caspase-3, -6 or -7. Caspase-10 is also known to be involved in the formation of DISC. It is thought that caspase-10 is activated by the DISC, but only signals apoptosis weakly.

TRAIL has been steadily researched in the field of cancer therapies. It is widely expressed in many tissues without causing harm, but can selectively kill cancerous cells while leaving the surrounding non-malignant cells intact and undamaged (Ricci *et al.*, 2004). Originally it was suspected that TRAIL was tightly regulated by its death receptors, therefore controlling its apoptotic abilities (MacFarlane, 2003), but as always seems the case when relating to cell death it was found to be more complicated than initially thought as 5 death receptors have been identified to date.

TRAIL has been identified in numerous tissues and is thought to have a role in immune surveillance, where it first identifies tumourgenic cells before initiating cell death (Wang *et al.*, 2004). In 2002, Lee *et al* published a paper on ocular TRAIL expression, though they excluded the lens from this investigation they found TRAIL (and Fas ligand known for its role in tumour surveillance) were expressed on tissues that were the boundary between the eye and the surrounding environment, for example the corneal epithelium and RPE, it was noted that tumours of the eye tended to be found outside the boundary formed by TRAIL and Fas expression (Lee *et al.*, 2002).

1.12 TNF receptor-associated factors (TRAFs)

Tumour Necrosis Factor receptor-associated factors (TRAFs) are a sub-family of adaptor proteins that mediate a wide range of cellular processes from proliferation to apoptosis (Lee & Lee, 2002). The TRAFs continue signal transduction through to the

activation of NF-κB and JNK transcription factors therefore mediating differentiation, survival and inflammatory responses (Dempsey *et al.*, 2003). This intracellular protein family interacts with many cell surface receptors, such as TNFRII. TRAF1 and TRAF2 were originally identified through their interaction with this receptor (TNFRII) and this relationship is where they got their name (Arch & Thompson, 1998).

Of the 7 TRAFs that have been identified to date, TRAF1 and TRAF2 were the first identified (Rothe *et al.*, 1994). The most recent, TRAF7 began to be characterised by Xu *et al* in 2004 (Xu *et al.*, 2004). During this short time-frame, copious amounts of research have been undertaken on these signal transduction molecules, but there is yet much to be understood. Though the TRAFs are a family of cytoplasmic proteins with structural similarities, each is unique. A diagrammatic representation of TRAFs structure can be viewed in figure 1.5.

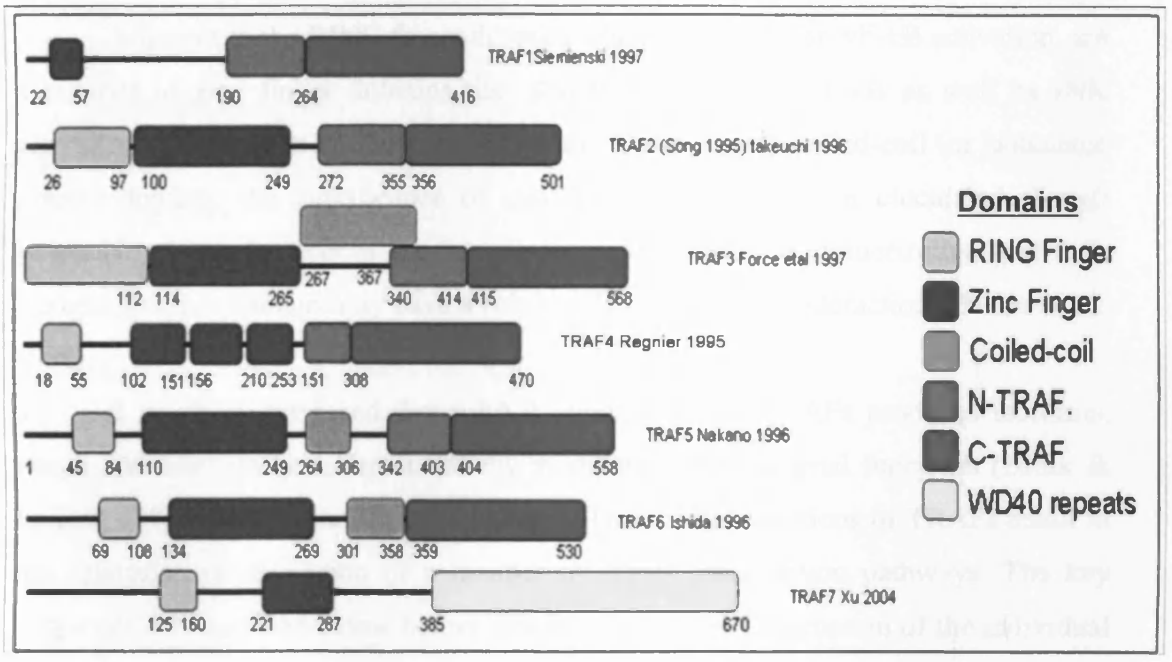


Figure 1.5 Proposed structures of the known TRAFs. TRAF1 does not have a RING domain and only has one zinc-finger repeat similar to TRAF7. TRAF2, 3, 5, and 6 contain 5 zinc-finger repeats while TRAF4 has 7 zinc-finger repeats. TRAF3 and TRAF5 contain a coiled-coil domain otherwise known as an isoleucine zipper. TRAF1 to TRAF6 contain the TRAF domain containing the sub domains N-TRAF and C-TRAF. TRAF7 is unique among the TRAFs as it does not contain the TRAF domain, but in its place has seven WD40 repeats.

The TRAF domain or carboxyl terminus is conserved in TRAFs1-6 and is split into two regions: The TRAF-C region, which is known to "bind downstream signalling molecules such as TANK", the second region is TRAF-N which can bind c-IAPs, which are anti-apoptotic molecules (Dempsey *et al.*, 2003). In TRAFs 1-6, the whole TRAF domain is required for receptor interactions and also hetero- and homo- di/ trimerization (Rothe *et al.*, 1995; Pullen *et al.*, 1999). TRAF7 does not have this domain; instead it has seven WD40 repeats that are involved in MAKK3 signalling. All TRAFs, barring TRAF1, contain a RING finger domain at the N terminus of the TRAF. The RING finger is now known to be involved in ubiquitin modulation of the TRAFs and, in the case of TRAF2 and TRAF6, does not signal degradation, but changes their subcellular localisation which in turn regulates the TRAFs' action on their respective downstream signalling cascades; e.g. TRAF2 activation of MAPK and JNK (Laine & Ronai, 2005).

Adjacent to the RING finger domain, which is crucial for NF-κB activation, are a number of zinc finger domains that also have a role in NF-κB as well as JNK activation (Dempsey *et al.*, 2003). TRAF3 and 5 also have a coiled-coil (or isoleucine zipper) domain; the significance of this domain has not been elucidated though isoleucine zipper sequences are known to mediate homo-oligomerization and it is thought that this domain may have a role in TRAF3/ TRAF5 interaction (Pullen *et al.*, 1998).

It has been proposed that mRNA splicing of the TRAFs produces isoforms, which are structurally different thereby modulating their normal functions (Brink & Lodish, 1998; van Eyndhoven *et al.*, 1999b). The normal functions of TRAFs result in the activation or mediation of a number of signal transduction pathways. The key pathways are described below before moving on to a brief discussion of the individual TRAFs.

1.12.0.1 NF-κB

NF-κB is a homo- or heterodimeric transcription factor that has been most studied in its role in the immune system. NF-κB also has key roles in its transcriptional regulation of signalling cascades influencing proliferation, differentiation and cell survival

(Hayden & Ghosh, 2008). TRAFs are involved in the activation of both the canonical, involved in inflammation and protection from cell death, and non-canonical pathways, which occurs during the development of lymphatic tissue (Bonizzi *et al.*, 2004). TRAFs activate IKK (IκB Kinase), which phosphorylates IκB (Inhibitor of NF-κB); this phosphorylation signals the ubiquitination and proteosome-dependant degradation of IκB. This results in the nuclear localisation of NF-κB, which was previously sequestered in the cytoplasm. NF-κB is then activated and induces transcription (Brown *et al.*, 2003). Though TRAFs activate IKK, which results in a selected response from the NF-κB pathway, it is the way that NF-κB interacts (cross-talks) with the upstream, initially unrelated signalling pathways, that will result in the ultimate NF-κB response (Hayden & Ghosh, 2008).

1.12.0.2 MAPK pathways

Mitogen-activated protein kinases (MAPK) are involved in three different signalling pathways: JNK, ERK and p38 of which the TRAFs are involved in signal one or more. At the core of each of the pathways is a signalling cascade: MAPK, MAPK kinase (MAPKK) and MAPK kinase kinase (MAPKKK/ MAP3K). MAPK3, when activated via phosphorylation, in turn activates MAPKK via phosphorylation. MAPKK activates MAPK by its phosphorylation ability as a dual specificity kinase. MAPKs then phosphorylate one of many possible targets; examples include proteins, transcription factors or other kinases (Uhlik *et al.*, 2004; Krishna & Narang, 2008).

The resulting pathways mediate many activities, such as cell cycle, survival, differentiation and apoptosis. The p53/ MAPK pathways have a role in cell cycle events and respond to extracellular stress; they are known to respond to the same stimuli that activate JNK, for example growth factors such as FGF and IGF and stress (Tan *et al.*, 1996). P53 regulates cell cycle arrest, apoptosis (downstream of caspase activation) and differentiation (Bourdon, 2007).

Extracellular signal-related kinases (ERKs) are activated by many extra- and intracellular stimuli, for example growth factors, serum, and hormones and they are also activated by osmotic stress and cytokines. ERK is a regulator of cell proliferation and, though it is mostly known for its cell survival roles, it is now thought to have a role in apoptosis through cross-talk with apoptosis-regulator factors such as Bax and p53 (Rubinfeld & Seger, 2005).

JNKs were renamed to highlight their activation and phosphorylation of transcription factor c-jun, which regulates gene expression by targeting specific DNA sequences (Yujiri *et al.*, 1998). JNKs are activated by growth factor deprivation, cytokines and external stressors such as UV irradiation. JNK's proposed pro-apoptotic roles include ubiquitin-mediated degradation of p53 and the inhibition of anti-apoptotic mitochondrial proteins Bcl-2 and Bcl-xl through phosphorylation, though this is in question *in vivo*. JNK is also thought to have a role in survival through the activation of pro-survival pathways and in the mediation of apoptosis after extended activation. JNK knockout mice are not embryonically lethal like ERK2 and p38α knockouts are and it has been suggested that JNK has roles in insulin response and obesity (Hirosumi *et al.*, 2002).

1.12.1 TNF receptor-associated factor 1 (TRAF1)

TRAF1 is unique among the TRAFs as it does not contain the RING finger domain that all the other TRAFs have and which is required for their interactions with other proteins (Zapata & Reed, 2002). TRAF1 was initially identified through its interaction with TNFRII, which was thought to occur via a heterodimeric complex of TRAF1 and TRAF2 bound to the C-terminal region of the cytoplasmic domain, which then allowed the transduction of signals into the cell (Rothe *et al.*, 1994). TRAF1, the smallest adaptor protein, can bind directly to the cytosolic domain of a number of TNFRs such as CD30 and RANK; to bind to other TNF receptors, it requires hetero-oligomerisation (forming associations/complexes) with other TRAFs. For example TRAF1/ TRAF2 heterodimers can bind to TNFRI, which then recruit anti-apoptotic proteins, which in turn suppress TNFRI induced apoptosis (Wang *et al.*, 1998). TRAF1 has restricted expression and has only been identified in lymphocytes, dendritic cells and some epithelia (Zapata & Reed, 2002). Experiments using transgenic and knockout mice have suggested that TRAF1 has a role in inhibiting apoptosis (Speiser *et al.*, 1997; Tsitsikov *et al.*, 2001). Inhibiting apoptosis is thought to be undertaken by various interactions, such as binding IAPs (which can suppress caspase-8 activation) (Wang *et al.*, 1998), or by increasing NF-κB activation, via TRAF2, which could then increase the transcription of anti apoptotic proteins (Duckett & Thompson, 1997). Recent research has also implicated TRAF1 in a pro-apoptotic role when cleaved by caspase-8

(Irmler *et al.*, 2000). This is supported by the finding of increased JNK activation in *TRAF1*-deficient mice. It may be that deficiency or cleavage of TRAF1 slows the activation of NF-κB, tipping the scales in favour of JNK, which is known to be pro-apoptotic. The varied participants interacting in TRAF1 signalling can result in both inhibition or increase in apoptosis and there are many factors (some about which we have little understanding) that affect the final cellular response, such as cell type, protein interactions and TRAF1 cleavage (Zapata & Reed, 2002).

1.12.2 TNF receptor-associated factor 2 (TRAF2)

TRAF 2, along with other TRAFs, has been demonstrated to mediate activation of NF-κB and JNK as described above. TRAF2 binds with TNF receptors, such as TNFR-II, RANK and CD40. TRAF2 has been identified in most tissues, making it the most ubiquitously expressed of the TRAFs (Rothe *et al.*, 1994). Deficiencies in TRAF2 expression lead to a slight reduction in TNF-induced NF-κB activation, but a large decrease in JNK activation which results in an increase in apoptosis that has been confirmed in knockout mice (Lee *et al.*, 1997; Yeh *et al.*, 1997). Recent studies have highlighted that TRAF2 is regulated by ubiquitin signals and its ability to form poly-ubiquitin chains, thought to be due to co-localization with a number of E2 and E3 complexes, result in its various functional abilities (Wu *et al.*, 2005).

TRAF2 is thought to be involved in B cell homeostasis and regulates proliferation and survival within lymphocytes. TRAF2 is necessary for JNK activation signalled by TNFR family members within lymphocytes where it is also thought to initiate anti-apoptotic signals independently of NF-κB on which TRAF2 seems to have no regulatory effect (Lee *et al.*, 1997).

TRAF2 is thought to be essential in CD40 (B cell proliferation and activation) signalling leading to NF-κB canonical pathway activation, while TRAF2 is also known to negatively regulate the non-canonical pathway of NF-κB (Nguyen *et al.*, 1999; Jabara *et al.*, 2002).

TRAF2 has also been shown to bind to the intracellular transmembrane endoplasmic reticulum (ER) receptor IRE1 (Urano *et al.*, 2000), which is a part of one of three signalling pathways activated by ER stress when misfolded proteins have accumulated in the ER. The IRE1 pathway is normally active at the same time as two

other stress pathways, but is the first one to switch off if ER stress does not abate, studies have suggested that the IRE1 pathway protects cells and, once this signalling pathway switches off, cell death increases. Following IRE1 switching off, ATF6 is then down regulated while PERK signalling was maintained (Lin *et al.*, 2007). IRE is known to interact with Bcl-2 family members who regulate apoptosis, activate IRE1α signalling and link ER stress and apoptosis pathways (Hetz *et al.*, 2006). IRE also activates the JNK signalling pathway by binding TRAF2 (Urano *et al.*, 2000).

TRAF2 knockout mice die within a few weeks of birth, in the small percentage of embryos that are not embryonic lethal. This suggests a key role in embryonic development and a protective role, suggested to either come from transcriptional activation of survival genes via NF-κB, or TRAF2 activates an, as yet unknown, pathway that supports the survival signals from NF-κB (Yeh *et al.*, 1997).

1.12.3 TNF receptor-associated factor 3 (TRAF3)

TRAF3 (LAP-1, CD40bp, CRAF-1) was described independently as a cytoplasmic factor that interacts with CD40 and LMP-1 (Hu *et al.*, 1994; Cheng *et al.*, 1995; Mosialos *et al.*, 1995; Sato *et al.*, 1995). Though TRAF3 can bind to CD40, it is not essential (unlike TRAF2 above), though it does have the ability to inhibit CD40 signalling through an unknown interaction (Hostager & Bishop, 1999). TRAF3 is also recruited in a ligand-dependent manner to the lymphotoxin- receptor (LTβR) and can have an inhibitory effect on NF-κB activation and it has a role in cell death signalling (Force *et al.*, 1997; Kuai *et al.*, 2003a; Kuai *et al.*, 2003b).

MIP-T3 (the only NF-κB protein that binds to only one TRAF) may provide a novel mechanism in sequestering TRAF3 to the cytoskeletal network. In experiments, using 293 cells in culture, TRAF3 is released when localised to microtubules allowing it to bind to CD40 after stimulation by CD40L (Ling & Goeddel, 2000).

TRAF-3 appears to negatively regulate protein activation and expression, which was confirmed in a study using embryonic kidney cells; the over-expression of TRAF-3 suppressed TNF-RII and CD40-dependent activation of NF-κB dependent reporter genes (Pullen, 1999).

Unlike TRAF2, 5 and 6, which are activators of the NF-κB canonical pathway, TRAF3 inhibits the non-canonical NF-κB pathway through interaction with its RING finger domain and C-terminal TRAF domain which is necessary to bind with NIK (NF-κB-inducing kinase)(He *et al.*, 2007).

TRAF3 is expressed in B cells, dendritic cells, macrophages and monocytes, endothelial cells, smooth muscle cells and fibroblasts. TRAF3 null mutant mice, though visually normal at birth, die around 10 days after birth and research suggests that TRAF3 is required for an active immune system and postnatal development. Though major organs were not obviously faulty, glucose levels dropped continuously, which has been previously associated with runting. Further research suggested that TRAF3 may be important in B cell development and survival (Xu 1996).

1.12.4 TNF receptor-associated factor 4 (TRAF4)

TRAF4, first known as CART1, (C-rich motif associated with RING and TRAF domains 1) was isolated by differential screening of a cDNA library of lymph nodes that contained metastatic tumour cells (Regnier *et al.*, 1995). TRAF4 has been shown to be expressed in the neuroeplithelium, limb buds, and sensory organs including the neuroretina of the developing mouse; there has been no comment on TRAF4 expression in the lens (Masson *et al.*, 1998). In the zebrafish, two cDNA constructs *traf4a* and *traf4b* have been identified; the latter was expressed ubiquitously and at low levels, while *traf4a* was expressed at higher levels in the early developing eye, becoming more restricted during development to the lens outer layer and proximal retina (Kedinger *et al.*, 2005). By the time a fully functional lens has developed, *traf4* expression was restricted to the equatorial region where secondary fibre cell differentiation begins, which was corroborated by the statement that TRAF4 (*traf4a* zebrafish orthologue) expression was "restricted to post mitotic cells away from proliferative zones" (Kedinger *et al.*, 2005).

Intracellularly, TRAF4 is mostly found in the nucleus and in undifferentiated cells; it promotes JNK activation through binding to MEKK4 and increasing MEKK4 kinase activity (Abell & Johnson, 2005).

TRAF4 deficient mice are embryonic lethal, though there are some who survive through to adulthood, they have skeletal malformations, such as spina bifida and

respiratory problems due to the incorrect formation of the trachea (Regnier *et al.*, 2002).

1.12.5 TNF receptor-associated factor 5 (TRAF5)

The discovery of TRAF5 was published in 1996. It was identified by degenerate oligonucleotide PCR amplification with primers that were homologous to a highly conserved region of the TRAF-C domain that is known to interact with CD40, LTβR, CD27 and CD30 (Ishida *et al.*, 1996b; Nakano *et al.*, 1996).

TRAF5 binds to CD40 via TRAF3, though is not necessary for CD40 signalling as shown in research using CD40 mutants with truncations in its cytoplasmic tail where the TRAFs bind (Leo *et al.*, 1999). TRAF5 is also thought to have a role in modulating OX40 expression, which regulates T cell differentiation (So *et al.*, 2004).

TRAF5 is known to be expressed in the lungs, thymus and spleen (Abdalla *et al.*, 2004) and TRAF5 null mice are healthy, though there is impaired activation of lymphocyte activation mediated by CD40 and CD27 (Nakano *et al.*, 1999). The key pathways that TRAF5 is involved in have not yet been clarified.

1.12.6 TNF receptor-associated factor 6 (TRAF6)

TRAF6 was isolated independently by the screening of an EST expression library and by utilizing CD40 as bait for a yeast two-hybrid screen (Cao *et al.*, 1996; Ishida *et al.*, 1996a). Overexpression of TRAF6 can result in activation of JNK, p38 and NF-κB (Baud *et al.*, 1999; Song *et al.*, 2006). The RING finger domain of TRAF6 can act as an ubiquitin ligase that is thought to have a regulatory role in IKK activation (Deng *et al.*, 2000).

IL-1 is known to regulate cytoskeleton reorganisation in osteoclast-like cells. IL-1 signalling stimulates TRAF6 to move with c-Src to actin ring structures around the sides of the cell (Nakamura *et al.*, 2002).

TRAF6 null mice suffer severe bone formation problems as well as defects in tooth eruptions and these mice become runted and die early on in postnatal life (Naito

et al., 1999). TRAF 6 is thought to be necessary in osteoclast differentiation factor signalling (Naito *et al.*, 1999).

1.12.7 TNF receptor-associated factor 7 (TRAF7)

From the figure 1.5, TRAF7, can be seen to have an N-terminal RING finger domain found next to a zinc finger domain, while, on the other end of the protein, the C terminus contains 7 WD40 repeats. Published in 2004 the discovery of TRAF7 occurred through an investigation of novel proteins containing TRAF-like ring finger domains (Xu *et al.*, 2004). It was found (via transfection studies) that TRAF7 interacted with MEKK3 through the WD40 repeats at its C-terminus. This interaction increased MEKK3 autophosphorylation, which in turn induced AP1 and, to a lesser degree, CHOP activation which is involved in the JNK and p38 kinase pathways, respectively. It was also found that the RING finger domain was highly involved in TRAF7-induced caspase dependant apoptosis, causing cellular condensation and DNA fragmentation. This was proven through inhibition of TRAF7-induced apoptosis by crmA, a caspase inhibitor (Xu *et al.*, 2004). TRAF7 (with TRAF6) is also involved in Toll-like receptor2 (TLR2) signalling, which causes activation of the IKKs-IκBα and MKK3/6-p38 pathways. These pathways then induce the transcription of proteins such as TNFα and CLYD (cylindromatosis), a tumour suppressor. CLYD, likely involved in "a deubiquitination-dependant mechanism", will then inhibit TRAF6 and 7, which will negatively regulate the TLR signalling. The study showed that TRAF7, together with TRAF6, was required to mediate TLR signalling through auto- and negative regulation (Yoshida *et al.*, 2005). TRAF7 has been shown to affect the cellular localization of molecules such as c-Myb via sumoylation when bound (Morita *et al.*, 2005). Other roles of TRAF7 include E3 ubiquitin ligase activity for self-ubiquitination and possible links with proteins involved in epithelial cell growth and polarity (Bouwmeester *et al.*, 2004).

1.13 Summary of Introduction

In this introduction, the current knowledge of lens development and a number of the TNFs and TNF-related factors of interest have been discussed. It has been highlighted that chick is an excellent model for this research due to the ease of collection of the samples and the relatively simplistic, quick and cheap cultivation of the fertilised eggs.

1.13.1 Objectives:

The objectives of this thesis are:

1) To obtain information on the temporal expression patterns of the TNFs and TNF-related factors (Chapter 3):

2) To characterise the spatiotemporal and protein expression patterns of TRAIL and TRAF2 and TRAF3 in the developing chick lens across the developmental stages E6- E16 (Chapter 4).

3) Characterisation of whole E10 chick lens culture as a model system and development of an optimised methodology for further functional studies on the role of apoptosis-related factors, including TNFs, on chick embryo lens development (Chapter 5).

The Overall hypothesis for the thesis is:

In this thesis, the hypothesis is tested that TNFs and TNF related factors (specifically TRAIL and TRAFs) are expressed in the developing chick lens at both the mRNA and protein levels and that characterisation of a whole chick embryo lens *in vitro* system is important and useful for potentially dissecting the roles of TNFs and TNF-related factors in embryonic lens development in future studies.

Chapter 2:

Materials and Methods

2.1 Tissue Collection

2.1.1 Egg collection and incubation

Fertilised white leghorn eggs (Henry Stewart & Co, Lincolnshire, UK) were placed in an Octagon 100 incubator set at 37.5°C ± 0.5 °C (Brinsea, Sandford, UK) as shown below in figure 2.1. The date and time at the start of incubation were noted on each egg using a pencil. Water levels in the incubator were checked twice a day to ensure water was continually available to maintain a humid environment of 55%.

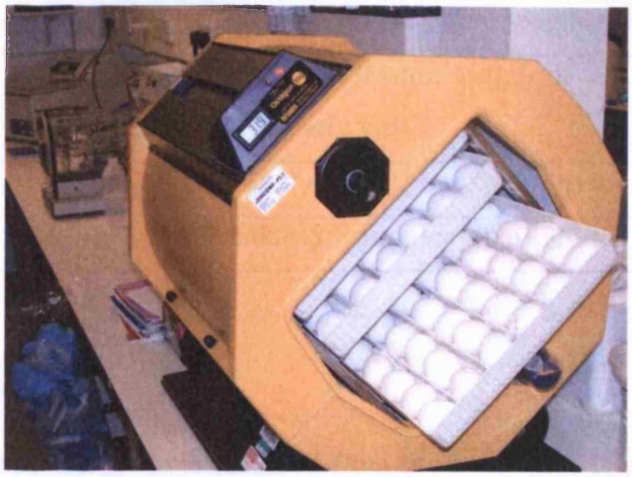


Figure 2.1 Octagon 100 incubator containing eggs.

The eggs were removed on specific days of embryonic development (E) corresponding to embryonic stages as described by Hamburger & Hamilton (1992) and shown in the table 2.1.

Table 2.1 Hamburger & Hamilton stages selected for study of lens development.

Embryonic day (E)	Hamburger & Hamilton Stages and description of development.
6	29: 3 digits, 4 toes rudiment of 5th toe. Lens secondary fibre cell differentiation has begun
8	34: Nictitating membrane
10	36: Length of 3rd toe from tip to middle of metatarsal joint = 5.4±0.3mm; Length of beak from anterior angle of nostril to tip of bill = 2.5mm; primordium of comb; labial groove; uropygial gland, Lens OFZ begins to form
12	38: Length of 3rd toe = 8.4±0.3 mm; length of beak = 3.1 mm
14	40: Length of beak = 4.0 mm; length of 3rd toe = 12.7±0.5 mm, Lens OFZ is clearly visble
16	42: Length of beak = 4.8 mm; length of 3rd toe = 16.7±0.8 mm
18	44: Length of beak = 5.7 mm; length of 3rd toe = 20.4±0.8 mm

2.1.2 Tissue collection

At specific time points, eggs were removed from the incubator. The upper side of the egg shell was sterilised using ethanol and a circular hole was made to open the egg, where the embryo was found as it floated on the surface of the albumen. The embryo was removed from the egg then placed in a 100mm diameter Petri dish (Sigma, UK) with PBS (Sigma, UK), on a dissecting microscope (Swift Optics Europe), as shown in figure 2.2.

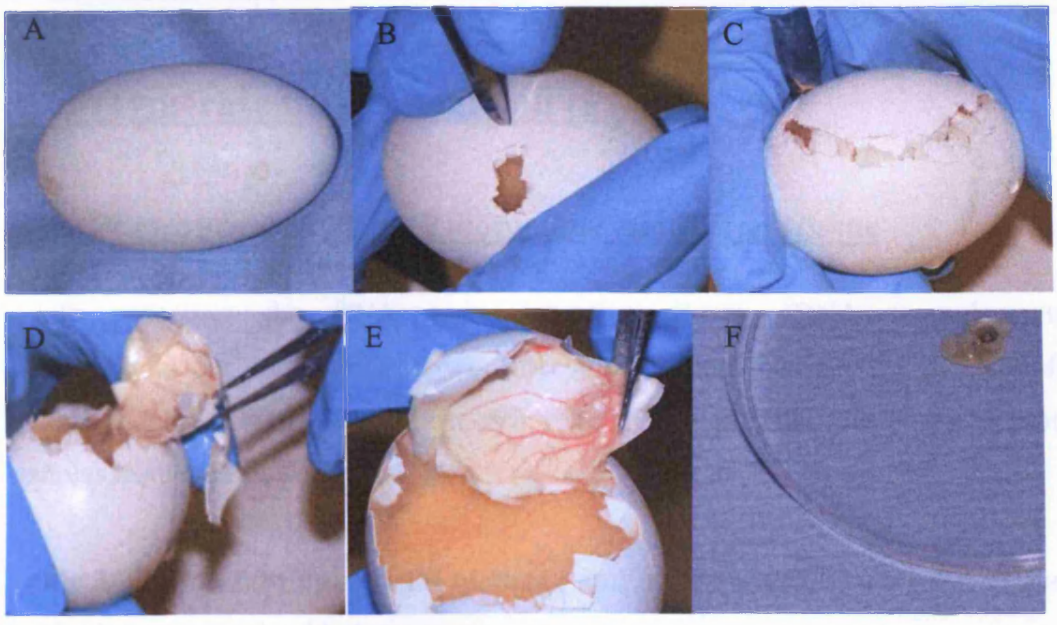


Figure 2.2 Stages in obtaining embryos. A, Whole fertilised egg. B, The first incision into the egg shell using the pointed reverse end of tweezers (Sigma, Dorset, UK). C, Large hole being made in the egg shell. D, The shell being removed. E, At E4-E6 the embryos tend to be found on the inside of the egg shell instead of floating. F, Life size image of a chicken embryo at E6.

Embryos were sacrificed in accordance with ARVOs recommendations for the use of animals in ophthalmic and vision research. Briefly, embryos were placed in ice cold double autoclaved (da) PBS followed immediately by decapitation. At this point tissue being collected for histology would be treated as explained in 2.4.1. If samples were being collected for RNA or protein the eyes were removed with #5 dissecting tweezers (R.A.Lamb, UK) and placed under a Nikon SMZ800 (Jencons Plc, UK) dissecting microscope for the more intricate dissection, any waste tissue was placed in a biological waste container for proper disposal.

An incision was made at the junction between the cornea and the sclera using fine bow spring scissors (R.A.Lamb, UK). The vitreous was removed with the lens attached. The vitreous allows the lens to be held in place without causing damage, enabling lenses to be cleared of remnants of surrounding tissue e.g. iris. The lens was removed from the vitreous and placed in fresh ice-cold PBS. Once lenses were removed and cleaned the subsequent methodology changed depending on the future use of the tissue i.e. for PCR (polymerase chain reaction) or Western blotting.

2.2 Polymerase Chain Reaction (PCR)

2.2.1 Preparation of RNA (Ribonucleic Acid) from tissue

While preparing the RNA and converting it to cDNA (complementary Deoxyribonucleic Acid), RNase-free filter pipette tips (Thistle, UK), RNase-free ependorff tubes (Sigma) and RNase-free water (Ambion, UK) were used to protect samples from contamination.

Table 2.2 Grid showing amount of tissue and solution values used for TRIzol® methodology explained below.

	E6	E8	E10	E12	E14	E16	E18
Number of lenses	30	20	20	18	18	16	14
Number of Embryos	15	10	10	9	9	8	7
TRIzol (ml)	0.5	0.5	0.5	0.5	1	1	1
Chloroform	0.1	0.1	0.1	0.1	0.2	0.2	0.2
Isopropyl alcohol	0.25	0.25	0.25	0.25	0.5	0.5	0.5
75% Ethanol	0.5	0.5	0.5	0.5	1	1	1
RNase-free water	30	40	40	50	50	50	50
10x DNase I buffer	0.1 vol. = 3	0.1 vol. = 4	0.1 vol. = 4	0.1 vol. = 5	0.1 vol. = 5	0.1 vol = 5	0.1 vol. = 5
DNase I	1	1	1	1	1	1	1
DNase Inactivation Reagent	5	5	5	0.1 vol. = 5	0.1 vol. = 5	0.1 vol = 5	0.1 vol. = 5

Shown in the table above were the numbers of lenses pooled to collect the required concentration of sample and the volumes of solutions used for each of the aforementioned samples. When a suitable number of lenses had been collected, the sample was placed in a sterilised glass Dounce homogeniser (Wheaton, UK) using a Pasteur pipette (Sigma-Aldrich, UK) with a widened end, the PBS pipetted up during collection was subsequently aspirated using sterile pipettes (Sigma, UK) of decreasing sizes, leaving the lenses intact.

2.2.2 TRIzol Preparation

Depending on the quantity of lenses, between 0.5 and 1µl of TRIzol® (Invitrogen, Paisley, UK) was added, as shown in table 2.1, the lenses were then homogenised using a Dounce homogeniser. The homogenate could then be stored for up to a week at -20°c.

The homogenised sample was incubated at room temperature for 5 minutes before 0.2ml of chloroform (Fisher Scientific, UK) was added for every 1ml of TRIzol® used. The mixture was vortexed and incubated at room temperature for 2 minutes then centrifuged for 15 minutes at 12,000 x *g* at 4°C in a Sorvall® *fresco* centrifuge (Jencons, UK). The mixture separated into three phases during centrifugation: the lower red phase, which contained the phenol chloroform; an interphase, containing cellular debris; and the upper colourless, aqueous phase containing the RNA. The colourless RNA phase was transferred, via sterile filter pipette, to a new, sterile eppendorf tube (Sigma, UK) insuring that neither of the other phases was transferred.

The RNA was precipitated from the aqueous phase by adding 0.5ml (per 1ml TRIzol®) of isopropyl alcohol (Fisher Scientific, UK) and incubating at room temperature for 10 minutes before centrifugation was carried out at 12,000 x *g* at 4°C for 10 minutes. The pellet on the side/ bottom of the eppendorf tube was the RNA precipitate. The supernatant was removed by pipetting and the RNA pellet washed by adding 1ml (per 1ml TRIzol®) of 75% ethanol (Fisher Scientific, Leicestershire, UK). The sample was mixed gently and centrifuged at 7,500 x *g* at 4°C for 5 minutes.

Most of the liquid was removed using a pipette and the remaining liquid was left to air dry for up to ten minutes. Depending on the amount of RNA left in the tube, between 30- 50µl of RNase-free water (Ambion, UK) was added to the eppendorf tube. The RNA was dissolved in the RNase-free water by pipetting up and down 5-10 times. The mixture was then incubated at 55°C for 10 minutes using a GRANT, Q.B.T. Block Heater (Jencons, UK).

2.2.3 Quantification and Integrity of RNA

Quantification of the RNA samples was undertaken using Gene Quant II spectrophotometer (Pharmacia, BioTech, UK). 2µl of sample was added to 98µl of nuclease free water in a sterile cuvette. The absorbance was read at 260 and 280nm. Nuclease free water was used as the reference before each samples absorbance reading. RNA concentration was obtained by multiplying the 260nm absorbance by the dilution (50) and then by 40 (RNA constant).

The integrity of RNA was confirmed by running the RNA out on a formaldehyde denaturing gel. The composition of the gel can be found in appendix 1. The gel was left to set under the fume hood for 1 hour. The RNA samples were denatured before being run on the gel. The components of the RNA reaction were 4µl RNA sample, 2µl 10xMOPS electrophoresis buffer (composition found in appendix 1), 3.5µl Formaldehyde (Sigma, UK), 10µl Formamide (Ambion, UK) and 0.5µl Ethidium Bromide (Sigma, UK). The components of the reaction were added to an eppendorf tube and incubated at 55°C for 15 minutes in a heating block. The samples were then placed on ice and 5µl of loading buffer (composition in appendix 1) was added to each of the samples. 200mls of running buffer was then added to the gel dock covering the pre-set gel by 1-2mm. The samples were then loaded onto the gel and the gel was run for 5mins at 40V followed by 80V for 30 minutes using the Consort E122 Mini Power Pack (Jencons Plc, UK). The bands on the gel were visualised and photographed using a UV transilluminator using a Gel Doc system (UVP BioDoc-It™ System).
Intact RNA gives two bands on the gel, one corresponding to the 28S and the other to the 18S ribosomal species (example shown in figure 3.1). If there was smearing of the bands then it suggested that the RNA had degraded and the sample was disposed of.
Samples that were intact were all diluted to a concentration of 10µg/50µl using RNase-free water (Sigma, UK).

2.2.4 DNase digestion

Within 24 hours of collecting the RNA sample, DNase digestion was carried out to remove any contaminating genomic DNA. This was done using DNA-*free*™ (Ambion, Cambridgeshire, UK): To each sample of RNA, 0.1volume of 10x DNase I Buffer and 1µl of DNase I was added. This was mixed by gentle pipetting and then incubated for 25 minutes at 37°C. DNase Inactivation Reagent, 0.1 volume or 5µl (whichever was greater) was added to the sample, which was mixed and incubated at room temperature for 2 minutes. The mixture was then centrifuged at 10,000 x *g* for 1 minute. The resultant solution containing the RNA was pipetted into a sterile eppendorf tube making sure that none of the pellet was also transferred. The pellet was discarded, while the RNA solution was stored at -20°C.

2.2.5 Reverse Transcriptase (RT) Reaction

RT reactions were carried out using the Superscript™ II Reverse Transcriptase kit (Invitrogen, Paisley, UK):

From the concentration calculated earlier, the volume of RNA required for between 1-5ng was noted. Up to 10µl of DNase digested RNA was used depending on concentration and placed in a nuclease free micro-centrifuge tube (Triple Red, UK); a replicate tube was also begun so it could be the No RT control. All the steps were carried out on both tubes, with the exception of the addition of the Superscript™ II RT. The no RT control is to insure there is no reagent contamination causing false positive results.

In brief, 1µl of Oligo dT (Invitrogen, Paisley, UK) and 1µl of 10mM dNTP mix (Invitrogen, Paisley, UK) was added to both tubes. If less than 10µl of RNA was used then the difference up to 12µl, all inclusive, was filled using DNase free water. The mixtures were heated at 65°C for 5 minutes and then chilled on ice. While they were on ice 4µl of 5x First Strand Buffer and 2µl of 0.1M DTT (Dithiothreitol) was added to each tube. The solution was then mixed and incubated at 42°C for 2 minutes. 1µl of Superscript™ II RT was added and mixed by pipetting up and down into the RT tube while 1µl RNase free water was added to the No RT tube. RT and NO RT were marked

on the respective tubes. The tubes were both incubated for 50 minutes at 42°C. The reactions were then inactivated by incubation at 70°C for 15 minutes. The final step carried out was to add 1µl RNase H and incubate the solutions at 37°C for 20 minutes. The samples were then stored at -20°C until use.

2.2.6 Primer design

The primers for GAPDH and δ crystallins were taken from Faulkner-Jones *et al.*, (2003). All other primers were designed using primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To design the primers, the genes were selected and identified in the chicken, which was carried out using Entrez Gene on the NCBI website: (http://www.ncbi.nlm.nih.gov/entrez). The accession numbers for the selected genes can be found in table A.2.1 in appendix 2. Once the selected genes were identified in the chicken, the mRNA sequence was noted and selected; the coding sequence (cds) was then selected and displayed in FASTA format. The cds was then cut and pasted into primer3. The selections made on this website were:-

To tick both the boxes for the 3' end (forward primer) and 5' end (backward primer).

Pick a sequence ID e.g. "chick TRAF".

Standardise the set up as below:

Primer size: Min 20, Opt 21, Max 24.

Primer annealing temperature: Min 58, Opt 62, Max 64.

Maximum temperature difference: 2.

Primer GC% Min 40, Opt 50, Max 60.

Maximum Self complementation: 3.

Maximum self 3' Complementation: 0.

Maximum poly "X" tail: 3.

GC Clamp: 0.

Select button PICK PRIMERS.

Selections of possible primers were then shown and the most suitable i.e. the one that most closely matched to the optimums was chosen. Both the forward (3') and backward (5') were copied and then pasted into the Operon > Custom Oligos ordering page (https://www.operon.com/store/oligo_order.php).

The primers ordered (in 10ng aliquots) were sent dry and were then rehydrated by adding the stated amount of TE buffer (Ambion, Cambridgeshire, UK) and thoroughly vortexing (Vortex-Genie, Jencons Pls, UK). The amounts advised for rehydration varied from primer to primer. The re-suspended primers were stored at -20°C.

The relative concentrations of the primers were revealed by running the primers out on a 1.5% agarose gel. The gel was prepared by adding 1.5 g of agarose to 100ml of TAE (Tris Acetate buffer) (Sigma, Dorset, UK) and then heating until the agarose dissolved. 5µl of ethidium bromide (Bio-Rad, Hertfordshire, UK) was added and mixed gently. The gel was then poured into a horizontal electrophoresis unit (Jencons, Bedfordshire, UK) and allowed to set for a minimum of 1 hour. The primers were diluted before being run on the gel. 1µl of primer was added to 2µl of loading buffer (Sigma, Dorset, UK) and 9µl of double autoclaved water. The 12µl mixture was then pipetted into a well in the gel. The gels were run at 40W for 5minutes going up to 80W for a further 20 minutes. The gels were then visualised. The primers were normalised so that the concentration for all primers was similar. The quantities used for each primer, after normalisation, are shown in table A.2.1 in appendix 2. The table also shows the primer sequences, expected PCR product size, optimised temperature and cycle number as explained below.

2.2.7 Optimisation of Primers

Though a recommended temperature for annealing was supplied with each primer set purchased, these and the optimum cycle number were confirmed before use. These optimums were found by carrying out PCRs at various temperatures around that recommended by Operon (Sigma, UK). The optimum temperature was decided by the temperature that offered a single, sharply defined band when visualised. Once the temperature was optimised the optimum cycle number was decided by running 5-7 PCRs of ascending cycle numbers and forming a standard curve by analysing the band intensities as explained in section 2.2.10. A cycle number was chosen from the bottom of the curve. An example of this is shown in the image and corresponding graph below which shows the GAPDH standard curve.

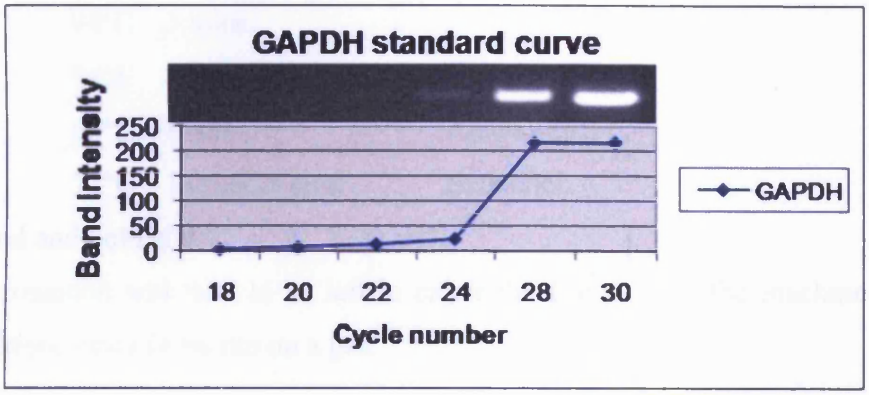


Figure 2.3 Graph showing GAPDH standard curve with corresponding PCRs.

2.2.8 Polymerase Chain Reaction (PCR)

PCR was carried out using GoTaq® Flexi DNA Polymerase (Promega, UK). The amounts used are found in table 2.3.

Table 2.3 Amounts used for GO Taq system.

Green Buffer	10µl	$MgCl_2$	4µl	Primer 1F	Various
dNTPs	1µl	Go Taq	0.25µl	Primer 1R	Various
		cDNA	various	H_2O	Make up to 50µl

For each sample used, the quantities in table 2.3 were added. The amount of cDNA varied between 0.5-2.5µl depending on the sample concentration and the amount of primer varied between 1- 3µl (as shown in table A.2.1 in appendix 2). The amount of RNA-free water used depended on the quantities of cDNA and primers.

Once the PCR mix was made up in PCR tubes (Ambion, UK), 2 drops of mineral oil (to prevent evaporation) were placed into each PCR tube, the lids of the tubes were closed and the samples were agitated by flicking the tubes. The tubes were then placed into a Techne *flexigene* PCR machine (Jencons, Bedfordshire, UK). The heated lid was switched off.

An example of a PCR programme used was:-

For 30 cycles with annealing temperature of 60°C.

94°C	3 mins.	
94°C	1 mins.	Melting.
60°C	1 mins.	Annealing.
72°C	1 mins.20secs.	Extension.

End and hold at 4°C.

N.B. The reaction was then to be left in either the fridge or in the machine until the reactions were ready to be run on a gel.

2.2.9 PCR Visualisation

A DNA gel of 2% agarose was left to set, for 1hour, while PCRs were running. In this time a DNA ladder (Invitrogen, Paisley, UK) was prepared by mixing 1µl of DNA ladder with 2µl of Green Buffer (GO Taq kit) and then adding in 9µl of RNA-free water. A stock of DNA ladder was made up and stored in the freezer until required.

Once the PCR had run and the gel was set, 12µl of diluted DNA ladder and each sample were placed into separate wells in the gel. It was standard practice to load the NO RT samples first therefore reducing the risk of cross-contamination. The loaded gel was run for 5mins at 40V followed by 80V for 40 minutes using the Consort E122 Mini Power Pack (Jencons Plc, UK). The bands on the gel were visualised and

photographed using a UV transilluminator using a Gel Doc system (UVP BioDoc-It™ System, Jencons Plc, UK).

2.2.10 Normalisation of GAPDH

After the GAPDH primer was optimised as stated in 2.2.7 a sample at each time point was selected and a PCR was carried out at the optimum temperature and cycle number. The resulting PCR for each sample was run out on an agarose gel as described previously in 2.2.9. The image of the gel was analysed using Scion Image software (freeware). The image analysis allowed the intensities of the bands to be valued numerically. These values were then compared and the concentrations of the samples were adjusted accordingly. The PCRs were repeated with the new sample concentrations and the band intensities re-analysed. When the concentrations were found to be within 10% of each other and the band intensities were visually indistinguishable, the sample set was said to be normalised using the housekeeping gene GAPDH.

2.2.11 Analysis of Genes

Once the primers had been optimised in temperature and cycle number as detailed in 2.2.7, the primers for each genes were run with a normalised sample set (E6, 8, 10, 12, 14, 16 and positive control Whole E8 embryo). The resulting PCR samples were run out on an agarose gel and visualised as described in 2.2.9. The bands from the images of the PCRs were analysed using Scion Image software; a section of the background image intensity was measured and subtracted from a same size section, measuring the intensity, from the centre of the bands giving a numerical value that was then normalised to the GAPDH measurements produced from the same sample set. This was carried out in triplicate, using different sample sets, and values were placed into SPSS 11 software and statistical analysis was carried using one-way ANOVA with Tukey's post-hoc test out to identify any significant difference of RNA expression between the developmental stages.

2.3 Western Blotting

2.3.1 Protein Isolation

Table 2.4 Number of embryos required for each time point.

Embryonic day	E6	E8	E10	E12	E14	E16
No. Embryos	26	18	14	12	12	10
No. of lenses	52	36	28	24	24	20

When a suitable number of lenses (shown in table 2.4, above) had been collected for a protein sample they were placed in a glass Dounce homogeniser (Sigma, Dorset, UK) and the PBS was aspirated leaving the lenses. Immediately after PBS was removed between 500μl-1ml of an ice cold RIPA buffer mixture (depending on quantity of lenses) was added to the homogeniser. 1x RIPA (Upstate, USA) was made up before dissection by adding 4.5mls distilled water to 500μl (10x) RIPA, this was stored on ice. Just prior to adding RIPA to the lenses, 50μl Protease Inhibitor Cocktail (Sigma, UK) was added to the diluted, ice cold RIPA. The lenses were homogenised in the RIPA buffer and the sample was pipetted into an eppendorf and placed for 30minutes at 4°C on a Spiramix 5 rotating platform (Denley, UK). The sample was then centrifuged at 13,000 x g at 4°C for 30 minutes. The supernatant was removed in 20μl aliquots into fresh eppendorf tubes and stored at -20°C. The pellet was disposed of.

2.3.2 Subcellular Fractionation

Subcellular fractionation was carried out to distinguish the cellular localisation of distinct bands identified using the TRAF3 antibody. Pooled E16 lenses were placed in a cooled Dounce Homogeniser, removing PBS, as stated above. 500 μl ice cold fractionation buffer (composition appendix 1) was added to the lenses which were then homogenised lightly (10 to 12 strokes of the homogeniser). The homogenate was transferred to an eppendorf tube. A 40μl aliquot was taken and 40μl 2x Laemmli buffer was added to produce the Homogenate (Ho) fraction. A 10μl aliquot was also removed for protein quantification. These were labelled and frozen immediately. The remaining homogenate was then centrifuged at 1000 x g for 5 minutes at 4°C. The (post-nuclear)

supernatant was transferred to a new eppendorf tube and aliquots were taken as above i.e. a 10µl aliquot was removed for protein quantification and a 40µl aliquot was taken and and added to 40µl 2x Laemmli buffer to produce the post-nuclear (pn) fraction. They were labelled and frozen immediately.

The pellet that remained from the previous spin was resuspended in 500µl of fractionation buffer. The aliquots were taken as described as above, labelled nuclear (n) fraction and frozen.

The post-nuclear supernatant was centrifuged at 14000x g (or max speed) for 15 minutes at 4°C. The post-mitochondrial supernatant (cytosol) was transferred to a fresh eppendorf tube. Aliquots were again taken as above and labelled cytosol (c) faction then frozen. The final pellet was resuspended in 50µl fractionation buffer. The aliquots were taken as above and labelled- mt- this faction contained membranes, cytoskeleton and mitochondria.

The flow diagram in figure 2.4 clarifies this procedure:

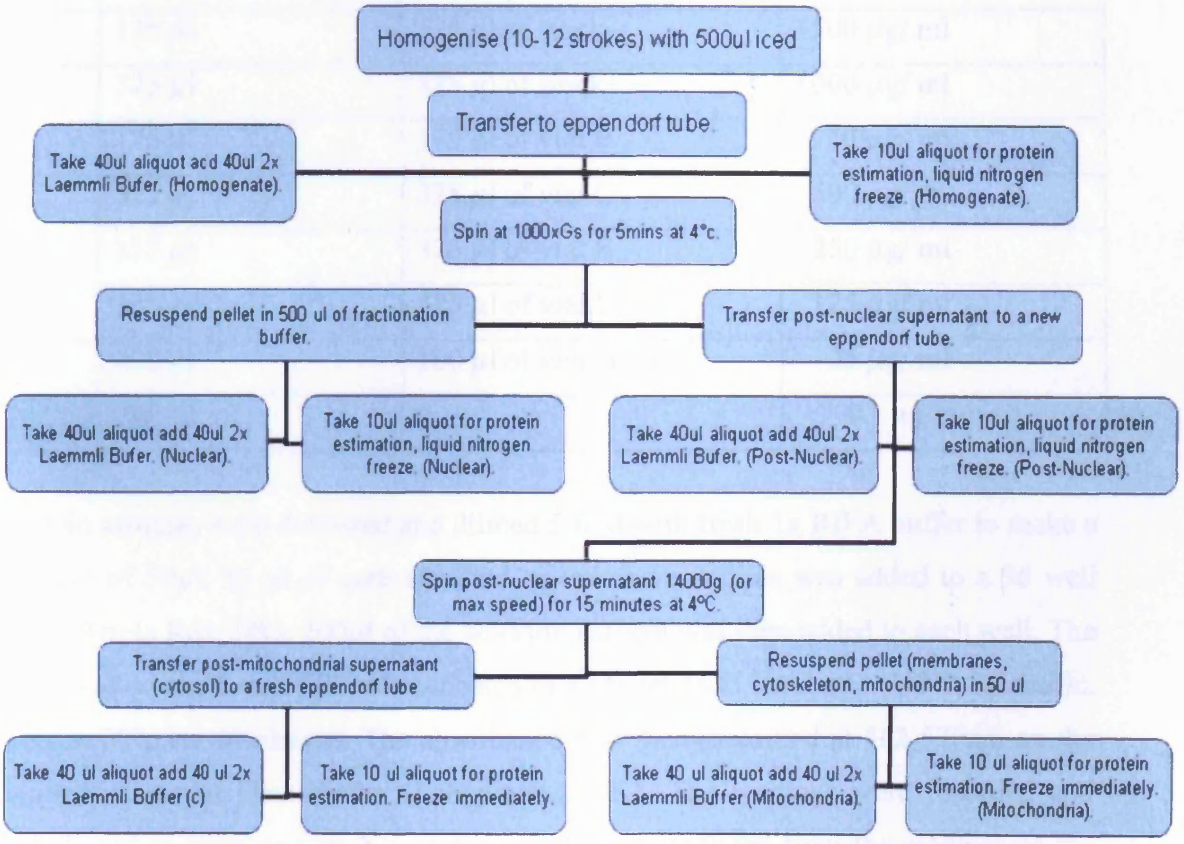


Figure 2.4 Flow diagram explaining the stages undertaken during subcellular fractionation.

2.3.3 Protein Quantification

Protein samples were quantified via the protocol obtained with the BCA (bicinchoninic acid) assay (Pierce, UK). BSA (bovine serum albumin) standards were produced in 1ml microcentrifuge tubes and labelled A to I. Various volumes of BSA stock and distilled water were mixed as shown in table 2.5, below, the final BSA concentrations were between 2000μg/ ml and 0μg/ ml. The quantity of working solution required was calculated by adding the number of samples to be quantified to the number of BSA standards, multiplying by 2 (duplication for accuracy) and then multiplying by 200 (μl). Once the amount of working solution needed was known this was made up using 50parts reagent A to 1part reagent B from the BCA assay.

Table 2.5 Volumes for BSA standards.

Vial	Vol. Diluents (H2O)	Vol. BSA	Final BSA conc.
A	0 μl	300 μl of stock	2000 μg/ ml
B	125 μl	375 μl of stock	1500 μg/ ml
C	325 μl	325 μl of stock	1000 μg/ ml
D	175 μl	175 μl of vial B	750 μg/ ml
E	325 μl	325 μl of vial C	500 μg/ ml
F	325 μl	325 μl of vial E	250 μg/ ml
G	325 μl	325 μl of vial F	125 μg/ ml
H	400 μl	100 μl of vial G	25 μg/ ml
I	400 μl	0	0 μg/ ml

Protein samples were defrosted and diluted 5 fold with fresh 1x RIPA buffer to make a volume of 50μl. 25 μl of each standard or unknown sample was added to a 96 well plate (Triple Red, UK). 200μl of the working reagent was then added to each well. The plate was covered with foil and incubated in a Model 1525 Incubator (VWR Scientific, UK) at 37°c for 30minutes. The absorbance was then measured at 562-570nm on the Multiskan ascent plate reader (Labsystems, UK). The readings were recorded and transferred to excel and an XY scatter graph was produced from the readings of the standards. A trend line and its equation were inserted into the graph. Rearranging the

formulae for the trend line allowed the calculation of the protein samples' concentrations.

2.3.4 SDS-Page

For SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) the kit used was the Mini-Protean®3 cell system (Bio-Rad, UK). Every piece of kit was cleaned with warm soapy water then sterilised with ethanol (Fisher Scientific, UK) before use. A set of glass plates were locked in to the casting frame, which was then put in its holder, distilled water was poured into the casting frame to ensure there were no leaks.

12% resolving gels were used for all proteins and was made up of 3.3mls Acrylamide-30% (Bio-Rad, UK), 4mls water, 2.5mls 1.5M Tris pH 8.8 (composition in appendix 1), 100µl of 10%SDS (Bio-Rad, UK), 100µl of 10%APS, (composition in appendix 1) and 20µl of TEMED (Bio-Rad, UK) which was added last and used in the fume hood (Bio-Rad, UK). The solution was lightly mixed and poured into the casting plates leaving 1.5cm at the top which was filled with distilled Water.

The resolving gel was left to set for 20 minutes and the stacking gel was made up using 1.67mls Acrylamide-30%, 5.83mls water, 2.5mls 0.5M Tris pH 6.6, 100µl of 10%SDS, 50µl of 10%APS, and 10µl of TEMED which again was added last and used in the fume hood. The water was removed from the casting plates and the stacking gel was then poured in, a casting comb was inserted between the casting plates to produce the sample wells while insuring no bubbles formed. The casting frame was covered with soaked tissue to keep the gels moist while they were left to set for 1 hour.

The casting plates containing the set gel was carefully removed form its frame, the comb was also removed. Gels, within their casting plates, were placed into the electrode assembly with the short plates facing towards each other. The electrode assembly was placed, securely into the clamping frame and placed into a mini-tank. 200mls running buffer (composition in appendix 1) was added to the mini tank and 125mls were added to fill the chamber formed by the gel plates. The protein samples (preparation described in 2.3.5) and a molecular weight marker (Bio-Rad, UK) were loaded into individual wells in the gel and the lid was placed on the mini tank. The gel was run for 30 minutes at 200 volts, 300mA using a Consort E122 Mini Power Pack.

2.3.5 Preparation of protein sample sets

Protein samples from E6 to E16 were all diluted to the same concentration (10μg/ 4μl) using the calculations from protein quantification, 2.3.3 the lysis buffer (1x RIPA) used to extract the protein initially was used to dilute the samples. 50μl of β-mercaptoethanol was added to 950μl sample loading buffer and the same volume of the sample loading buffer was added to each sample i.e. if loading 4μl protein sample, 4μl sample loading buffer was added. The proteins in loading buffer were boiled for 5 minutes on a Grant QBT2 heating block (Jencons Pls, UK) at 100°C under a fume hood.

2.3.6 Protein transfer

Once the SDS-Page was complete the gel was removed from its casting plates and placed in transfer buffer (composition in appendix 1) Filter paper, nitrocellulose membranes and fibre pads were soaked in transfer buffer and left on a Stuart platform rocker STR6 (Jencons Pls, UK), for 15minutes. A gel sandwich was then made up in a gel holder cassette consisting of the gel and transfer membrane in between two filter papers which were then sandwiched between two fibre pads. The sandwich was smoothed out using a tube hard enough to remove bubbles but gentle enough not to damage the gel. The cassette containing the gel sandwich was placed in a mini tank, on magnetic cooling unit, with a magnetic stirrer and 600mls transfer buffer. The lid was placed on the tank and the transfer was carried out using a Consort E122 Mini Power Pack at 100V, 350mA for 1 hour. After completion the sandwich was unpacked and the membrane, now containing the transferred protein samples, was placed in 1x TBS/Tween (composition in appendix 1) in the fridge until use (maximum 2 days). At all times, throughout this protocol it was ensured that the membrane was never allowed to dry out and the 1x TBS/Tween was maintained at 4°C by replacing in the fridge between washes. Before blocking the membrane the 1x TBS/Tween was poured away and the protein samples were visualised using 20mls Ponceau S (Sigma, UK). The membrane was left, in Ponceau S, until staining developed (5-10minutes) and was then photographed using the white light source on the Gel Doc system to confirm equal

loading of protein samples. The membrane was replaced on the rocker and washed multiple times (3minutes each) with 1x TBS/Tween until all staining had been removed.

2.3.7 Membrane blocking

Blocking solution was made up using 5% milk (Fluka, UK) in 1x TBS/Tween (5g in 100mls), 20mls was added to the membrane once the 1x TBS/Tween had been poured away. The membrane, in solution, was left on a rocker for 1 hour at room temperature. The blocking solution was then disposed of and the membrane was washed 6 x 5 minutes in 1x TBS/Tween.

2.3.8 Primary antibody incubation

After optimisation of the antibodies (which was carried out by using various dilutions, incubation times and temperatures for each antibody) the antibody was diluted appropriately in 1% milk (0.2g in 20mls 1x TBS/Tween). The 1x TBS/Tween was poured off the membrane and the antibody solution, 20mls, was added to the membrane and left on the rocker for an hour at room temperature. For smaller dilutions, membrane and antibody solution, 5mls, was placed in a 40ml Falcon tube (BD Biosciences, UK) and placed on rotating platform therefore reducing the amount of antibody required. The antibody solution was removed and stored in the refrigerator for up to 2 days as each antibody solution could be used twice. The membrane was washed 6 x 5 minutes in 1x TBS/Tween.

2.3.9 Secondary antibody incubation

Secondary antibody solution was made up in 1% milk (as above). 20ml of secondary antibody solution was added to the membrane and left on the rocker for an hour at room temperature. Again, the antibody solution was removed, stored and the membrane was washed 6 x for 5 minutes in 1x TBS/Tween.

2.3.10 Developing films from the membranes

The membrane was placed into a plastic wallet in a developing Hypercasstte™ (Amersham, UK) removing excess liquid from around the membrane using small pieces of blue roll (Newhall Janitorial, UK). In a dark room, ECL (enhanced chemiluminescence reagents; ECL Plus Western Blotting Detection Reagents, Amersham, UK) were made up – 50parts reagent A to 1 part reagent B. 1ml of ECL solution was added to the membrane, allowed to incubate for 3 minutes and excess solution around the membrane was removed. The membrane was then covered by the plastic wallet, removing all air bubbles. A Hyperfilm™ (Amersham, UK) was placed on top of the plastic wallet and the Hypercassette™ was sealed for either 2, 5, or 10 minutes. The film was then removed form the cassette and put into a developing solution (Photosol Ltd, UK) for 3minutes followed by a fixing solution (Photosol Ltd, UK) for 3 minutes. The film was then rinsed in water and dried using a RCD3200 print dryer (Durst, Germany).

2.3.11 Stripping membrane

Once the initial western blot was completed the membrane was re-probed with an Actin antibody (I-19, Santa Cruz, USA). To do this the membrane was first stripped by washing the membrane for 2 x 10minutes with a medium strength stripping buffer (composition appendix 1). The stripping buffer was poured away and the membrane was washed twice with 1x PBS for 10 minutes each.

2.3.12 Re-incubation with Actin antibody

After stripping and washing with 1x PBS the membrane was washed with 1x TBS/Tween. The methods in 2.4.7 to 2.4.10 were then repeated using the actin antibody to confirm concentration of samples was equal.

2.3.13 Western Blotting Analysis

Films containing the results from the western blots (carried out in triplicate) were scanned into the computer using an Epson Expression 1680 Pro (Office Direct, UK). The images were saved using the software obtained with the scanner and then opened in the software Labworks™ (Media Cybernetics, UK). Densitometry analysis was then carried out by obtaining numerical values for the intensities of each of the bands observed. The results were then imported into Microsoft® Excel (Microsoft, USA) and the intensities of the experimental bands were normalised to the band intensities obtained in the corresponding reprobe using Actin. The mean band intensities (normalised) for each protein at each time point were calculated along with the standard error. Statistical analysis was then carried out to identify any significant difference of protein expression between developmental stages using one-way ANOVA with Tukey's post-hoc test.

2.4 Histology

2.4.1 Preparing tissue for histology

The embryo heads were collected and placed in 1xPBS as stated in 2.1.2 and were then only dissected further only if they were over E8 (H&H 34). E6 (H&H 29) heads were embedded whole. At E8 or over the eyes were removed from the surrounding tissue. The posterior half of the eye was then removed using bow sprung dissecting scissors, therefore allowing easier penetration of the fixative and wax into the lens. The tissue from this point on was treated the same no matter what the developmental stage. Once the tissue was dissected as necessary it was fixed in 4% PFA (Paraformaldehyde purchased from Sigma, UK), made as described in appendix 1, and stored in the fridge overnight. The 4% PFA was removed (taking care to put the PFA into a glass storage bottle marked for chemical disposal) and the tissue was washed 3x 30minutes in 1x PBS. The tissue was then placed in 50% ethanol (Sigma, UK) and stored overnight. To continue dehydration, the tissue was washed in fresh 50% ethanol for 30 minutes and

then 2x 30 minutes each of 70, 90 and100% ethanol. The tissue was then transferred to small glass containers and washed 2x 30 minutes in 50:50 ethanol: xylene followed by 2x 1hour washes in xylene (Sigma, UK). The tissue was then placed in molten wax (RALamb, UK) and stored in the oven for 30 minutes before being transferred to fresh glass containers of molten wax and soaking overnight in a 60°C oven (Weiss Gallenkamp, UK).

2.4.2 Embedding tissue

Following incubation overnight in molten wax the tissue samples were removed individually using tweezers and placed into a mould tray (Disposamould, RALamb, UK) full of molten wax. Once the tissue was orientated as shown in image 2.5, below, a paper tab was added to the wax giving details of the age of tissue and date of collection, the tab was placed on the side of the wax block the lens was facing and fixed in place with an embedding ring (RALamb, UK). The tray was placed on a cold plate (RALamb, UK) and the wax was allowed to set. After ~30 minutes the mould trays containing tissue were placed overnight in the refrigerator allowing them to completely harden. The wax blocks were then removed from the mould trays and stored at room temperature until use.

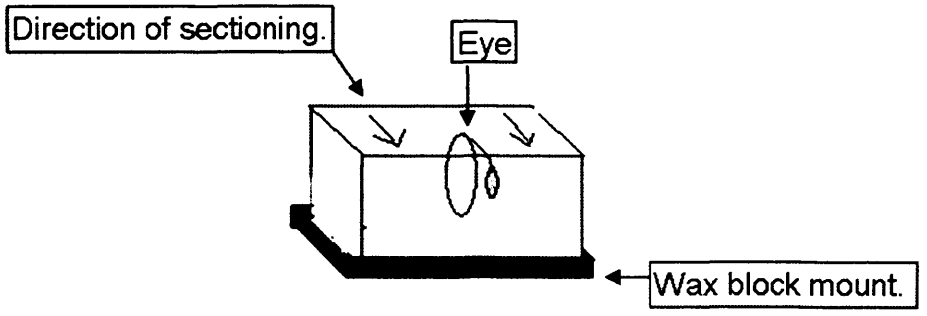


Figure 2.5 Diagram of the orientation of the tissue within a wax block.

2.4.3 Sectioning tissue

Excess wax was removed from wax block using a Gem Razor Blade (LabX, USA) leaving around 0.5cm of wax surrounding the tissue. The wax block was then positioned on the microtome (HM325, Microm, Germany) and thick sections (~30μm)

of wax were removed from the top of the block, until tissue was revealed, using a microtome blade (Nakura systems, Japan). Just before sectioning the lens, the thickness of the sections was reduced to 7μm. Sections containing lens tissue were then placed on cold water to ensure there were no air bubbles before being transferred via slide to a warm water bath (RALamb, UK), containing a solution of 20mls Mayers Albumen (Histolab, Gothenburg) in boiled water, where any folds or waves in the wax would soften away. Once the wax sections had warmed they were lifted from the water bath using a clean Histobond® slide (RALamb,UK) ensuring that the sections were flat and that there were no air bubbles. Excess water/solution was removed from the slide using soft tissues and the slide was placed on a Photax dishwarmer 2 (Photax, UK) at 40°C for a few minutes evaporating the water. Once the slides were dry notations were made on the slide stating the age of lens, date, slide number and thickness of section. The slides were stored in a slide container until use.

2.4.4 Selecting sections/ slides for staining

Slides were chosen for each developmental stage. These were judged by eye under a Olympus BH-2 light microscope. If the lens was in the right orientation when fixed, the sections at each stage with the largest lens gave a cross-section through the centre of the lens showing the equatorial region and the OFZ (if formed).

2.4.5 Haematoxylin and Eosin Staining

Haematoxylin and eosin stains were used to highlight lens morphology. The selected slides were placed in slide holders and dipped in 2x 5minutes xylene, then decreasing concentrations of IMS (Industrial Methylated Spirit purchased from Fisher Scientific, UK) 2x 5minutes 100% followed by 2 minutes 90%, 70, 50% IMS followed by 2 minutes in water. The slides were placed in Haematoxylin (RALamb, UK) for 3 minutes then placed in a bowl of gently running tap water until the water ran clear (~4minutes) the slides were then placed for 2 minutes in Eosin (RALamb, UK) before again being washed in a bowl of gently running tap water. Once the water ran clear the sections were dehydrated using fresh containers of IMS at the above concentrations for the same lengths of time but in reverse order, finishing in fresh containers of xylene.

The xylene containing the slides was transferred to a mounting hood where all mounting was carried out. Slides were removed from the xylene containers one at a time and the excess xylene was removed with tissue. Depending on the number of sections on a slide, between 1-3 drops of DPX (RALamb, UK) was placed onto a coverslip (Fisher Scientific, UK). A slide was then gently placed on top of the coverslip, at an angle therefore producing fewer air bubbles. The slide and cover slip were immediately turned over and any remaining air bubbles are teased out by applying gentle pressure to the coverslip. The slides were then left under the mounting hood overnight before being viewed under the Leica DMRA2 microscope (Leica, UK). Images were recorded using the Leica software.

2.4.6 Peroxidase staining

The wax clearing, hydration, dehydration and mounting stages are the same as stated in 2.4.5., above.

Before wax clearing and hydration, 15mls of Antigen Unmasking Solution (Vector Labs, Peterborough, UK) was added to 1600mls of distilled water and placed in a pressure cooker and heated on a heating block until it boiled. Once the solution was heating, selected slides were cleared of wax and hydrated as above. The slides were then transferred to a metal holder and stored in distilled water (ensuring the slides did not dry out at any point) until the Antigen Unmasking Solution was boiling. Once boiling the slides were transferred to the solution and, once pressurised, timed for 1 minute. After the cooker cooled the slides were stored in distilled water until they were individually removed, excess water dried from the slide and using a PAP pen (a hydrophobic marker pen, Vector Lab, UK) a circle was drawn around the sections on the slide. The slide was then placed in a moisture chamber and the area circled on the slide was filled with PBS (again ensuring the sections never dried out). This area was then filled with a quench solution, 1:1:8 parts Methanol: Hydrogen Peroxide (Fisher Scientific, UK): distilled Water, for 5 minutes. The slides were then washed 3x 5minutes in 1x PBS before blocking with Goat Serum (Vectorstain ABC elite Kit, Vector Lab, UK), 150µl in 10mls PBS for 1hour. Excess goat serum was removed from the slides before incubation with the Primary Antibody. After optimisation it was

found that all antibodies worked well at room temperature, incubating for 4hours at concentration of: primary antibody: 1x PBS, TRAIL 1:300, TRAF2 1:250 and TRAF3 1:350, all antibodies were purchased from Santa Cruz Biotechnology Inc, USA. After 4 hours the primary antibody was removed by washing for 3x 5minutes in 1x PBS. Again, after optimisation it was found that the secondary antibody worked best for TRAIL at 1:750, TRAF2 at 1:1000 and TRAF3 1:750. The sections were incubated with secondary antibody at room temperature for 1hour before being washed for 3x 5minutes in 1x PBS. Vectrastain Elite ABC Reagent (Vector Lab, UK) was then added to slides for 30 minutes before the sections were again washed for 3x 5minutes in 1x PBS. Very intense purple (VIP) peroxidase (Vector Lab, UK), composition in appendix 1, was added until the appropriate intensity of staining developed (~2minutes). The sections were dehydrated, dried, mounted and images were taken as in 2.4.5.

2.4.7 Immunofluorescence

The wax clearing, hydration, antigen retrieval and slide preparation (PAP pen) were carried out as stated in 2.4.5. Sections were then incubated for 1 hour in 1.5% Goat Serum (Vector, UK) in 1x PBS containing 0.2 % Tween20 (Sigma, UK). Excess serum was removed from the slides before the primary antibody was added (diluted in 1x PBS containing 0.5% goat serum and 0.05% Tween20). The primary antibody was left overnight at 4°C before being washed with 1x PBS for 3 x 5 minutes. The sections were then incubated with 1:1000 Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, UK) diluted in 1x PBS (containing 0.5% goat serum and 0.05% Tween20) and 3ul/ml of Hoescht 33342 (Invitrogen, UK) was also added. The sections were incubated with the solution for 1-2 hours in the dark, before being washed with 1xPBS 3x 5minutes and were then mounted using Vectrashield (Vector, UK). The slides were then left under the mounting hood overnight (in the dark) before being viewed under the Leica DM6000B microscope (Leica, UK). Images were recorded using the Leica AF6000 software.

2.5 Organ Culture

2.5.1 Preparing Medium

Medium for organ culture was prepared under sterile conditions in a Hera Safe D3505cell culture hood (Thermo Electro Corporation, UK). All solutions used to make the medium were passed through a 0.2μm filter (Triple Red, UK) before addition. Two solutions of medium were prepared, one with glucose (Sigma, UK) and one without. The 199 Medium (Gibco, UK) was placed in a water bath (Jencons Pls, UK) at 37°C until warmed. The solution for Medium with glucose was 106.13mls Medium 199, 670ul Glucose, 12mls FCS (10% total vol.) (Sigma, UK), 1.2 mls (1 in100 dilution) solution of penicillin (Penna-100mu, Sigma, UK) and streptomycin (s-6501, Sigma, UK) (composition of solution in appendix 1.xxii).The solution for Medium without glucose was 106.8mls Medium 199, 12mls FCS and 1.2 mls pen-strep. When putting medium onto lenses the medium was warmed in the water bath before use. After use the medium was sealed before being stored in a refrigerator.

2.5.2 Preparing Tissue

Whole eyes at E10 were removed as stated in 2.1.2 (being careful not to damage the eye) and they were placed in sterile PBS on ice. The eyes were then transferred to a sterile Petri dish containing a 1: 8 solution of Betadine (Seton Healthcare Group, UK) in distilled water. The Petri dish was sterilised with ethanol and placed under the cell culture hood. Within 2-5 minutes the eyes were transferred into a sterile 50ml pot of sterile 1x PBS this was repeated twice to remove the betadine. Individually the eyes were removed to a sterile Petri dish of 1x PBS and the lens was dissected as required. Three different dissections were carried out, while maintaining the sterile environment. Lenses were either removed with the iris and vitreous attached, with only the vitreous attached or the lens was dissected without any of its surrounding tissue. Once dissected using sterile implements the tissues were placed in either medium with or without glucose. Once all dissection had been completed the tissue/ organ was placed in individual wells of a culture plate containing 1.5mls of either medium with or without

glucose. The cultures were then transferred to a Biohit incubator (Wolf Labs, UK) at 37°C and with 0.5% CO2.

2.5.3 Assessment of tissue quality

After incubation for 4 hours, cultures were checked under a microscope for opacifications which would indicate that the lens had been damaged during dissection. If opacifications were observed then the lens was disposed of. The cultures were then returned to the incubator.

2.5.4 Tissue collection

Every 2 days, for a total 8 days, 3 replicates of each of the 6 different cultures (with or without glucose in the medium, with iris and vitreous, with vitreous or lens alone) were taken from culture. The remaining cultures were placed in fresh, sterile and warmed mediums and replaced in the incubator. The lenses collected were photographed under dark field using a Leica MZ10F microscope (Leica, UK).

2.5.5 Analysis of cultured lens opacities

The images of the cultured lenses were then placed in Scion Image software and the opacity of the lens measured. This was done by measuring the intensity of the lens image in three places; the centre, the periphery and the centre of the radius of the lens. The background reading of the image was subtracted from the average of the above three readings to give an idea of the overall opacity within the lens. The final amounts were then converted into a reading between 0 and 5, where 0 was no opacity going up to five where there was the most extensive opacification.

2.5.6 Fixing cultures

After photography the lenses were immediately placed in 4% PFA overnight and then transferred to fresh tubes containing 1x PBS, after 30mins the lenses were placed in

eosin for 10-15 seconds (very light stain) to allow the lenses to be orientated and embedded correctly. The lenses were then washed 3x 30mins in 1x PBS before dehydration, embedding, sectioning and selection of tissue was carried out as stated in sections 2.4.4.

2.5.7 Haematoxylin and Eosin Staining

To characterise the morphology of the lens cultures haematoxylin and eosin staining was carried out as stated in 2.4.5. Images of the sections were taken using the Leica DMRA2 microscope (Leica, UK). Images were recorded using the Leica software and the sections photographed from the centre of the lens (the largest lens sections) were then used to take measurements of the lens using using Image-Po Plus (Media Cybernetics, UK). The length and height of the whole lens was taken and where appropriate (after the development of the OFZ at E12) the size of the OFZ was measured as well as the distance of the OFZ from the anterior and posterior of the lens was taken to measure any variations in the development of the lens in culture. A diagram showing the measurements taken can be seen in Chapter 5, figure 5.2.

Chapter 3:
TNF family member expression in developing Chick Lens

3.1 Introduction

In 1998, Wride and Sanders characterised the expression of TNFα and its receptors TNFR1 and TNFR2 in the developing chick lens (Wride & Sanders, 1998). Since then, no research has been carried out to identify the TNFs and associated family members found in the chick (*Gallus gallus*) lens. Primers for all TRAFs and TRAIL were designed as stated in the general methods section 2.2.6. Briefly, the sequence of selected genes identified in *Gallus gallus* were placed in the primer3 online program where primers can be designed from published DNA sequences.

Embryonic stages (on even days) from E6 to E16 were used to examine the gene expression of TRAIL and TRAFs in the lens. From previous studies, it is known that organelle degradation, accompanying fibre cell differentiation, begins at around E12, though mitochondrial fragments have been noted as early as E8 (Bassnett & Beebe, 1992). Also at E8, nuclei in cells at the centre of the lens condense. At E10, mitochondria are noticeably enlarged and, from E12, both nuclei and mitochondria are absent from the cells (Bassnett & Beebe, 1992). The ER has been shown to be removed at around the same time as nuclei (around E12) but the Golgi apparatus fragments much earlier, when the cells begin to differentiate at the equatorial region (Bassnett, 1995). As these organelles degrade and the OFZ forms, it has also been found that transcription ceases, forming an "RNA-depleted zone", first identified at E13 by Faulkner-Jones *et al.* (2003).

The development of the OFZ is a key part of lens development and without this region light is scattered as it enters the lens, inhibiting the progress of the light onto the retina. The organelle degradation, which allows light to pass through the lens, was suggested to be similar to an attenuated from of apoptosis (Dahm, 1999), but it has also been published that the ubiquitin-proteasome pathway is required for lens proliferation and differentiation (Shang *et al.*, 1999). There is recent data showing relationships between TNF family members and ubiquitination (Wertz & Dixit, 2008). Though the molecular pathways involved in lens development/ differentiation have not been elucidated, it is shown here that TNFs and related family members, known to be involved in apoptosis signalling pathways, have been identified in the developing chicken lens.

The aim of this chapter is to determine whether TRAIL and the TRAFs are present in the developing chick lens using semi-quantitative RT-PCR.

3.2 Experimental design

The experimental design has been discussed in greater detail in subchapter 2.2. Briefly, RNA was collected from lenses of chick embryos on even days of development from E6 to E16. The RNA samples were purified using SV Total RNA Isolation system resulting in the removal of genomic DNA. cDNA was then synthesised from the RNA using Superscript™ II Reverse Transcriptase kit (Invitrogen, Paisley, UK). The RT and no-RT samples produced using the kit were used in semi-quantitative PCR reactions using the primers designed as stated in section 2.2.6. The primers were resuspended in volumes of sterile TE buffer. Once resuspended, the primers were run on an agarose gel to check their concentrations. When visualised, the primers were diluted as necessary and rerun on a gel to confirm that they were of equal concentration.

Each set of samples were initially normalised using the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This housekeeping gene is an evolutionarily conserved glycolytic enzyme, expressed in all organisms and most cells. It is involved in glycolysis and also in DNA base excision repair. It has a low variability in expression, which makes it a better housekeeping gene than actin (Hirono *et al.*, 2000). After normalisation, sample sets were then used in experimental PCRs. PCRs were replicated three times with different cDNA samples collected from separately obtained sets of pooled lenses. The products of the PCR reactions were visualised on an agarose gel and the intensities of the bands were quantified using Scion image software (Freeware). The three replicates gave confirmation of gene expression for the genes examined and also the variation involved in the PCRs. This approach accounted for both biological and experimental variation.

3.3 Integrity of RNA samples

Once lenses were pooled and RNA isolated, the integrity of the sample was examined by running an aliquot of the sample on a formaldehyde denaturing gel as described in the general methods 2.2.3. If RNA was intact, the RNA produced two distinct bands on the denaturing gel. The higher band, the 28S ribosomal band should be brighter by a ratio of 2:1 to the lower 18S ribosomal band. Examples of RNA samples obtained are shown in figure 3.1. Any samples showing a smear were discarded as they were degraded.

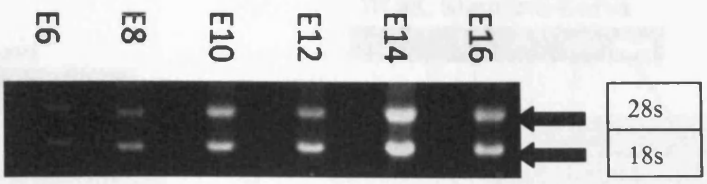


Figure 3.1 Image of RNA denaturing gel.

This RNA was collected on alternate days for six days and stored short-term at -20°C. The longest length of storage for RNA was 8 days before DNase digestion was carried out this was followed by synthesis of cDNA and the reverse transcriptase (RT) reaction, using the Superscript II kit. Though the concentration of RNA was calculated (as described in 2.2.3), variations in the RT step efficiency lead to inaccuracies that make true quantification impossible using this method. Semi quantitative RT-PCR will only produce information on the relative changes in gene expression, when levels of expression are normalised with respect to a housekeeping gene, such as GAPDH.

3.4 PCR primer optimisation

Before gene expression could be quantified via RT-PCR, the primers used were optimised by finding the most favourable conditions, including concentration of $MgCl_2$ (which influences the productivity and fidelity of the polymerase); this was done by carrying out a titration of 2, 4, 6 and 8µl of 25mM $MgCl_2$ (results not shown). The optimum annealing temperature was also established by trying various temperatures between 55 and 68°C (results not shown). An E12 sample was selected to plot annealing temperatures and also to obtain the most suitable cycle number. To insure that the expression levels found using RT-PCR were of quantifiable intensity an increasing number of PCR cycles was used to produce a standard curve.

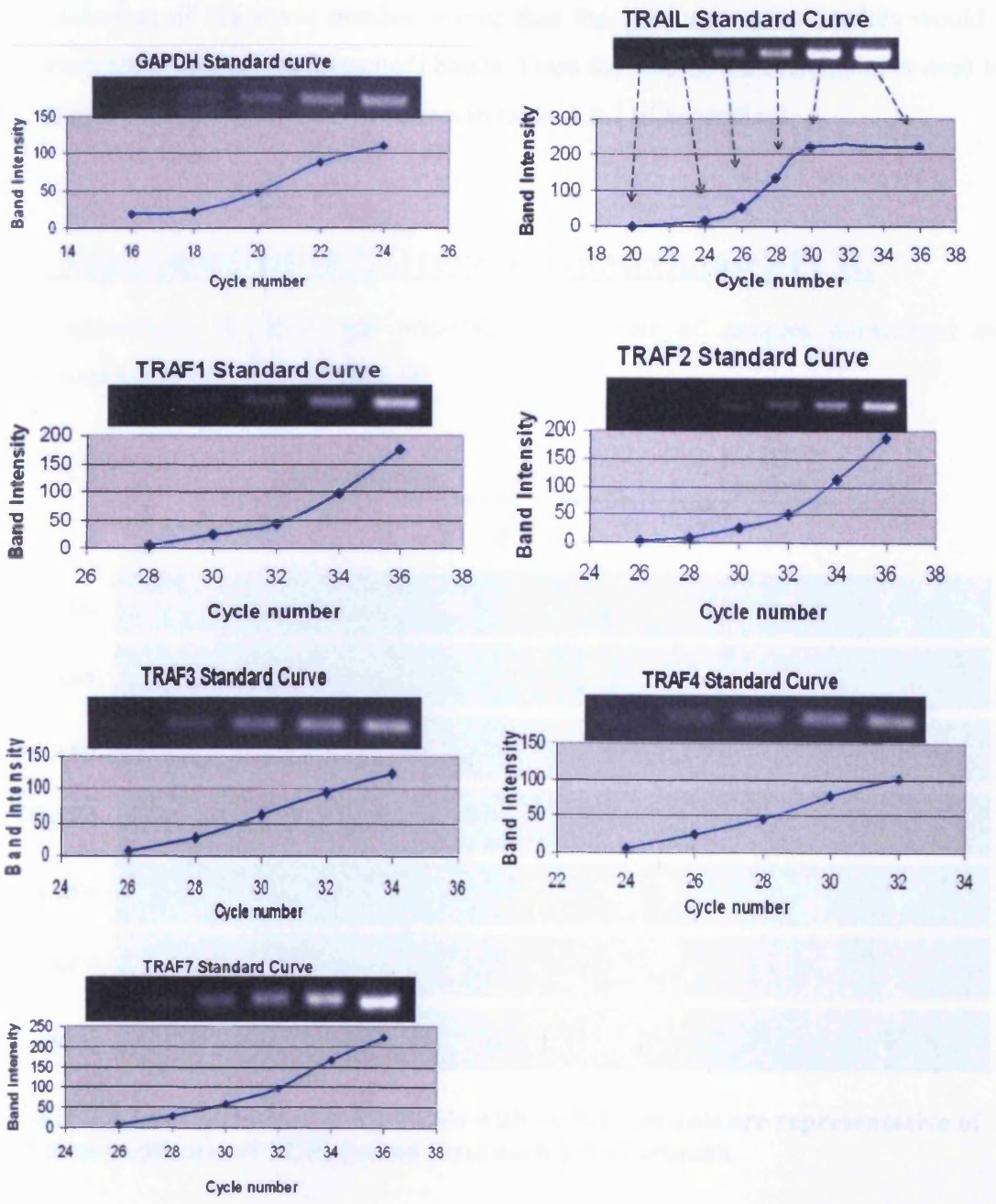


Figure 3.2 Shows PCR standard curves for selected genes. In the TRAIL standard curve the arrows from the bands point to their corresponding points in the graph.

Standard curves (shown in figure 3.2) were completed for all genes, shown in figure 3.3, below. The PCR reactions were carried out at different cycle numbers to produce the standard curves shown above each graph. The visualised PCRs were then quantified using Scion Image and a graph of the resulting band intensities plotted. The PCR cycle number used in all subsequent reactions was selected from the lower region of the exponential phase of the curve.

The selection of the cycle number meant that the gene expression studies would not be incorrect from over-bright (bleached) bands. From the results, the cycle number used for each set of primers was selected and is shown in table A.2.1 of appendix 2.

3.5 Semi-quantitative RT(reverse transcriptase)-PCR.

Semi-quantitative RT-PCR was undertaken with sets of samples normalised as described above and in chapter 2.2.10.

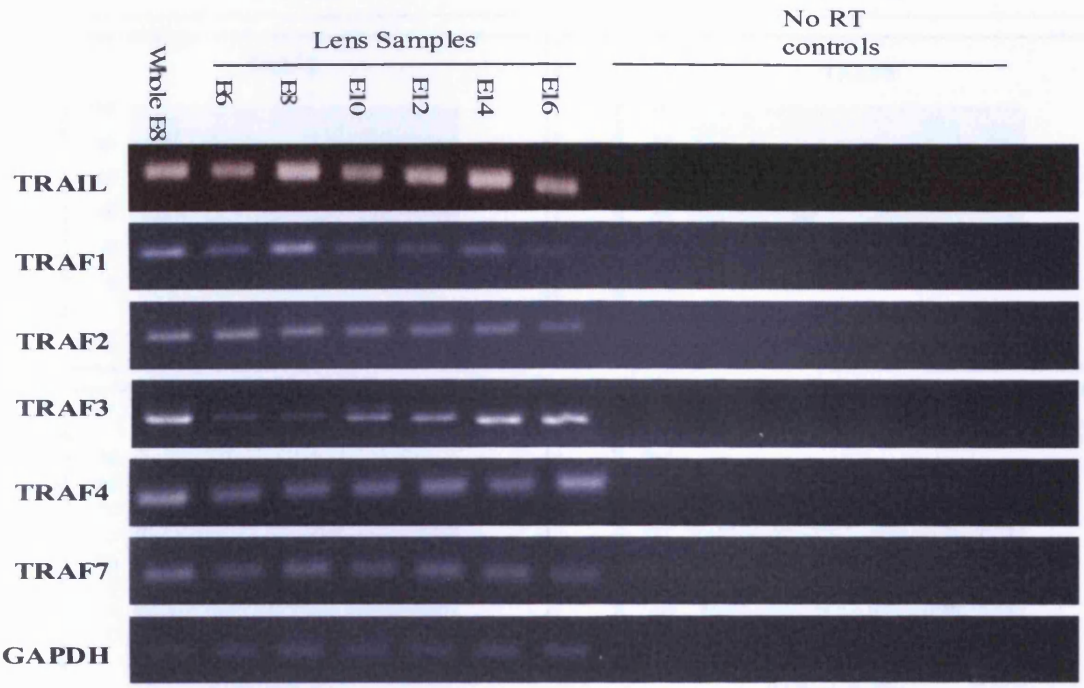


Figure 3.3 Semi-quantitative RT-PCRs with no RT controls are representative of the three replicates of PCRs gained form each set of primers.

3.6 Analysis of Semi-quantitative RT-PCR.

The analysis of the differential expression of genes was undertaken using the housekeeping normalisation method stated in section 2.2.10. Briefly, in Scion Image, the background was measured and subtracted from the band intensities measured. The band intensities were then normalised with respect to GAPDH. The mean and the

standard error of the mean for the three replicates for each of the PCRs were calculated and graphs are shown in figure 3.3. The densitometry table for these results is shown in table A.2.2, appendix 2.

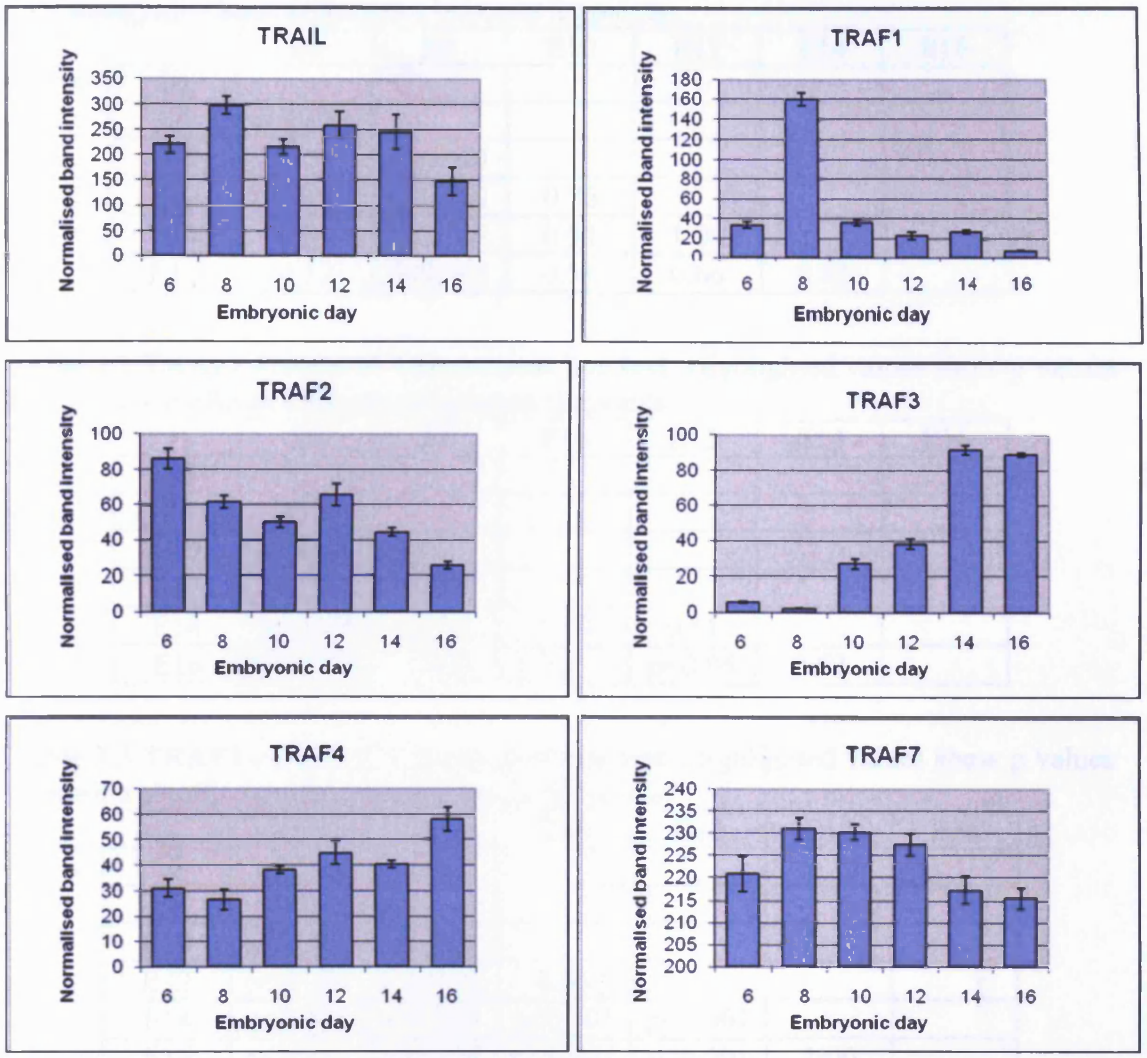


Figure 3.4 Graphical representation of RT-PCR results obtained. These graphs show the results of the mean from PCR reactions carried out in triplicate. The error bars shown represent the standard error of the mean.

3.7 Statistical analysis of RT-PCR

Table 3.1 TRAF1 results of Tukeys post hoc test. Highlighted values show p values indicating significant differences between timpoints.

	E6	E8	E10	E12	E14	E16
E6						
E8	p<0.001					
E10	1.00	p<0.001				
E12	0.89	p<0.001	0.76			
E14	0.99	p<0.001	0.94	1.00		
E16	0.17	p<0.001	0.11	0.66	0.42	

Table 3.2 TRAF2 results of Tukeys post hoc test. Highlighted values show p values indicating significant differences between timpoints.

	E6	E8	E10	E12	E14	E16
E6						
E8	0.38					
E10	0.09	0.91				
E12	0.56	1.00	0.76			
E14	p=0.04	0.70	1.00	0.51		
E16	p<0.001	0.09	0.37	p=0.05	0.62	

Table 3.3 TRAF3 results of Tukeys post hoc test. Highlighted values show p values indicating significant differences between timpoints.

	E6	E8	E10	E12	E14	E16
E6						
E8	0.99					
E10	p=0.02	p=0.01				
E12	p<0.001	p<0.001	0.36			
E14	p<0.001	p<0.001	p<0.001	p<0.001		
E16	p<0.001	p<0.001	p<0.001	p<0.001	1.00	

Once normalised to GAPDH, statistical analysis was carried out on the PCR results. One-way ANOVA with Tukey's post-hoc test was used to detect any significant changes in expression between the developmental timepoints used. All of the analysis can be found in appendix 2 and from this it was found that TRAF1 had significantly increased expression at E8 that was significantly different to any other time point ($p< 0.001$). TRAF2 expression at E6 was statistically higher than that at E14 and E16 ($p=0.04$ and $p< 0.001$ respectively). TRAF2 expression at E12 was also shown to be statistically higher than at E16 with a p value of 0.05. TRAF3 expression at E6 and E8

were not significantly different from each another; this was the same for the pair E10 and E12 and also pair E14 and E16. There was a significant difference of expression found between these sets increasing as development continued as shown in table 3.3, above. TRAIL, TRAF4 and TRAF7 were found not to have any statistically significant difference in expression.

3.8 Discussion

The semi-quantitative PCRs confirmed the expression of the genes shown in figure 3.3. Of those genes shown to be differentially expressed using semi-quantitative RT-PCR, TRAF1 was shown to have significantly increased expression at E8. At this stage in development, the earliest signs of organelle degradation can be seen such as mitochondrial debris.

TRAF2 had significantly decreased expression at E14 and E16 compared to at E6, it is possible that this suggests a role for TRAF2 in the maintanence of the cells, specifically in the maintenance of the organelles. It is known the deficiences in TRAF2 expression lead to an increase in apoptosis (shown in knockout mice) and in lymphocytes TRAF2 is involved in anti-apoptotic signals (Lee *et al.*, 1997). It can be suggested that the decrease in TRAF2 expression in the developing lens may indicate that survival/ maintenance signals are reducing allowing the signalling for the degradation of the organelles, which is known to be evident in the mitochondria by E8, to become active.

TRAF3 had statistically increasing expression at E10, which then increased again at E14. The continued increase in expression of this gene around the development of the OFZ could suggest a role in maintenance of the fibre cells once the OFZ begins to form. The increase in expression corresponds to the start and increase in size of the OFZ. The knowledge of the role of TRAF3 is limited though it is thought to be involved in B cell development and survival (Xu *et al.*, 1996) which suggests that the role of TRAF3 proposed here is plausible.

The developmental time points used were chosen to show genes expressed around the stage in lens development where organelle degradation is initiated and the genes above were found to be differentially expressed in the lens at these

developmental time points. However, these results do not confirm that they have significant roles in formation of the OFZ and the possible roles suggested above are hypothetical. These genes could be found in any or all regions of the lens and there is the possibility of their involvement in numerous other processes such as maintenance or remodelling of cell membranes at the equatorial region of the lens where epithelial cells begin to differentiate, proliferation or many other aspects of lens development. Semi-quantitative RT-PCR shows the expression of the genes at the developmental timepoints examined, but does not confirm the region of expression within the lens nor can it confirm that these genes are translated into proteins, which carry out the required functions within the cell. Protein studies including western blotting and immunohistochemistry, carried out in the next chapter for a selection of genes, will give insight into whether the corresponding proteins are expressed during the developmental timepoints studies and also supply information on the spatiotemporal pattern of expression and nature of the sub-cellular localisation of the proteins, further elucidating any potential roles for the genes.

All genes in the table shown in appendix 2, table A2.1, were expressed in the chick lens during development. Due to time, financial constraints and experimental problems, such as multiple bands (TRAF5) and degradation of the primers (TRAF6), not all PCRs were completed at all time points examined or in triplicate. Therefore these results were presented in appendix 2, table A.2.23. Future experiments may find statistically significant expression in these genes and other TNF-related factors not yet shown to be present in the lens during development.

Chapter 4:
TNF-related factors in the Developing Chick Lens: Spatio-temporally Regulated Expression of TRAF2, TRAF3 and TRAIL proteins

4.1 Introduction

Following the semi-quantitative RT-PCR in the previous chapter, TNF-related genes with interesting, statistically significant, differential expression were chosen for further study at the protein level.

TRAF2 is described in detail in section 1.12.2. Briefly, TRAF2 is one of the most highly studied members of the TRAF family and is important in many TNFR signalling pathways initiating downstream signalling that can activate transcription factors from the NF-κB family and either JNK or p38 though MAP kinase cascades (Song *et al.*, 1997). TRAF2 is known to initiate anti-apoptotic signals when the TNF pathway is activated and it is thought that the pathway is independent from NF-κB, though this pathway and the NF-κB anti-apoptosis pathway working in synergy are much more effective (Lee *et al.*, 1998).

TRAF3 (described in section 1.12.3) was first identified by its ability to bind to the cytoplasmic tale of TNFR and for many years its functions were unclear. Since the turn of the century, it has been found that TRAF3 negatively regulates the NF-κB non-canonical pathway (Liao *et al.*, 2004), though TRAF3 is known to have a number of splice variants that can induce NF-κB activation (van Eyndhoven *et al.*, 1999b). TRAFs are also required in the production of type 1 interferon via a number of the viral recognition pathways (Saha *et al.*, 2006).

TRAIL/ Apo2L is a type II transmembrane protein that initiates apoptosis when it binds to its active, non-soluble death receptors (DR4 and DR5). The death domain on these receptors then engages the cytoplasmic protein FADD (Fas associated death domain) and caspase-8, which forms the DISC (death inducing signalling complex) (Kamradt *et al.*, 2005). Caspase 8 proteolyses and then activates other caspases, which are termed effectors, such as caspase 3, 6 or 7. TRAIL's selective induction of apoptosis in a wide range of cancer cells (which most normal adult cells can resist) has made it popular in anticancer therapy research (Sayers & Murphy, 2006). Though TRAIL was not found to have differential expression during the stages of lens development studied during PCR, research by (Jordan *et al.*, 2001) highlighted TRAIL expression in cultured human lens epithelial cells (hLECs) with possibilities discussed for future use in PCO therapies. Only RT-PCR was carried out in the 2001 study

(Jordan *et al.*, 2001) leaving the question of the nature of TRAIL protein temporal and spatial expression in the lens unanswered.

The aim of this study is to show, for the first time, the protein expression of TRAF2, TRAF3 and TRAIL in the developing chick lens, there has been very limited information published of the expression and roles of these factors during embryonic chick development. The methods used were western blotting, which gave the intensity of protein expression at the same developmental time points used for PCR, and immunohistochemistry and/or immunofluorescence, which revealed the localisation of the corresponding antigen of the protein within the lens.

4.2 Experimental Design

Both western blotting and histology were carried out on lenses taken from the same time points as in the previous chapter (ED6, 8, 10, 12, 14 and 16). Western blotting analysis was used to show the temporal protein expression of TRAF2, TRAF3 and TRAIL in the developing chick lens. Immunohistochemical studies were also undertaken to elucidate the localisation of the above proteins at the selected time points.

All three antibodies used in this study were purchased from Santa Cruz, USA and were raised in rabbit. When selecting antibodies available for purchase the limiting factors included whether antibodies for the genes were available to purchase and if those antibodies were likely to work on *Gallus gallus* samples. To assess whether antibodies were likely to be appropriate for use in the chick, BLAST analysis was undertaken and comparisons between the chick and antibodies' animal of origin were made. BLAST alignments with 80% identity or greater were accepted as more likely to be successful. On this basis, three proteins of interest were suitable for further analysis: TRAF2, TRAF3 and TRAIL.

The two main methods used in this chapter were western blotting and histology; their methodologies have been explained in detail in sections 2.3 and 2.4, respectively. They will only be briefly described below.

4.2.1 Western Blotting

Western blotting showed the protein expression of the selected genes at the developing stages (E6 to E16) of the chick lens. Lenses were collected, pooled together and homogenised in a solution of dilute RIPA buffer and Protease Inhibitor Cocktail. The protein samples were then quantified using a BCA assay kit. The samples were diluted so each aliquot contained 10μg/ 4μl of protein. Three sets of samples were collected so as to insure reproducibility. Protein samples were run on denaturing polyacrylamide resolving gels in order to separate the protein content according to size. The protein bands were then transferred onto a nitrocellulose membrane using electrophoresis. The membrane was blocked with 3% semi-skimmed milk and then incubated with the primary antibody at a dilution of TRAF2 1:3000, TRAF3 1:2500 and TRAIL 1:1000, followed by the secondary antibody at a dilution of 1:10000 for all the primary antibodies. Between incubations, the membranes were thoroughly washed with 3% skimmed milk for 5 minutes, 3 times each. The membranes were then incubated in an ECL solution and a film was exposed to the membranes for between 2 and 10 minutes. The film was then placed in developer followed by fixer and allowed to air dry. After exposure of the membrane to film, the membrane was chemically stripped to remove the antibodies. Membranes were subsequently re-probed using an actin antibody raised in Donkey (which ensured that no previously used, i.e. rabbit, antibody was detected) at a dilution of 1: 2500 followed by an anti-donkey secondary at a dilution of 1:10000. The resulting film was developed and was used to confirm equal loading and for normalisation during analysis. The films were scanned into a computer (using an Epsom Expression 1680 Pro scanner) and band intensities were analysed using Labworks™ software (Media Cybernetics, UK). The data, collected in triplicate, were normalised against actin and statistical analysis was carried out to identify any significant changes in expression using a one-way ANOVA with Tukey's post-hoc test.

4.2.2 Subcellular Fractionation

In order to determine in which sub-cellular compartment the TRAF3s' isoforms were present, subcellular fractionation was carried out on pooled E16 lenses and samples were collected for; the whole homogenate, nuclear, post-nuclear, cytosol and the final

sample contained membrane, mitochondria and cytoskeleton. This method was used to determine in which sub-cellular compartments TRAF3 was found. A diagram of the protocol is shown on page 58, figure 2.4.

4.2.3 Histology

Section 2.4 explains the histological methodology in detail, but briefly, eyes were collected from chick embryos at even days of development from E6 to E16. The eyes were washed in ice cold 1xPBS and the back half of the eye was removed using dissecting scissors. The eyes were fixed overnight in 4% Paraformaldehyde in PBS, dehydrated using 2 washes of 30 minutes each of 50, 75, 90 and 100% IMS followed by washes of a 50:50 mix of IMS: xylene. Two washes of pure xylene, lasting 30 minutes each, were undertaken before the tissue was placed in molten wax. After 30 minutes, the wax was changed and the eyes were left in molten wax overnight. The eyes were then embedded in blocks of fresh wax with the front of the eye facing the side of the block (as shown in figure 2.5). The blocks were left to harden for at least 24 hours before the lenses were sectioned in 7μm slices onto slides using a Microm microtome.

Sections through the centre of the lens were selected for staining using the same antibodies as were used in Western blotting. Paraffin wax was removed and the tissues sections hydrated using 2x 5 minute washes of xylene followed by 100, 90, 75 and 50% IMS. The slides of sections were placed in a pressure cooker of antigen unmasking solution (2 minutes), for antigen retrieval, and then washed in tap water for 5 minutes. The tissue was then quenched to remove any endogenous peroxidase. The tissue was blocked in 5% BSA in PBS/Tween for 1 hour to inhibit non-specific binding of antibody. The sections were then incubated with the primary antibody (TRAIL 1:300, TRAF1 1:250, TRAF2 1:350) for 4 hours, washed 3x 5 minutes in 1x PBS/Tween and incubated with secondary antibody for 1 hour (the secondary antibody dilution for sections incubated with TRAIL, TRAF2 and TRAF3 were 1:750, 1:1000 and 1:750 respectively) then washed with 1x PBS/Tween for 3x 5 minutes. For peroxidase staining Vectastain Elite ABC Reagent was then added to slides for 30 minutes before VIP (very intense purple) peroxidase was added until the appropriate intensity of staining developed (~2 minutes). The sections were dehydrated and

mounted. Images were taken using a Leica microscope with attached digital camera using QWin V3 software.

For staining with immunofluorescence, a similar methodology is undertaken as above but without quenching and secondary antibody; Alexa Fluor 488 goat anti-rabbit IgG with 3µl/ml of Hoescht 33342 at a concentration of 1/1000 was used before being washed and mounted using Vectrashield. Images were taken using a Leica microscope and AF6000 software.

In all histological experiments a negative control of Rabbit IGg was used at the same dilution and incubated for the same time as the primary antibodies.

4.3 Results

4.3.1 Western Blotting

4.3.1.1 TRAF2

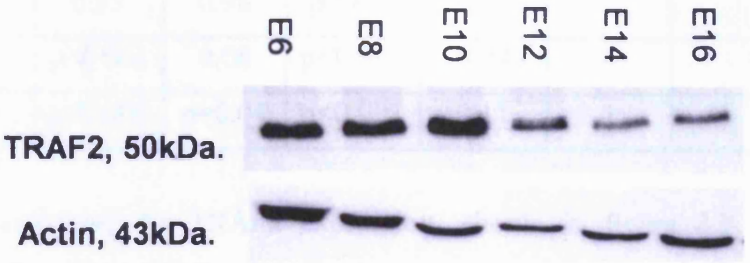


Figure 4.1 Representative western blot of TRAF2 protein expression with actin as a sample loading control. Expression of TRAF2 was high at earlier timepoints (E6-10) which then progressively decreased at E12 and E14. At E16, expression remained low.

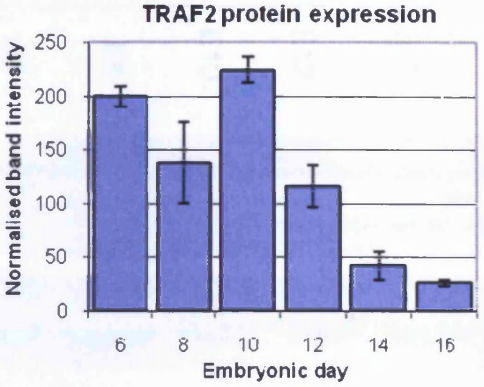


Figure 4.2 Graphical representation of densitometry results of TRAF2 protein expression. Expression of TRAF2 diminishes as development proceeds.

Western blots were performed in triplicate, expression of TRAF2, in the representative western, figure 4.3, was shown to be higher at earlier timepoints i.e. E6-10, expression progressively decreased at E12 and E14, at E16 expression remained low. Normalised densitometry results are shown above in figure 4.4 followed by the results of the statistical analysis in table 4.1.

Table 4.1 TRAF2 p values obtained from Tukey's post hoc test. Cells highlighted in yellow show values that are significantly different from each other.

	E6	E8	E10	E12	E14	E16
E6						
E8	0.4					
E10	0.96	0.13				
E12	0.15	0.98	p=0.04			
E14	p<0.001	0.08	p<0.001	0.24		
E16	p<0.001	p=0.04	p<0.001	0.11	1	

The analysis confirmed the TRAF2 expression, shown in figure 4.3, at E6 was significantly higher than at E14 or E16. At E8 expression was higher than at E16 and E10 expression was statistically, significantly higher than all later days of expression studied. More detail of the statistical analysis can be found in appendix 3.

4.3.1.2 TRAF3

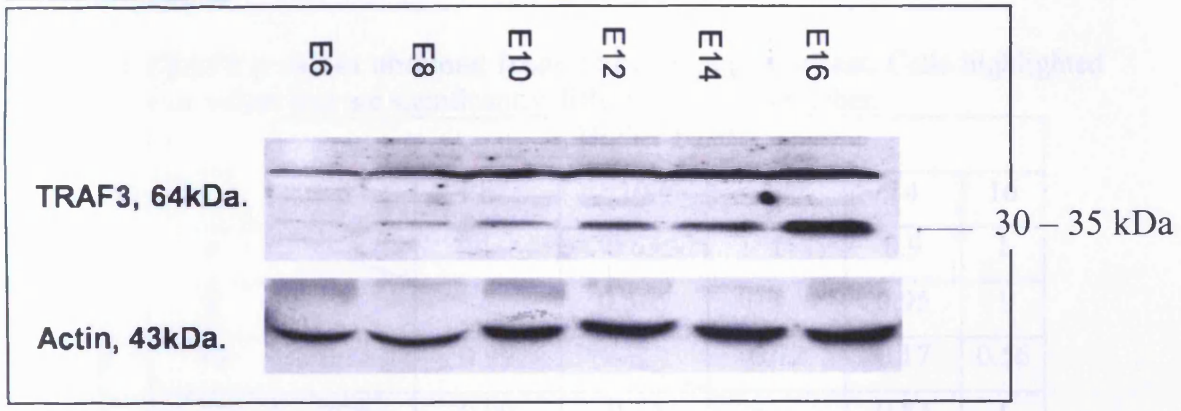


Figure 4.3 Western blotting of TRAF3 at all developmental time points with actin as a loading control. TRAF3 expression at 64 kDa remains relatively constant, while the 30 kDa TRAF3 shows minimal expression at E6 and is then up-regulated as development of the lens proceeds.

TRAF3 western blots were performed in triplicate, and expression in the representative western, figure 4.5 and the graphical representation in figure 4.6 show that overall, TRAF3 expression increases as the lens develops (within the stages studied) but that this expression is found in two different molecular weight bands. The 64 kDa band

(shown in the datasheet of the antibody) remains relatively constant while the lower band, at 30 and 35 kDa, was increasingly expressed as development continued.

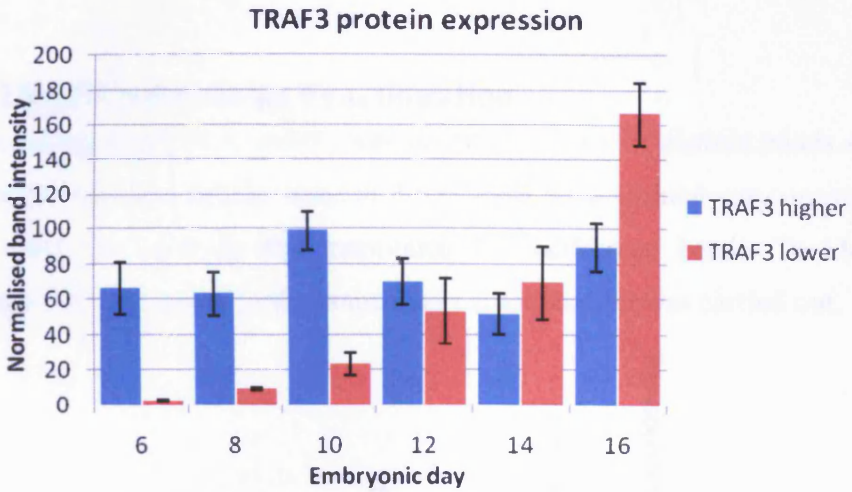


Figure 4.4 Graphical representation of densitometry results from TRAF3 protein expression. Expression of the short (30 kDa) form increases as development proceeds while the longer (64 kDa) form only shows minimal differences in expression.

Table 4.2 TRAF3 p values obtained from Tukey's post hoc test. Cells highlighted in yellow show values that are significantly different from each other.

		Higher band					
		6	8	10	12	14	16
Lower band	6		1	0.63	1	0.9	1
	8	1		0.545	1	0.95	1
	10	0.94	0.99		0.72	0.17	0.56
	12	0.06	0.09	0.24		0.83	1
	14	p=0.01	p=0.02	p=0.05	0.91		0.94
	16	p<0.001	p<0.001	p<0.001	p=0.01	p=0.02	

Though no statistical difference was found for the band representing full-length TRAF3 (64kDa), the lower weight band of TRAF3 (30- 35 kDa) had statistically, significantly increased expression at E16 in comparison to all earlier developmental time points.TRAF3 at E14 was also found to have higher expression than at E6, E8 or

E10 as shown in figure 4.5 and 4.6. Details of the statistical analysis carried out for TRAF3 can be found in appendix 3.

4.3.1.3 TRAF3 Subcellular Fractionation

Western blotting of TRAF3, above, was found to have two distinct bands at 64 kDa and also a substantially smaller band at 30-35 kDa. This seemed very unusual and no research using the antibody had mentioned any additional bands. To identify the expression of the two bands further subcellular fractionation was carried out.

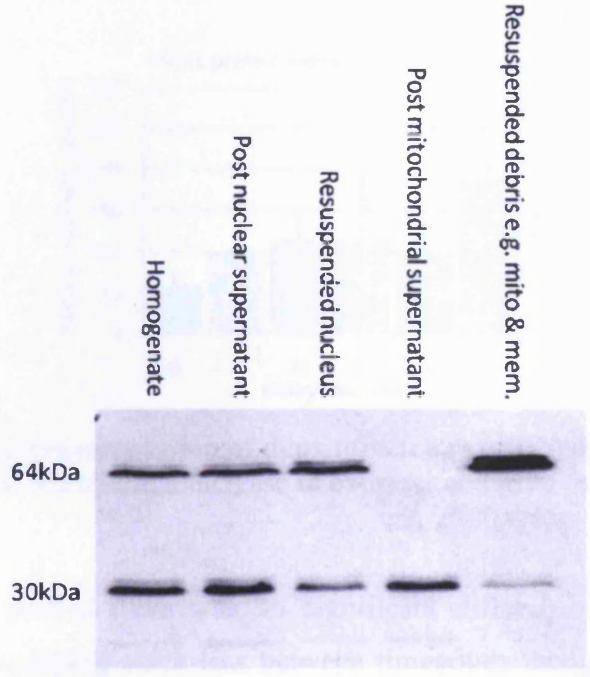


Figure 4.5 Subcellular fractionation of TRAF3 at E16. The full-length TRAF3 (64 kDa) is found associated with membranes and/or mitochondria, but not in the post-mitochondrial supernatant (cytosol), whereas the lower molecular weight isoform of TRAF3 (30-35 kDa), is found associated with all fractions but most highly expressed in fractions with higher concentrations of soluble proteins i.e. homogenate and cytosol.

Subcellular fractionation carried out on pooled E16 lenses shows that the full-length high molecular weight (64 kDa) TRAF3 is found in factions containing membrane material and organelles, but not in the cytosol suggesting it is associated, if not bound to membranes. The lower molecular weight isoform (30-35 kDa) is found in all factions, though there is a reduced amount in the re-suspended pellets of the nucleus and the mitochondria and membrane debris.

4.3.1.4 TRAIL

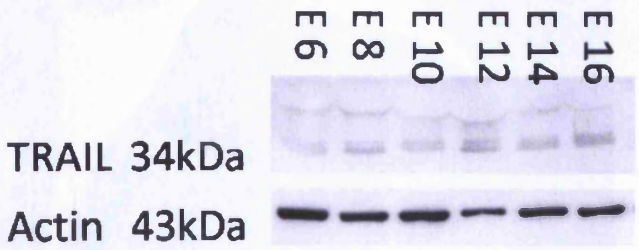


Figure 4.6 Western blot of TRAIL in the developing chicken embryo lens showing bands at 34kDa as stated on the Santa Cruz datasheet.

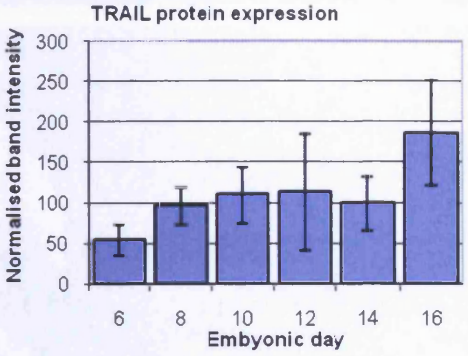


Figure 4.7 Graphical representation of densitometry results from TRAIL protein expression. The trend is a minimal increase in expression during the developmental stages shown.

Statistical analysis showed there was no significant difference in TRAIL protein expression in the developing chick lens between timepoints though there is a limited increase in expression as the lens develops.

4.3.2 Histology

Prior to localisation studies, using the antibodies from the western blots, the standard morphology of the lens at the developmental time points selected was confirmed in figure 4.10 and 4.11 shown below.

4.3.2.1 Haematoxylin and Eosin staining

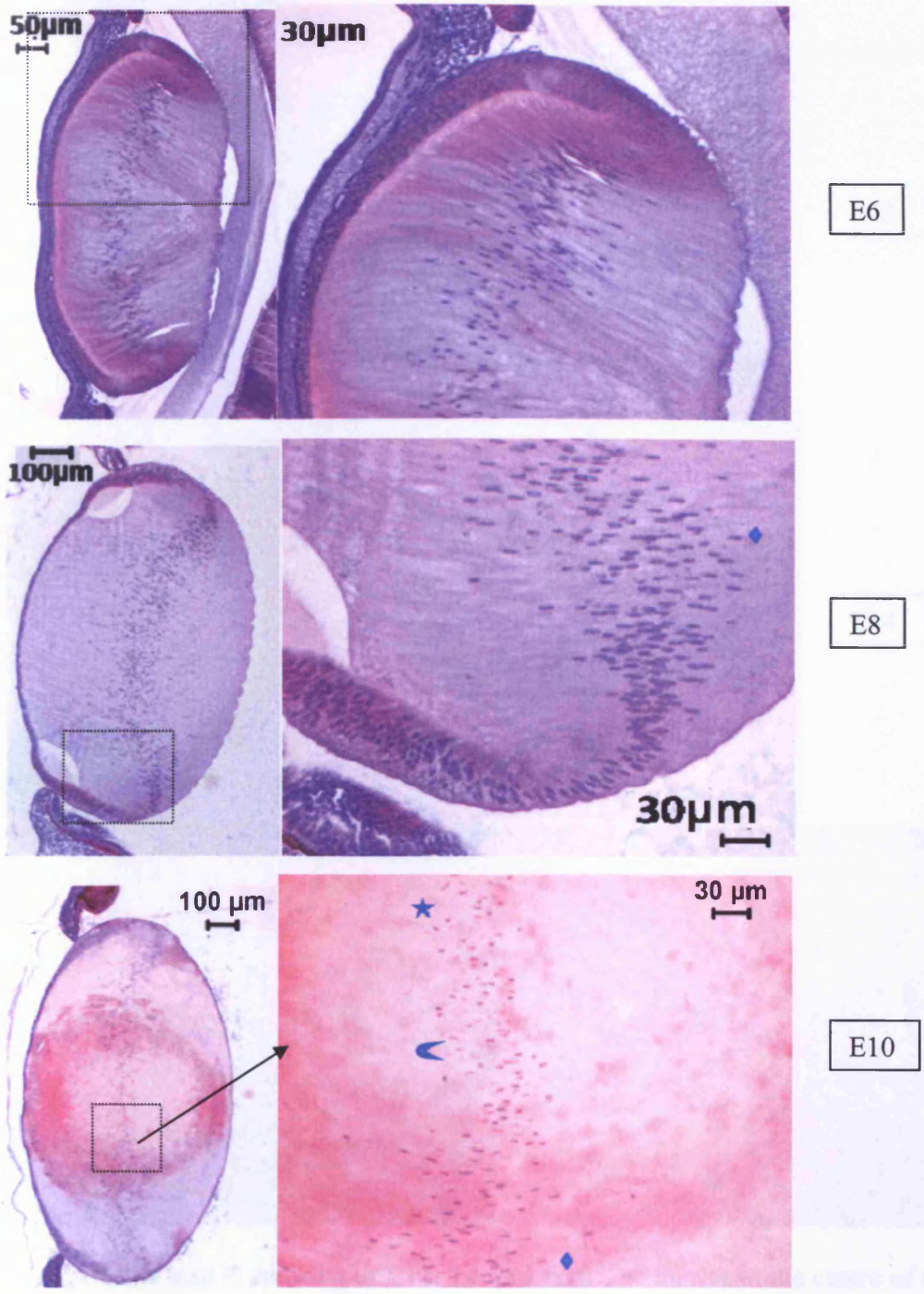


Figure 4.8 H and E staining at E6, E8 and E10. The ☆ indicates pycnotic nuclei, ◇ indicate elongated nuclei and ☾ highlight degenerating nuclei during early pycnosis.

Heamatoxylin and Eosin staining continued

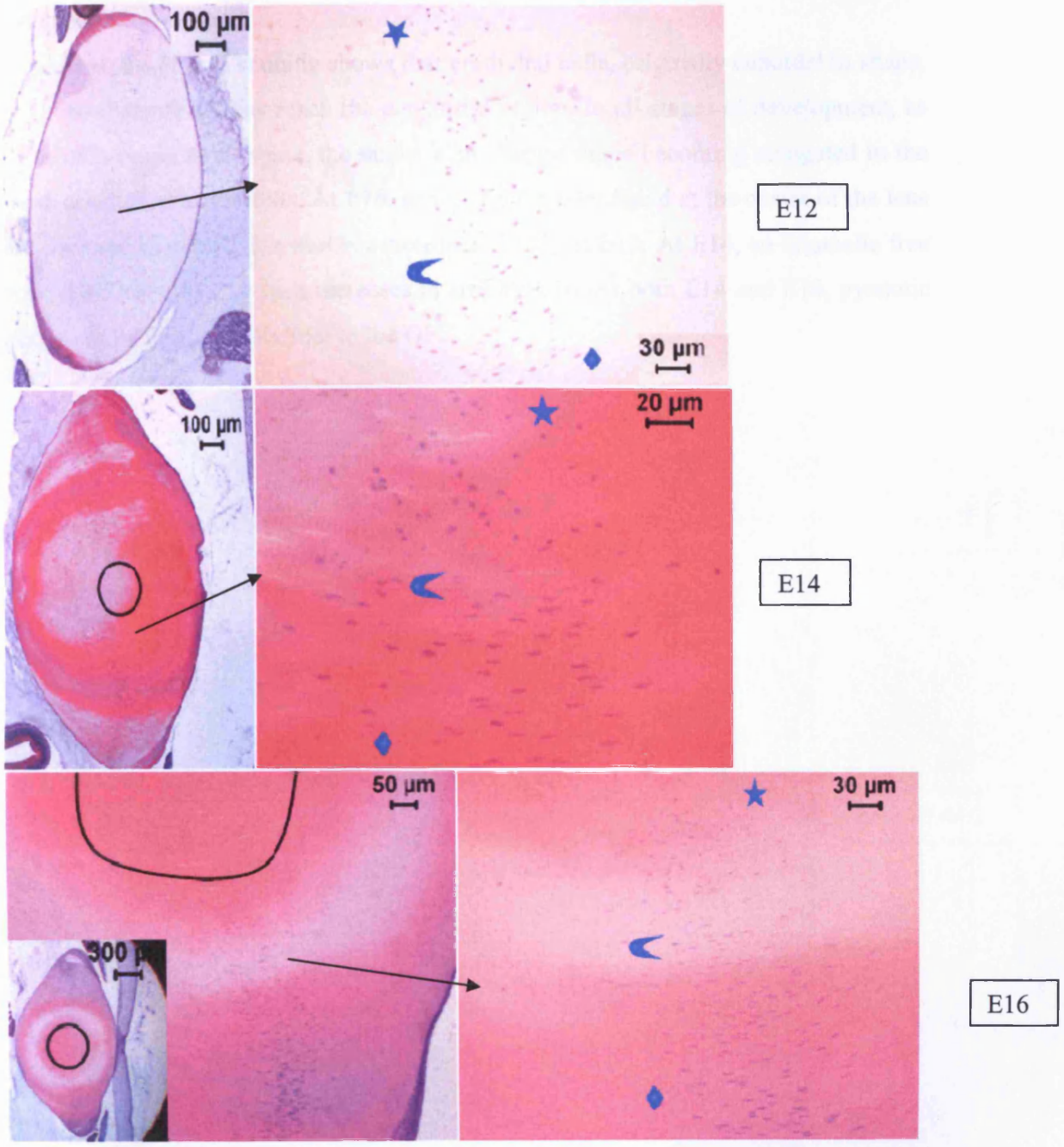


Figure 4.9 H and E staining at E12, 14 and E16 The circles at the centre of the lens show the organelle free zone. The ☆indicates pycnotic nuclei, ◇indicate elongated nuclei and ☾ highlight degenerating nuclei during early pycnosis.

The normal lens morphology during development was characterised through the use of Haematoxylin and Eosin (H & E) staining. The staining shows the morphology of individual cells within the various regions of the lens. The haematoxylin stains the

nuclei displaying any changes in their presence or shape, while eosin stains the cytoplasm.

In general, the H & E staining shows that epithelial cells, originally cuboidal in shape, begin to elongate as they reach the equatorial region. In all stages of development, as these cells begin to elongate, the nuclei also change shape becoming elongated in the same orientation as the cells. At E10, some of the nuclei found at the centre of the lens are pycnotic in nature; this number increases greatly at E12. At E14, an organelle free zone (OFZ) is visible, which increases in size by E16. At both E14 and E16, pycnotic nuclei can be seen at the border to the OFZ.

4.3.2.2 TRAF2

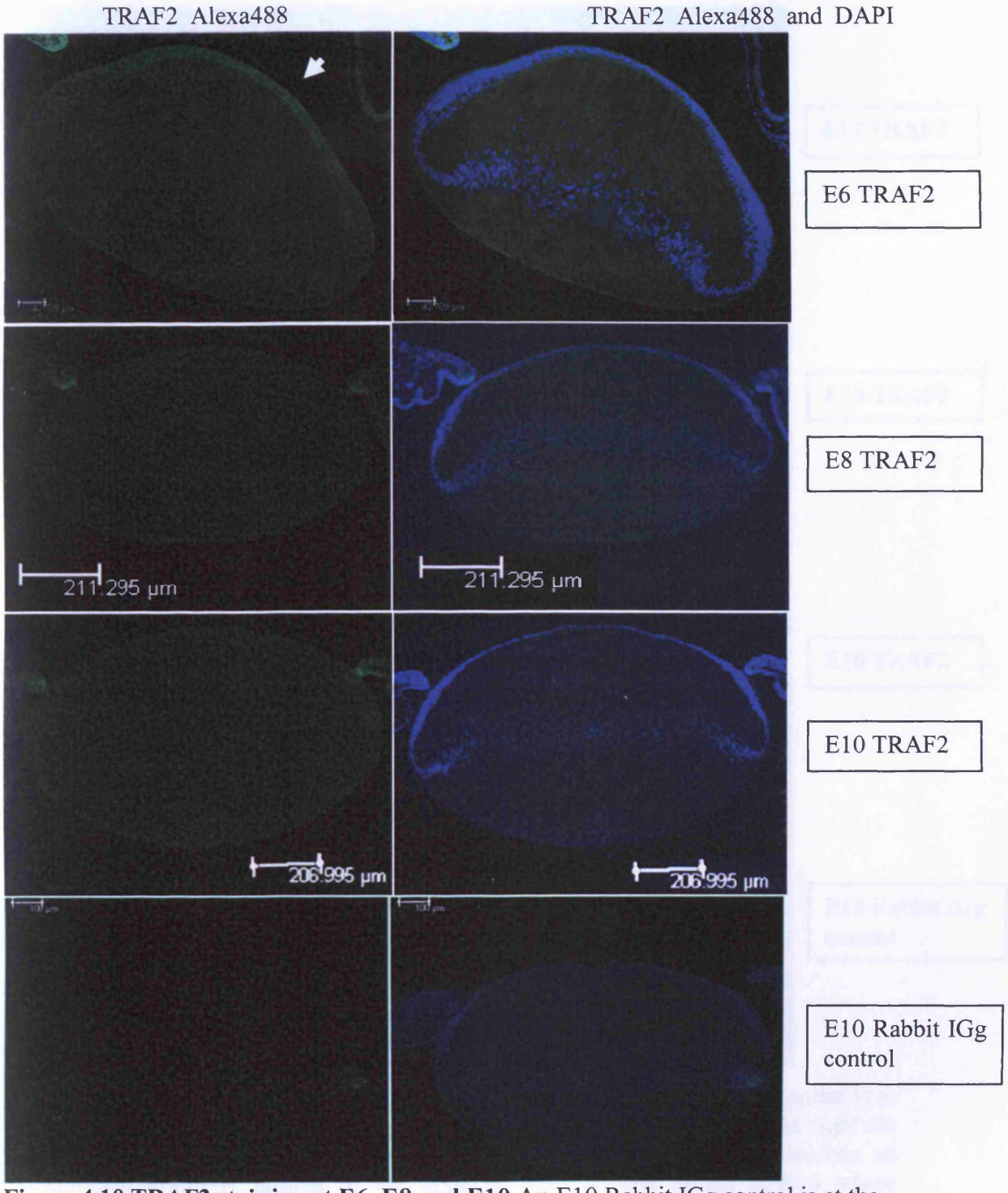


Figure 4.10 TRAF2 staining at E6, E8 and E10 An E10 Rabbit IGg control is at the bottom of the panel and is negative for staining. On the left are the TRAF2 alexa 488 images, on the right are the same images merged with DAPI staining. TRAF2 staining is associated with the lens epithelium at E6 (arrowhead) and then diminishes as development proceeds, similar to the Western blotting data (figure 4.3).

TRAF2 continued

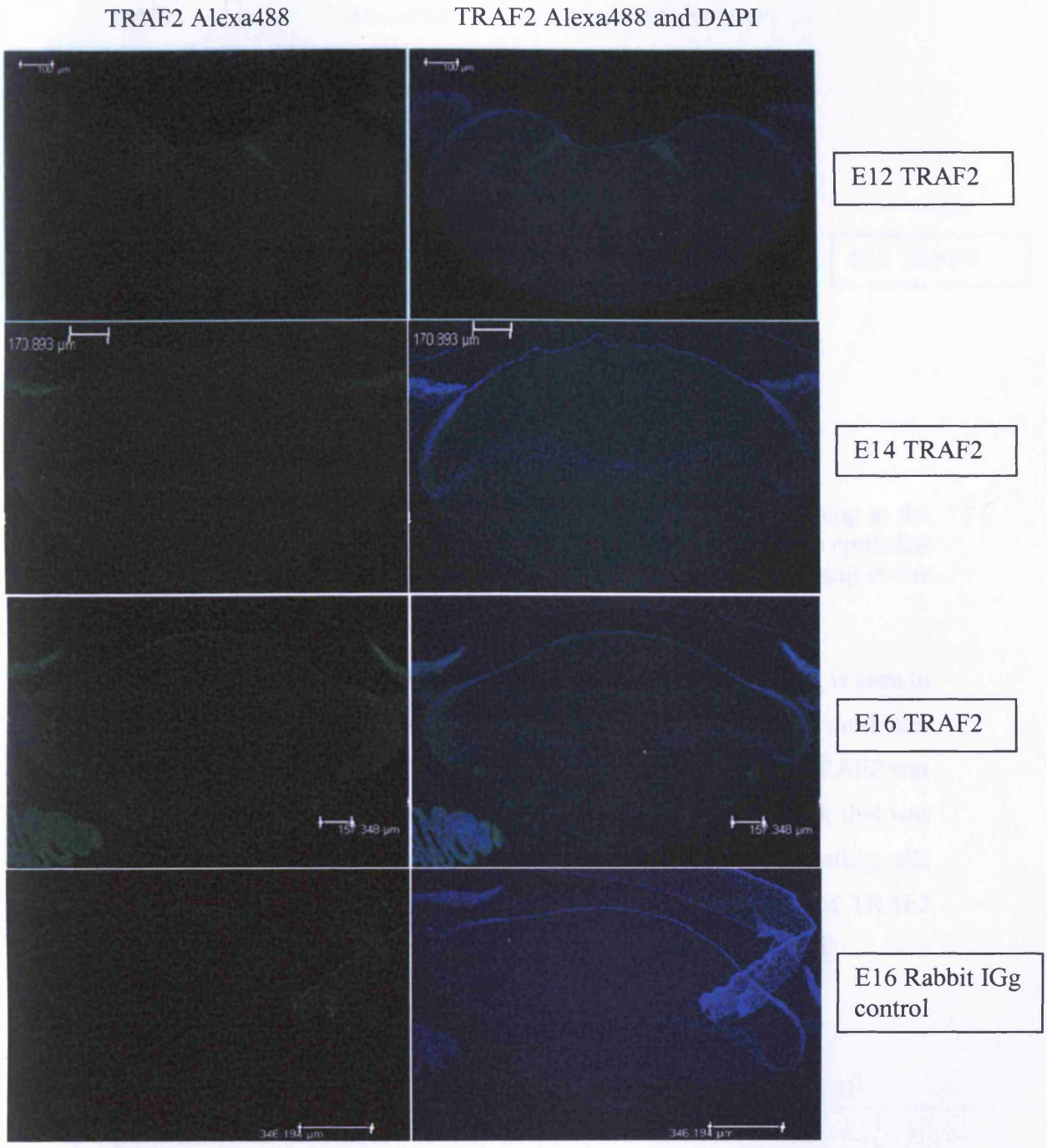
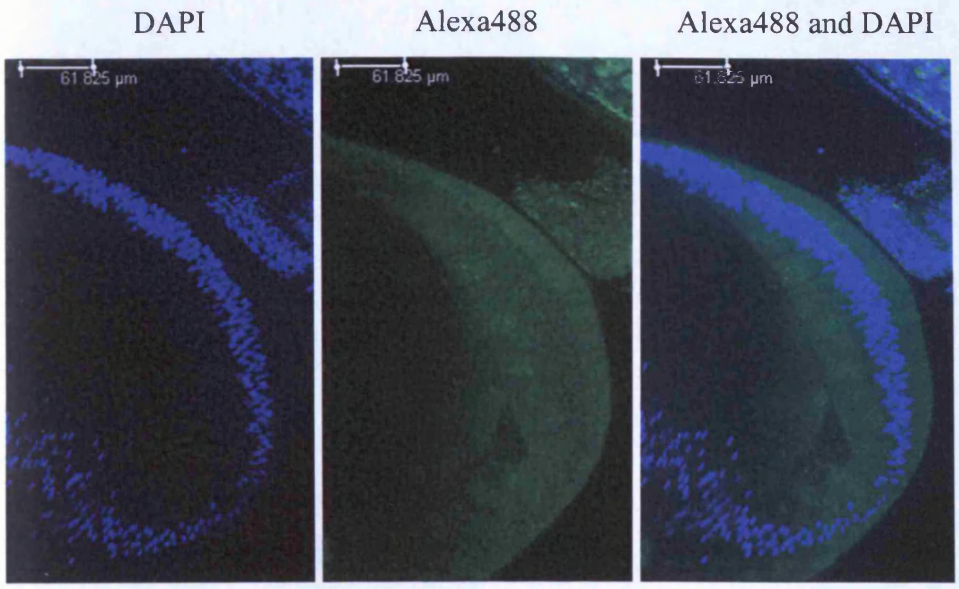


Figure 4.11 TRAF2 staining at E12, E14 and E16. An E16 Rabbit IGg control is at the bottom of the panel. On the left are the TRAF2 alexa 488 images, on the right are the same images merged with DAPI staining. TRAF2 staining diminishes as development proceeds, similar to the Western blotting data except at E16 where staining is more condensed to the equatorial region (figure 4.3).



E16 TRAF2

Figure 4.12 TRAF2 cytosolic staining at E16. Image shows TRAF2 staining at the equatorial region of an E16 lens. Staining is intense in the cytosol of the lens epithelial cells and in early differentiating lens fibre. There is a distinct lack of staining in the nuclei of both the lens epithelial cells and the lens fibre cells.

As can be seen in figures 4.12 and 4.13, using immunofluorescence, staining is seen in E6 lens epithelial cells, but diminishes thereafter, remaining low in the lens until later in development (E16) when it is found expressed at the equatorial region. TRAF2 was stated to be cytoplasmic in the datasheet and literature (Horie *et al.*, 2002); this was confirmed in figure 4.14 showing a lack of staining in the nuclei of differentiating cells in the equatorial region of an E16 lens which is representative of a lack of TRAF2 staining found in nuclei at all developmental time points.

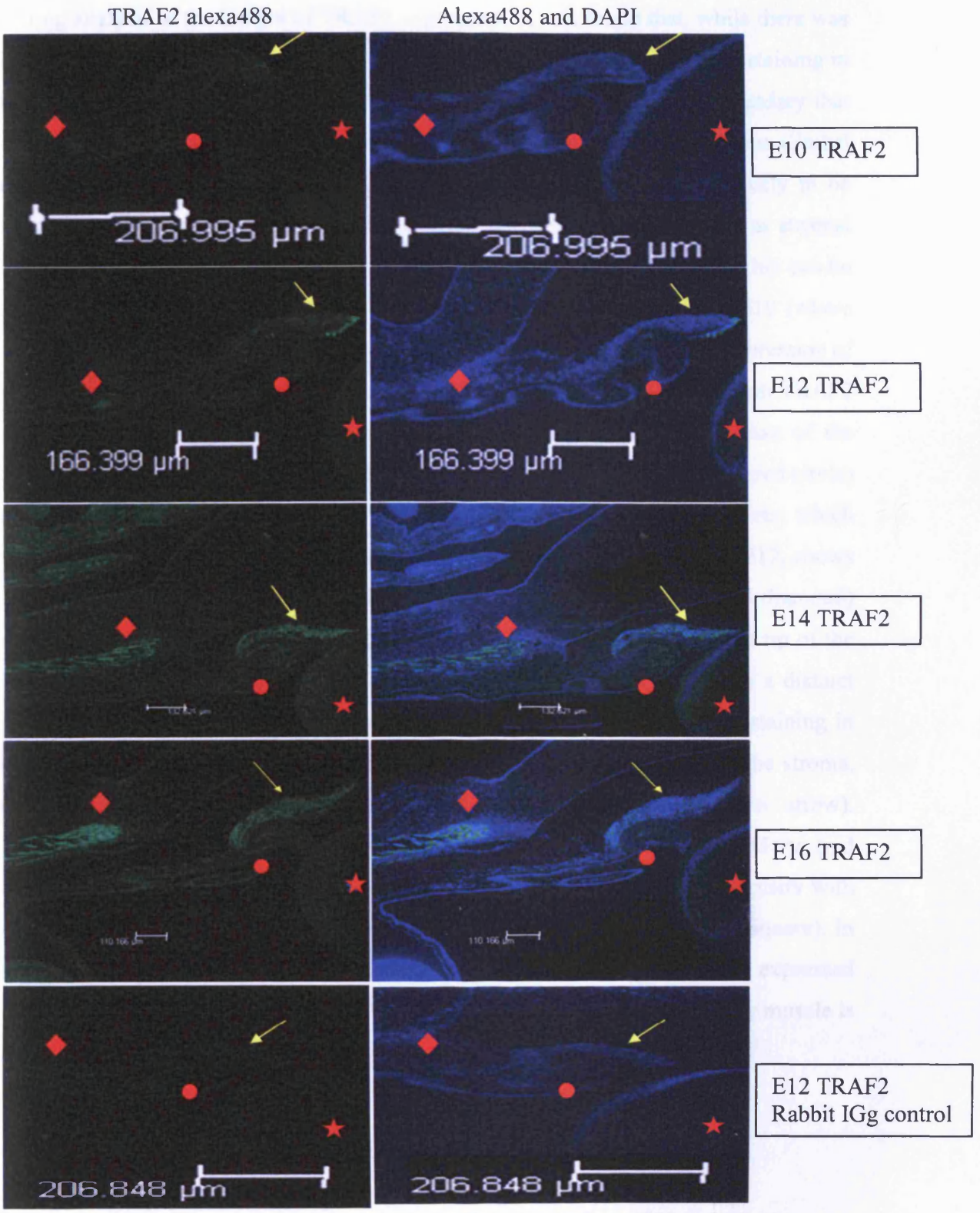


Figure 4.13 TRAF2 staining of the iris and ciliary body from E10 to E16. The symbols in the images show the iris (▬) corneal-scleral boundary(♦), ciliary body(●) and lens(★). Staining is found at increasing intensities in both the iris and corneal- scleral boundary as the development continues from E10 to E16 there is also limited expression in the ciliary body.

During analysis of the images of TRAF2 expression, it was found that, while there was little staining in the lens, at later stages of development there was increased staining in the iris and the ciliary body as well as in a region at the corneal-scleral boundary that could represent an early manifestation of the stem cell niche for the cornea (limbal stem cells). Research of past publications suggest the staining is more likely to be muscular as the staining is similar to that in the iris, which was identified as stromal muscle when comparing staining to past research (Ferrari & Koch, 1984). This can be seen in figure 4.15, which shows staining, in increasing intensities, from E10 (where TRAF2 expression is first seen at the tip of the iris) to E16 where intense expression of TRAF2 has developed throughout the iris. At stages prior to E10, there was no TRAF2 staining visible. At E10, the first suggestion of staining is seen at the base of the corneal-scleral boundary (red diamond) and in the presumptive ciliary body (red circle) and in the developing iris (yellow arrow) mostly found at the tip of the iris, which extends over the anterior of the lens as development continues. Staining at E12, shows a larger and slightly more intense stain at the corneal-scleral boundary (red diamond) and in the developing ciliary muscle (red circle) and also on the edge of the tip of the iris (the sphincter of the iris; yellow arrow). At E14, staining is present in a distinct 'crescent' shape throughout the ciliary body (above red circle), while the staining in the iris is no longer present at the sphincter and is now found throughout the stroma, though is still more highly expressed towards the tip of the iris (yellow arrow). Staining also becomes much more intense at the corneal-scleral boundary (red diamond). At E16, the staining at the corneal-scleral boundary gains in intensity with more condensed and higher expression at the end closest to the cornea (red square). In the iris, the staining is still intense at E16 though it is more evenly expressed throughout the stroma (yellow arrow), while TRAF2 expression in the ciliary muscle is more diffuse (red circle).

4.3.2.3 TRAF3

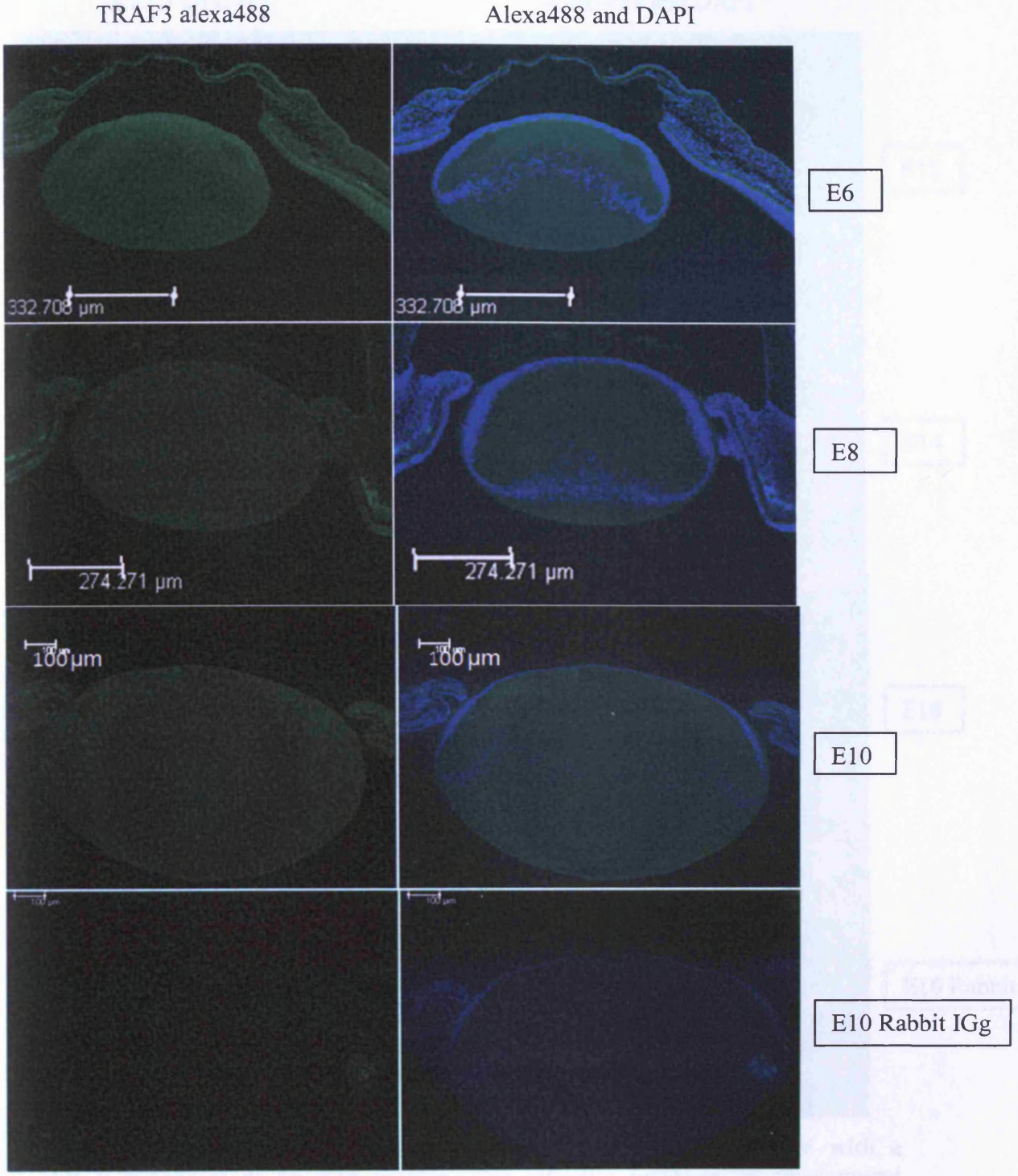


Figure 4.14 Immunofluorescent staining of TRAF3 at E6, 8 and 10 with a Rabbit IGg control of an E10 section. Staining is found throughout the E6 lens but with more intensity at the anterior. Staining intensity decreases and at E10 can be seen in the differentiating fibre cells.

TRAF3 continued

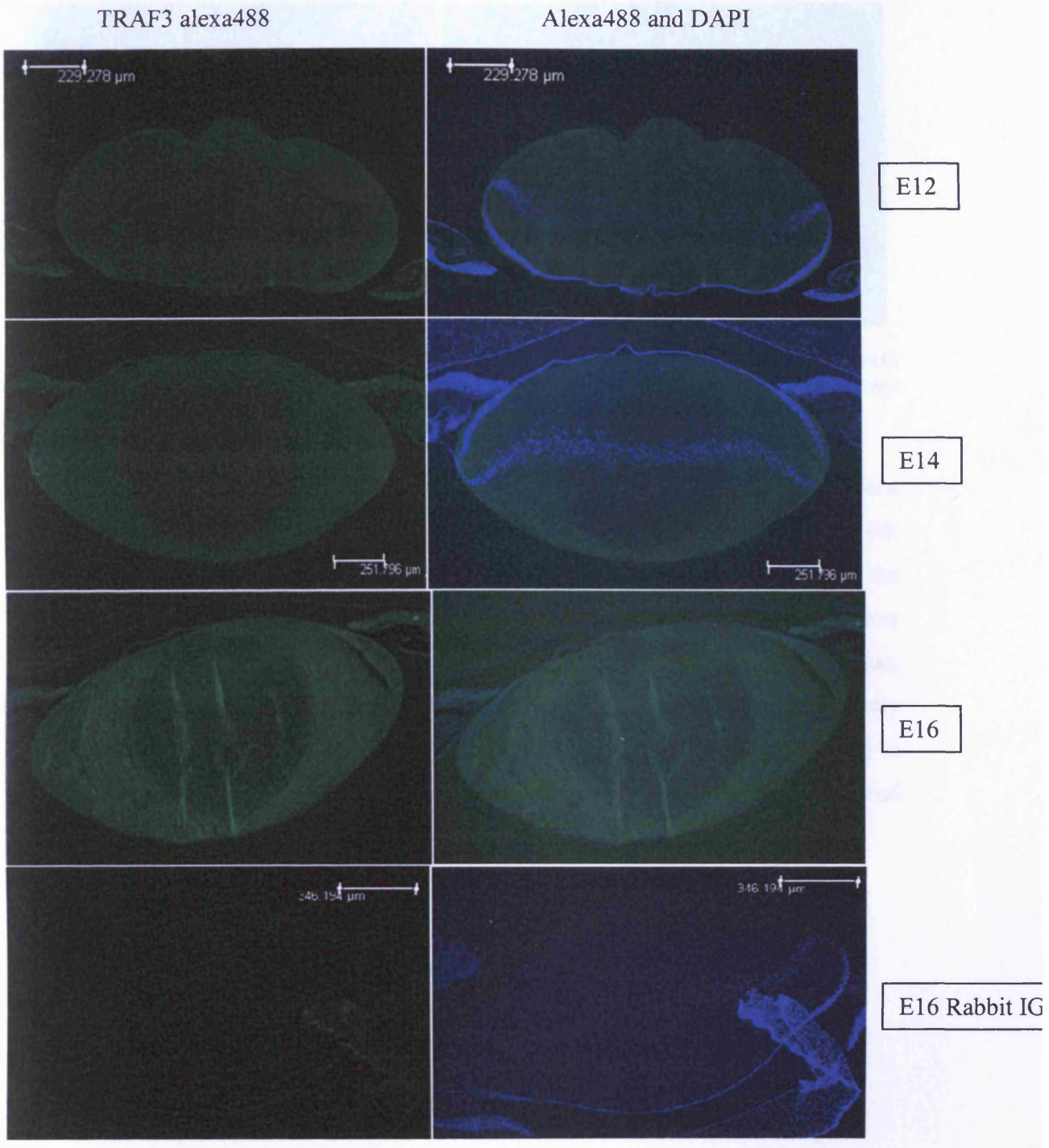


Figure 4.15 Immunofluorescent staining of TRAF3 at E12, 14 and 16 with a Rabbit IGg control of an E16 section. The intensity increases as development continues from E12 to E16. The staining is highly visible in the differentiating secondary fibre cells before they cross the boundary into the central fibre cell mass, at this point there is a clear decrease in staining which is visible as a darker circle at the centre of the lens.

TRAF3 alexa488 Alexa488 and DAPI

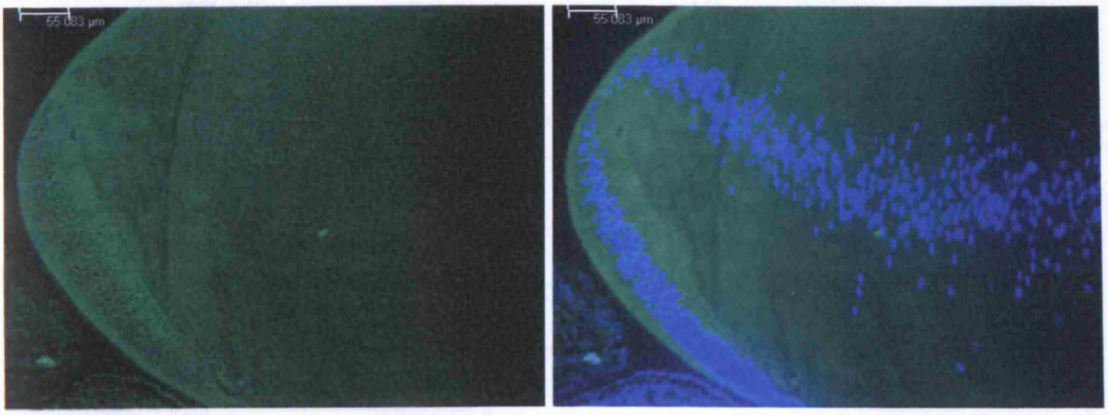


Figure 4.16 Immunofluorescent staining of TRAF3 at E14. Staining is clearly cytosolic (as indicated by the lack of nuclear staining) and ubiquitous, being absent only from the centre of the lens where the OFZ is forming.

In figures 4.16 and 4.17, the localisation of TRAF3 can be seen as the lens develops. At E6, staining was slightly more intense at the anterior of the eye. At E8, staining intensity has reduced. At E10 there appears a large area at the centre of the lens that contains considerably less staining than that found within the newer differentiating fibre cells, prior to their incorporation in to the central fibre cell mass, and the epithelial cells. As the lens develops the staining in the lens progressively increases while maintaining the same localisation. At E16, the brightest staining is found in the same location; i.e. the cortical fibre cells while absent from the central fibre cell mass.

4.3.2.4 TRAIL

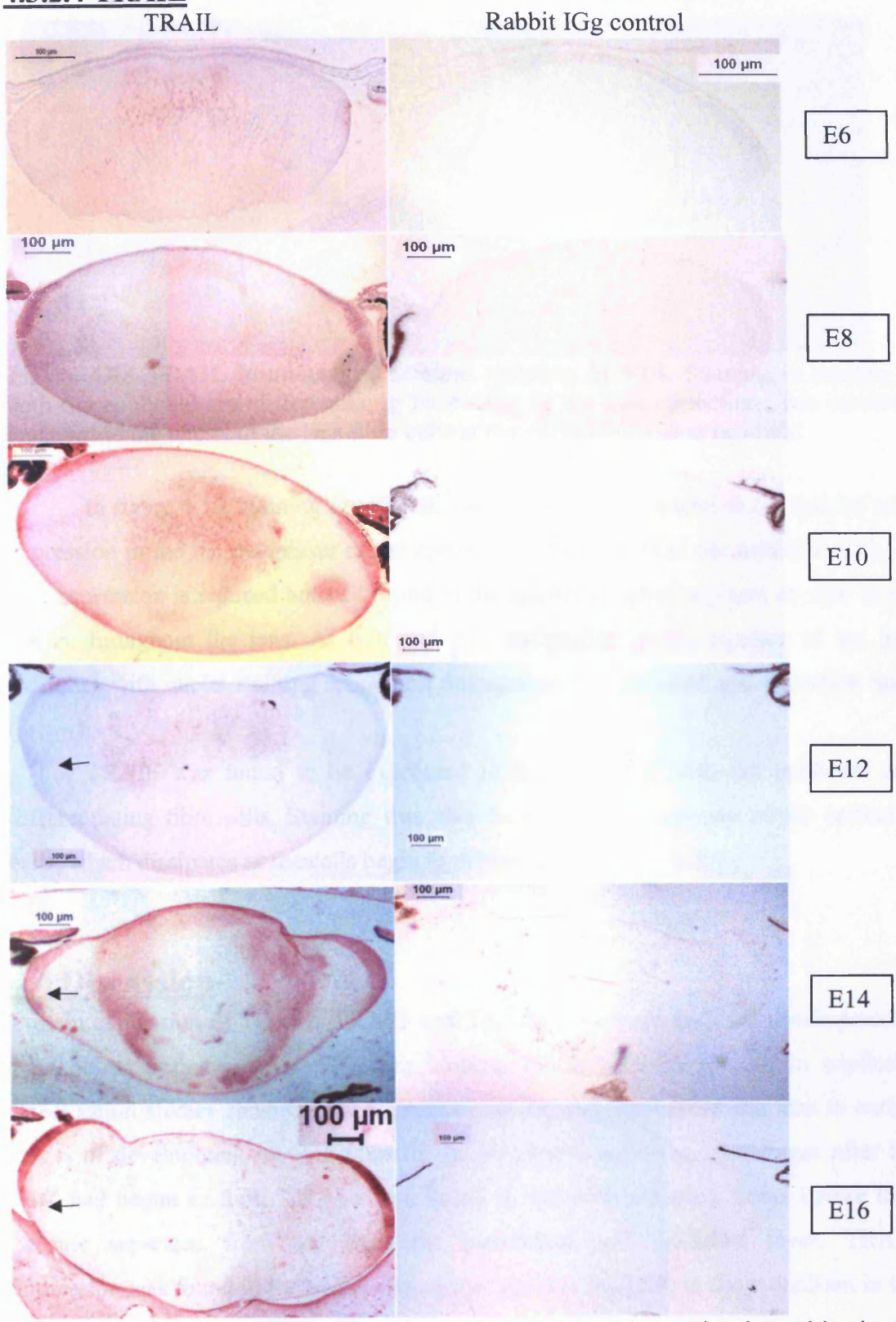


Figure 4.17 TRAIL staining from E6 to E16 visible at increasing intensities in the epithelial layer and into the transition zone at E12 and later. Staining at the transition zone (arrow).

E14 TRAIL E14 Rabbit IGg control

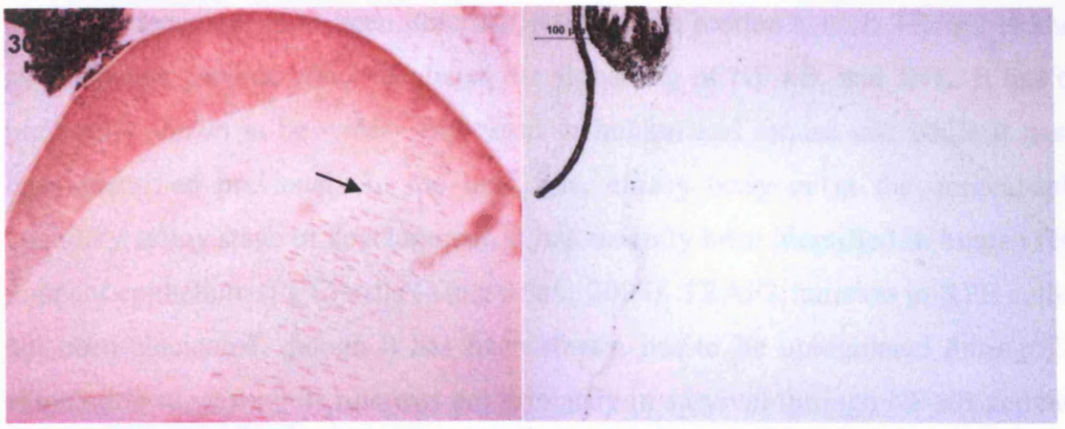


Figure 4.18 TRAIL immunocytochemical staining at E14. Staining is present in both the epithelial and differentiating fibre cells in the lens epithelium, but becomes localised to the nuclei of the lens fibre cells at the equatorial region (arrow).

In figure 4.19, staining for TRAIL can be seen in the nuclei at E6 and E8 with expression in the the cytoplasm of the epithelial cells as well as the nuclei at E10. At E12 expression is reduced but still found in the epithelial cell cytoplasm as well as the nuclei throughout the lens. At E14 and E16 expression at the equator of the lens increases with nuclei staining remaining throughout the epithelial and transition zone (arrow).

TRAIL was found to be expressed in the nuclei of both the epithelial and differentiating fibre cells. Staining was also found in the cytoplasm of the epithelial cells, which dissipates as the cells begin to differentiate (figure 4.20).

4.4 Discussion

Protein expression of TRAF2, TRAF3 and TRAIL was observed at all developmental time points examined using Western blotting which was carried out in triplicate. Localisation studies showed light expression of TRAF2 throughout the lens in earlier stages of development moving towards the equatorial region at later stages after the OFZ had begun to form. TRAF3 was found in the differentiating fibres before they became separated from the basement membrane and epithelial layer. TRAIL expression was found in the nuclei throughout the lens and also in the cytoplasm in the equatorial cells at later stages of development.

4.4.1 TRAF2

TRAF2 interactions have been described in detail in section 1.12.2. TRAF2 is known as an adapter protein, which mediates the signalling of NF-κB, and JNK. It has been previously shown to be widely expressed in human and mouse and while it has not been identified previously in the lens, iris, ciliary body or at the corneal-scleral boundary at any stage of development, it has recently been identified in human retinal pigment epithelium (RPE) cells (Yang *et al.*, 2005). TRAF2 function in RPE cells has not been elucidated, though it has been shown not to be upregulated during TNFα stimulation suggesting its role was not primarily in survival through NF-κB activation. When NF-κB was blocked, RPE cells were still resistant to apoptosis, likely to be due to the continued expression of survival factors such as TRAF2 (Yang *et al.*, 2005).

TRAF2 protein expression in the lens was found to vary slightly between western blotting and immunofluorescence. As all experiments were undertaken in triplicate, it is unlikely that the variations are due to experimental error. Western blotting is a more sensitive technique and showed a clear decrease in expression as development progressed in comparison only an overall reduction in expression in immunofluorescence which seemed to slightly increase at E16 was identified. This "increase" in expression is most likely to be a result of the localisation of staining to the equatorial region and lens epithelium.

Though TRAF2 lens staining was of low intensity in histological studies, a significant amount of expression was identified in the developing iris, ciliary muscle and in an intense band at the boundary between the cornea and sclera. Both the iris and ciliary body originally develop from a mixture of cells, including optic cup ectodermal cell, head migratory mesenchymal cells and in the chick the iris muscles have been shown to develop from neural crest cells (Johnston *et al.*, 1979; Creuzet *et al.*, 2005). The similarities of the iris and ciliary muscle in regards to the time and progress of development suggest that TRAF2 may have a role in the initial development of these structures, which occurs between E5 and E15 when the smooth muscles in the iris stop growing (Gabella & Clarke, 1983). Research into the transition of "smooth-to-striated" muscle in the iris identified similar patterns of expression between TRAF2 (as revealed here) and α-smooth muscle-specific actin (αSMA), used as the transition marker. The signalling for both was initiated at the papillary margin

(tip of the iris), which then spread peripherally and by E16 was expressed throughout the stroma, though αSMA was less intensely expressed at this time point (Link & Nishi, 1998).

These results suggest that TRAF2 maybe involved in the development of the muscle within these tissues, which is supported by research that has shown that TRAF2 knockout mice have decreased muscles mass though no eye defects have yet been identified (Lee *et al.*, 1997; Yeh *et al.*, 1997). TRAF2 is normally present in skeletal muscle, though is more highly expressed in terminally differentiated rather than undifferentiated forms (MacLachlan & Giordano, 1998). Also, TNF has been shown to modulate myogenesis (Szalay *et al.*, 1997) whose histological features were first used to identify iris muscle differentiation (Ferrari & Koch, 1984).

4.4.2 TRAF3

The complex and not fully understood roles of TRAF3 have been described in section 1.12.3. TRAF3 is important in signalling the activation of the immune response, NF-κB activation and also cell death (Chung *et al.*, 2002). Both TRAF2 and TRAF3 are necessary for postnatal survival (Xu *et al.*, 1996; Lee *et al.*, 1997; Yeh *et al.*, 1997). In this chapter, TRAF3 protein expression was found to be similar to that of the RT-PCR data, increasing as the lens develops. A band around 30 kDa is clearly visible and this is likely to be a splice variant or cleavage product of the 64 kDa native form of TRAF3.

Subcellular fractionation revealed that the larger 64 kDa isoform was found at the highest concentration, at E16, in the fraction containing re-suspended mitochondrial and membrane debris, figure 4.7. It was also present in all the other factions except the one containing the post-mitochondrial supernatant (i.e. the cytosolic fraction remaining after the mitochondria had been removed). The lower molecular weight 30 kDa isoform was expressed in all factions, though there was considerably less in the factions containing the resuspended nucleus and mitochondrial or membrane debris.

TRAF3 has previously been reported to bind p-62 Nucleoporin, a protein involved in the formation of pores and nuclear transport (Hurt, 1993) suggesting that TRAF3 has an adaptor function at the nuclear membrane (Gamper *et al.*, 2000) as well as at the

plasma membrane (Lee & Lee, 2002). Though TRAF3 is a cytoplasmic protein its interactions with membranes bound proteins support the finding of its increased expression in fractions containing membranous debris.

The immunofluorescent staining shows highest TRAF3 expression in cortical lens fibre cells. The finding that this expression does not pass to those fibre cells that have been covered by newer fibre cells at the centre of the lens, and certainly not into the OFZ, suggests that the lack of staining corresponds to the point in the differentiation of the fibre cell when the tips at the posterior and anterior of the lens fibre cells have reached the sutures. It is at this point that fibre cells first come into contact with the corresponding fibre cell from the opposite equator and is also when the fibre cell loses it attachment to the lens capsule and epithelial layer at the posterior and anterior of the lens, respectively (Menko, 2002; Zelenka, 2004). The staining pattern of TRAF3 is similar to that of N-cadherin and band 4.1, which were found to be expressed at the region of the lens where extensive remodelling of the cell adhesion complexes occurs (Beebe *et al.*, 2001). N-cadherin is a transmembrane protein known to form bonds between cells and its role is thought to be in holding cells together, stabilization and possibly the formation of the extracellular space found between fibre cells which has been proposed to be important in maintaining the transparency of the lens (Frenzel & Johnson, 1996; Ferreira-Cornwell *et al.*, 2000; Beebe *et al.*, 2001). Band 4.1 protein is believed to have a role in the interaction between the membrane and the actin cytoskeleton possibly being involved in the change in shape and elongation of the fibre cells (Hoover & Bryant, 2000). The similarities in the localisation of staining between TRAF3, N-cadherin and band 4.1 suggest that further functional studies might first look at a membrane-related role for TRAF3 in the differentiating fibre cells, indeed TRAF3 is known to be able to interact with LMP (latent membrane protein) (Sandberg *et al.*, 1997) which in turn has been shown to stimulate PI3K activation and actin stress-fibre formation associated with transformation (Dawson *et al.*, 2003). TRAF3 has also been identified to associate with TRAF3 interacting protein-1 (MIP-T3) which is proposed to form a link between the microtubule network and TRAF3, though its role has not been elucidated (Ling & Goeddel, 2000). Though these links are tenuous for identifying the role of TRAF3 in

lens differentiation, the collation of these interactions can propose a path for further research.

It has been shown here that TRAF3 (full length) is not found in the post nuclear supernatant (i.e. cytoplasmic fraction), suggesting that it is not found free in the cytoplasm and is membrane-bound which would explain its high expression in the resuspended debris, containing much of the membrane fragments from the cell. TRAF3 is known for its adaptor function in membrane bound proteins such as TNFRs, CD40, LTβ and OX-40 (Baker & Reddy, 1998) Its binding with the membrane bound proteins, along with its interactions with proteins involved in membrane remodelling and stabilisation suggest a reason for its presence in the subcellular fractionations containing membrane and an active role in the membrane of differentiating fibre cells, though whether its true purpose is structural, maintenance or something entirely different would require further studies.

4.4.3 TRAIL

TNF related apoptosis-inducing ligand (TRAIL) can bind two out five of its receptors to induce apoptosis; its three other receptors are decoys, which cannot transmit the apoptotic signal (Kamradt *et al.*, 2005). In this chapter, it was shown that while there were no statistically significant changes in protein expression, which is comparable to the results shown using RT-PCR in chapter 3, TRAIL was found to be present at the equatorial regions, at mildly increasing intensities, as the lens developed and also in the nuclei of all cells in the lens whether in the epithelia or the differentiating fibre cells (excluding the terminally differentiated fibre cells in the OFZ). The novel identification of TRAIL in the nuclei requires further study but it is possible that fragments of TRAIL are transported to the nuclei via an unknown nuclear transport protein. The expression of TRAIL at the equatorial region, and in the transitional zone, at later stages, suggest a possible role in early signalling of epithelial cells to begin differentiation into fibre cells.

Use of TRAIL was previously identified as a potential PCO therapy (Jordan *et al.*, 2001), but it was found that over-expression of TRAIL in lens cells remaining in the capsular bag after cataract surgery did not induce the apoptosis of those cells and could not prevent PCO (Malecaze *et al.*, 2006).

4.4.5 Summary

The western blotting results in this chapter showed the protein expression of TRAF2, TRAF3 (a prospective long and short isoform) and TRAIL. Spatio-temporal studies using immunofluorescence and immunocytochemistry illustrated the localisation in the lens of the proteins examined. While TRAIL had no statistically differential expression in either Western or immunohistological studies there was specific expression in the epithelium especially around the equatorial region which proposes a role for TRAIL in signalling the early stages of epithelial cell differentiation.

The short isoform of TRAF3 (30-35 kDa) exhibited increased expression during lens development in Western blotting, and in the subcellular fractionation studies was present in all the fractions, whereas the 64 kDa fraction was absent from the cytosolic fraction. Immunofluorescence found increasing expression of TRAF3 in the differentiating fibre cells of the lens cortex prior to them being incorporated into the central fibre cell mass, suggesting that when they no longer had any physical contact with the epithelial cells or the basement membrane the TRAF3 signal was abruptly lost.

TRAF2 had decreasing protein expression with development during Western blotting which, along with immunofluorescence expression suggests that expression at earlier stages of lens development merits further study, though the lack of expression at later stages suggests that it does not have a role during later stages of lens development. The considerable expression of TRAF2 in the iris, ciliary body and in the corneal-scleral boundary increased as development of the embryo continued. When compared to published expression of known muscle markers; eg αSMA, this suggests a role in ciliary muscle and iris stromal development. The expression at the scleral-corneal boundary may indicate a role in limbal stem cell differentiation.

The novel expression of TRAF2 and TRAF3 in the developing eye has given the opportunity to begin a new path in the discovery of the factors involved and subsequently their roles through functional studies in the future.

Chapter 5:
Optimisation of whole chick lens culture

5.1 Introduction

The lens in the eye is an accepted model for studying various developmental events including proliferation, cellular specialisation, differentiation and maturation (Hawse *et al.*, 2005). Menko *et al* (1984) developed lens epithelial culture. In the intervening time, the method has been well characterised and optimised in many species and has been useful in many studies (Taylor-Papadimitriou *et al.*, 1977; Taylor-Papadimitriou *et al.*, 1978; Wride, 1996). In comparison to epithelial cell culture, the experimental protocol for lens organ culture between laboratories in the published papers was seen to vary greatly.

Whole lens culture has been sporadically used since 1991 (Hightower & McCready, 1991). In this study, lenses from 4 week old rabbits were cultured in medium and used to identify the damage caused by Selenite in whole lens and whether the affect it has on membrane permeability and cell-cell transport is localised to specific regions of the lens.

Previous research has used lens organ culture in a number of studies including "The role of Src family kinases in cortical cataract formation" (Zhou & Menko, 2002) and the phosphorylation of connexins in lens organ culture to identify lens organ culture as a valid *in vitro* model in functional studies of connexins (Jiang & Goodenough, 1998). Lens organ culture has been used in various papers, but there are different methods for culture. Unlike lens epithelial cell culture, there has been no published research to show the optimum protocol for whole lens culture.

Previous research has used Medium 199 with the lens alone (Zhou & Menko, 2002), with the vitreous attached to the lens (Weber & Menko, 2005), with the iris and vitreous cultured with the lens (Jiang & Goodenough, 1998) and the lens cultured in medium 199 with glucose (Zhou & Menko, 2004) as an option in whole lens culture. In this chapter, we will characterise the optimum method for whole lens culture in order to provide an additional method for carrying out functional studies to investigate the role of various factors in lens development (including TNFs and TNF-related molecules). Undertaking a histological comparison between whole lens cultures and lenses removed from embryos at the corresponding developmental time points will

reveal any differences in development between the lens developing *in ovo* and the lenses grown in culture.

5.2 Experimental Design

Two cell culture media of different composition were made up prior to eye dissection: one with and one without 14mM Glucose (Sigma, UK). Other than this difference, both were made with Medium 199 (22350 Gibco, UK), 10% Foetal Calf Serum (FCS), (Biosera, UK) and 0.1μg/ml penicillin (Penna-100mu, Sigma, UK) and streptomycin (s-6501, Sigma, UK). While whole eyes were dissected from chick embryos, the media were placed in a water bath at 37°C. The eyes were then placed in a solution of 1:8 betadine: distilled water to sterilise them before being placed under a class II cell culture hood so that the fine dissection (e.g. removing the lens) would be carried out under sterile conditions. The lenses were prepared in one of three ways: either the lens was separated from all surrounding tissue; with only the vitreous attached or with both the surrounding iris and vitreous attached. Within 2 minutes, the betadine was washed from the eyes using 1x PBS, the eyes were further dissected in the medium they would be cultured in. Once the excess tissue was removed, the lenses were placed in fresh medium and transferred to a cell culture incubator (5% CO_2/ 95% air). After 4 hours, the lenses were checked under a microscope for any opacity, which would indicate the lens had been damaged during dissection; such lenses were removed from the experiment and disposed of. The lenses that remained were replaced in the incubator. The medium was replaced every two days and three lenses from each culture condition were removed to be fixed and embedded in paraffin wax at 2 day intervals throughout the period of culture every day for 8 days. The lenses were then sectioned and stained using H & E.

During this experimental chapter a number of issues were identified with the methodology. Due to the fragility of the lens, the size and softness of the organ was a problem, to minimise the mechanical trauma that could occur during dissection of the lens, extensive practice was undertaken to improve technique. After 4 hours of culture, lenses were examined using dark field microscopy to identify any lenses damaged during the process of dissection. Any damaged lenses were removed from the experiment and disposed of. To examine and identify any opacifications, the lenses had

to be isolated from any surrounding tissues cultured with the lens, in these situations the lens could not be examined for damage or opacifications until culture had been completed. Once completed the surrounding tissue was then removed from the lens and those with no visible damage were photographed using dark field microscopy and fixed as described in 2.5.6. When lenses had been fixed and were to be embedded in wax it was discovered that without the surrounding tissue for guidance it was very difficult to orientate the lens correctly. To overcome this problem the lenses were placed for 10 seconds in haematoxylin (after dehydration) to express a light stain making the lens more visible when placed in the molten wax block therefore making it less difficult to correctly orientate the lens. In a number of papers it was stated that experienced technicians were used for dissection and especially for embedding the lenses in the correct orientation due to the difficulty of the technique (Ghosh & Zigler, 2005).

5.3 Results

5.3.1 Darkfield visualisation of cultured whole lenses.

Before lenses were fixed overnight with 4% Paraformaldehyde in PBS at 4°C, they were washed with ice cold PBS and any surrounding tissue blocking the view of the lens was removed. Images of the lenses were taken with a Leica microscope on a dark field platform, this allowed the visualisation of any opacities found in the cultured lenses.

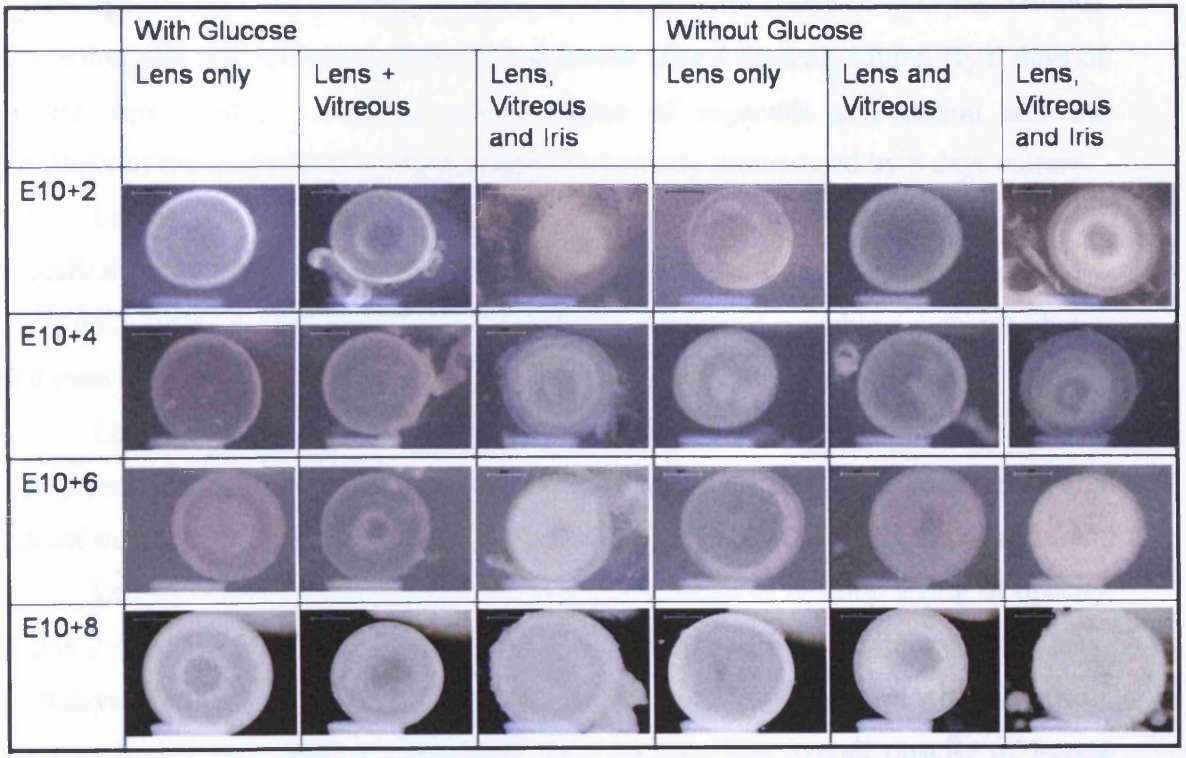


Figure 5.1 Dark field images of E10 lenses cultured for 2, 4, 6 and 8 days (as shown on left of table) with various methods (at the top of table), scale bar 0.5mm.

Table 5.1 Showing the amount of opacification in whole lens cultures, from zero = no opacity upto 5 = extensive opacity.

		With glucose			Without glucose		
		Lens only	Lens+ Vitreous	Lens, Vitreous+Iris	Lens only	Lens+ Vitreous	Lens, Vitreous+Iris
E10 lenses + days in culture	E10 + 2	1	1	3	1	1	2
	E10 + 4	1	1	3	2	1	2
	E10 + 6	2	2	3	2	2	3
	E10 + 8	4	3	5	3	5	5

The dark field images above (figure 5.1) show any opacification of the whole lens cultures. This data was obtained in duplicate, and the amount of opacity was measured as stated in section 2.5.5, briefly the overall opacity of the lens was measured in three places, the centre, the periphery and the centre of the radius of the lens. The background reading was then subtracted from the average of the above three readings. The final amounts (which can be found in Appendix 4, table A4.1) was then converted into a reading between 0 and 5, where 0 was no opacity going up to five where there was the most extensive opacification. For lenses cultured alone with glucose, there was a faint opacification around the periphery of the lens with flecks of opacities towards the centre; this was noticed in many of the lenses after 4 days of culture. By 6 days of culture, opacification at the presumed region of organelle degradation and the periphery of the lens becomes more defined and clearly pronounced by 8 days culture.

Lenses with vitreous in medium containing glucose showed a faint ring of opacification in the centre of the lens from 2 days in culture, this became gradually more noticeable as culture continued and this is confirmed by table 5.1 which shows that even by 8 days in culture there is only a three out of five opacity reading.

Lenses with both vitreous and iris cultured in medium containing glucose show clear opacification in a central ring of the lens, which became more widespread and intense during the period of culture resulting in extensive opacity by 8 days of culture.

Lenses cultured in medium without glucose begin to develop a ring of opacity within 2 days of culture that intensifies and spreads towards the periphery of the lens. At 6 days of culture, an obvious peripheral opacification has developed with the rest of the lens becoming slightly cloudy by 8 days culture. The overall opacity of lenses cultured this way by 8 days in culture was the least found in the lenses cultured in medium without glucose at a 3 out of 5 reading.

A light cloudiness is perceptible in a wide band around the lens at both 2 and 4 days of culture in medium without glucose, with vitreous attached to the lens. A vague secondary ring towards the middle of the radius of the lens is seen at 6 days in culture with a wide deeply opaque ring visible at 8 days in culture resulting in an extensive opacity reading of five.

Cultured lenses, with iris and vitreous attached, in plain medium show two clearly defined rings of opacification in the lens within 2 days of culture that becomes

wider after 4 days and denser until, at 8 days in culture, it is apparent that the whole lens is extensively opacified which is confirmed with an opacity reading of 5 out of 5 (table 5.1).

5.3.2 Haematoxylin and Eosin staining.

On the following pages, figures 5.3 – 5.7 show examples of H & E staining obtained from fixed and sectioned lenses cultured alone, with the vitreous or with the iris and vitreous attached, in medium 199 with or without glucose.

H & E staining was used to highlight any variations in the morphology of the lenses in culture and in comparison to lenses, at the corresponding time points, that had been fixed, embedded, sectioned and stained directly after being dissected from the embryo. In normal early lens development (prior to E10-E12), well characterised in previous research and described in section 4.3.2.1, nuclei are found to run through the centre of the lens in lens fibre cells from equator to equator. However, at E10 nuclei at the centre of the lens begin to condense and at E12 pycnotic nuclei are clearly visible at the centre of the lens. By E14, nuclei at the centre of the lens have disappeared and the organelle free zone (OFZ), characterised by the lack of nuclei, is clearly visible. As development continues, the OFZ expands in size as more fibre cells migrate towards the centre of the lens and the organelles and nuclei contained within them are degraded. At the border of the OFZ, nuclei condense and become pycnotic in nature until they are no longer visible (reviewed in Wride, 1996; Bassnett, 2002).

Any variations in the cultured lenses from the standard lens developmental process *in vivo* will be clearly visible in the following H & E images. The images were obtained from the sections through the centre of the lens which were found by measuring the size of multiple sections, from multiple slides, under the microscope. The largest one was taken as the section through the centre of the lens.

Measurements of the largest lens sections were carried out so comparisons between the various culture methodologies could be done. The measurements done are shown in figure 5.2. Briefly, the full width (fig. 5.2, line a) and anterior to posterior length (fig. 5.2, line b) was measured, the lens was then broken down to the width (fig. 5.2, line c) and length (fig. 5.2, line d) of the OFZ shown as a gray circle at the centre

of the lens in figure 5.2. The distance of the OFZ from the anterior and posterior of the lens was also measured, shown in figure 5.2 as line e and f, respectively.

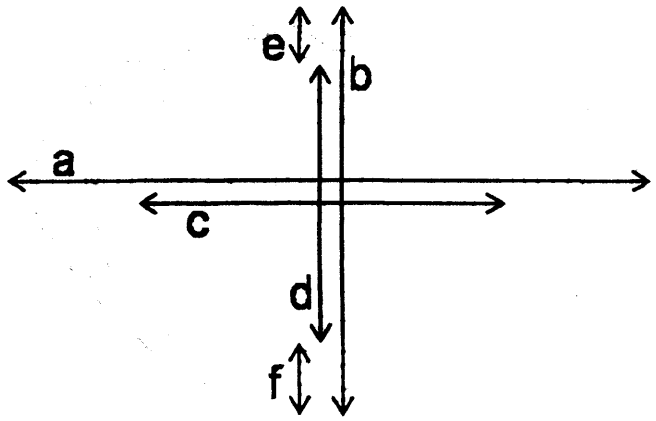


Figure 5.2 Diagram showing the measurements of the lenses used in this experiment. The first measurement taken is the grey circle at the centre of the lens showing the area of the OFZ, this was only done when relevant (ie after E12). a=length of lens, b=height of lens, c=length of OFZ, d= height of OFZ, e=distance between anterior of the lens and the top of the OFZ and finally f= distance between posterior of the lens and the lowest most point of the OFZ.

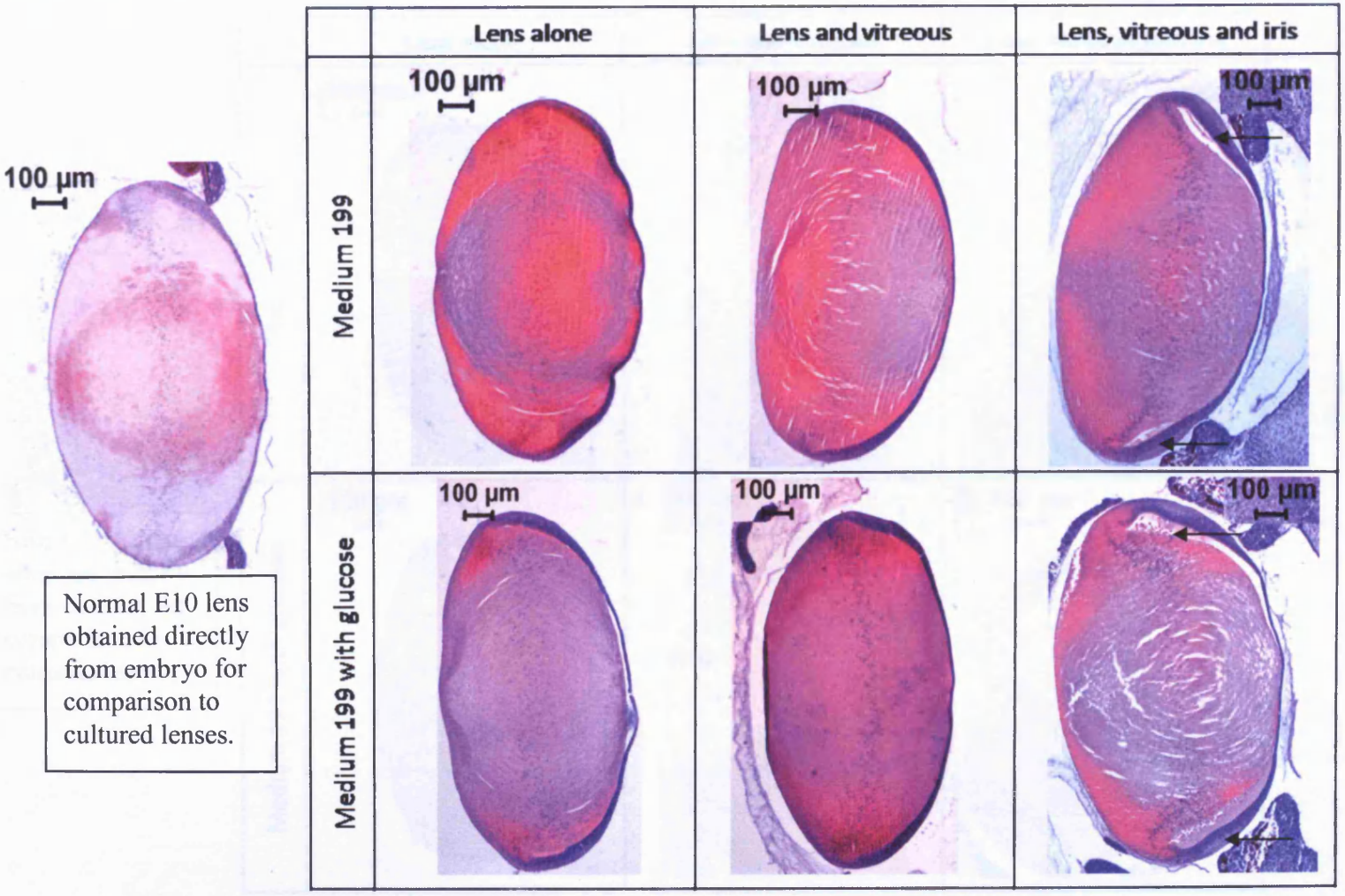
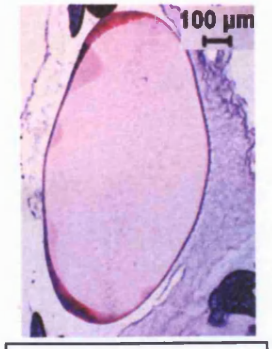


Figure 5.3 E10 lens cultured for 4 hours. E10 lens obtained directly from an embryo (far left), within the table, lenses cultured for 4 hours with or without glucose, either culturing the lens alone, lens with vitreous attached or lens with iris and vitreous attached. ← Showing the disassociation of the epithelium layer from the fibre cell mass.



Normal E12 lens obtained directly from embryo for comparison to cultured lenses.

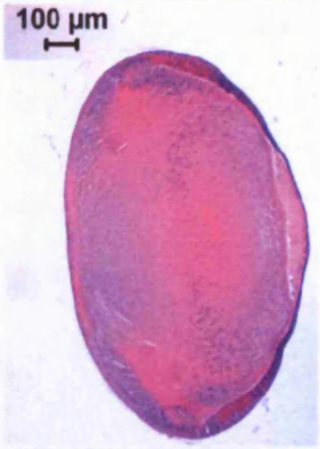
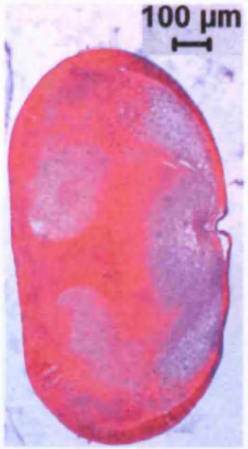
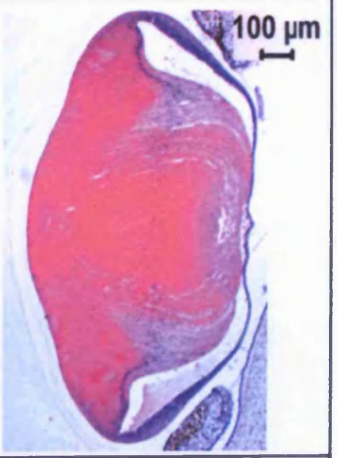
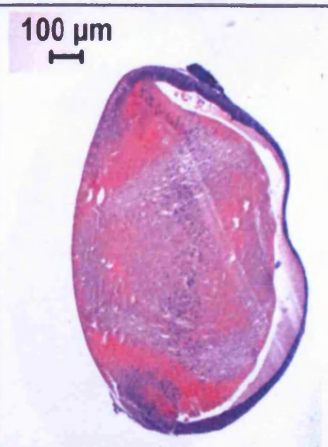
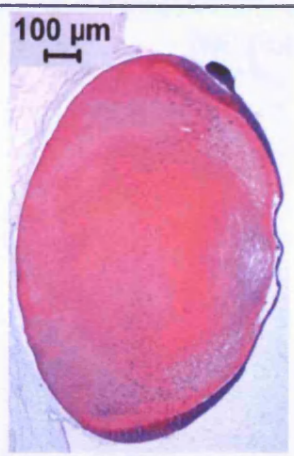
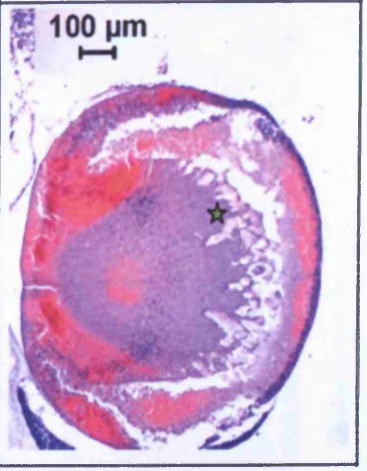
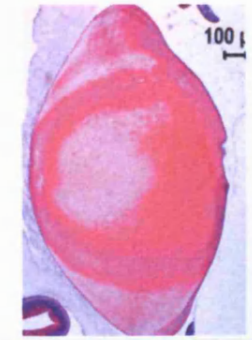
	Lens alone	Lens and vitreous	Lens, vitreous and iris
Medium 199			
Medium 199 with glucose			

Figure 5.4 E10 lens cultured for 2 days. E10 lens obtained directly from an embryo (far left), within the table, lenses cultured for 2 days with or without glucose, either culturing the lens alone, lens with vitreous attached or lens with iris and vitreous attached. ★Indicating the degradation at the anterior of fibre cells.



Normal E14 lens obtained directly from embryo for comparison to cultured lenses.

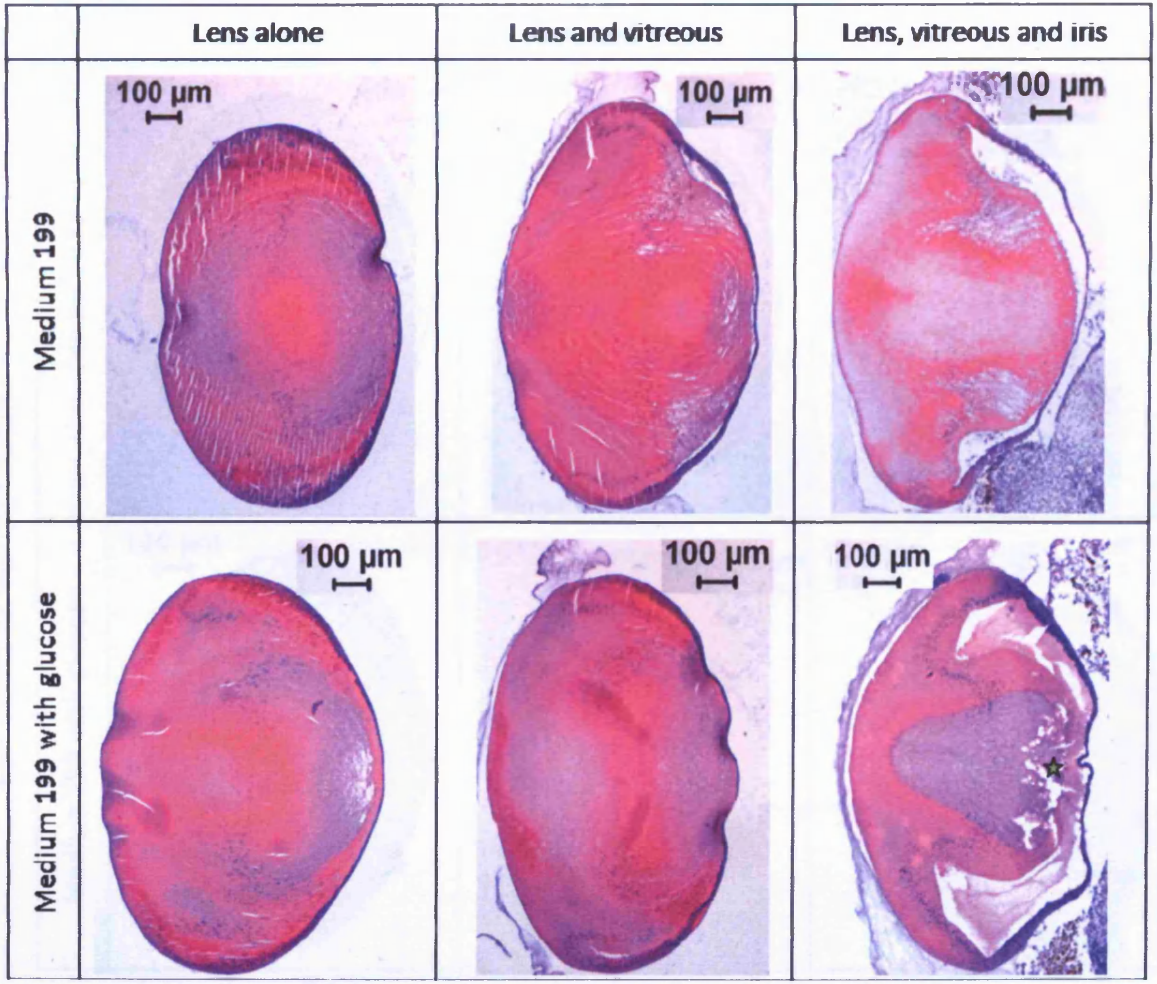
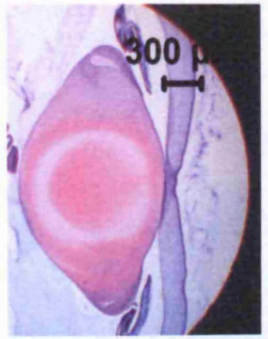


Figure 5.5 E10 lens cultured for 4 days. E10 lens obtained directly from an embryo (far left), within the table, lenses cultured for 4 days with or without glucose, either culturing the lens alone, lens with vitreous attached or lens with iris and vitreous attached. ★Indicates the degradation at the anterior of fibre cells.



Normal E16 lens obtained directly from embryo for comparison to cultured lenses.

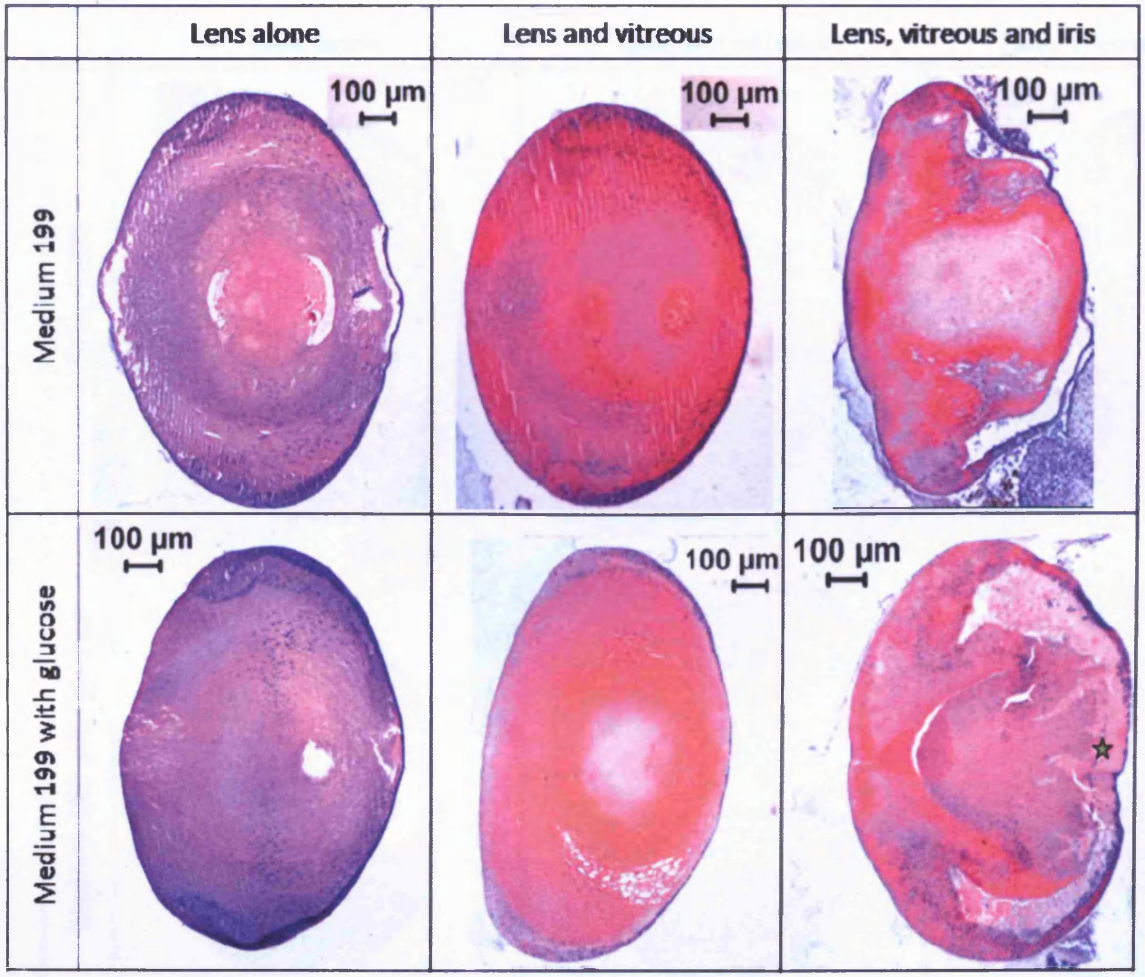
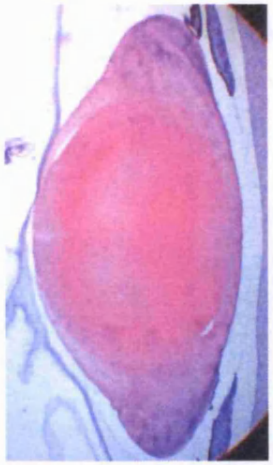


Figure 5.6 E10 lens cultured for 6 days. E10 lens obtained directly from an embryo (far left), within the table, lenses cultured for 6 days with or without glucose, either culturing the lens alone, lens with vitreous attached or lens with iris and vitreous attached. ★ Indicates the degradation at the anterior of fibre cells.



Normal E18 lens obtained directly from embryo for comparison to cultured lenses.

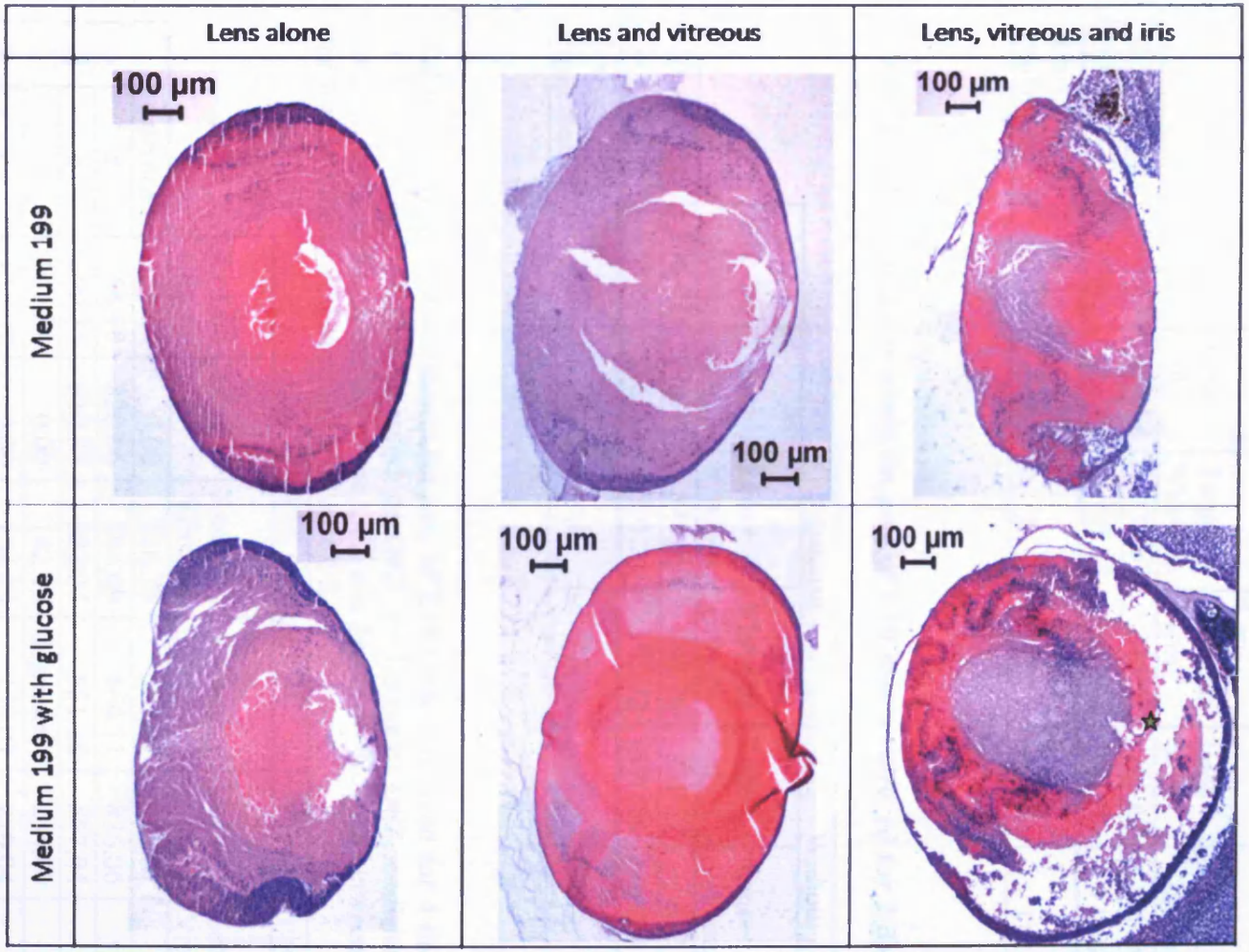


Figure 5.7 E10 lens cultured for 8 days. E10 lens obtained directly from an embryo (far left), within the table, lenses cultured for 8 days with or without glucose, either culturing the lens alone, lens with vitreous attached or lens with iris and vitreous attached. ★Indicates the degradation at the anterior of fibre cells.

Table 5.2 Mean measurements (in μm) of E10 lenses cultured for 4 hours. a=length of lens, b=height of lens.

			With glucose			Without glucose		
		E10 no culture	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris
E10 + 4hours	a	946.63	914.34	870.67	815.76	763.43	808.08	871.41
	b	595.45	518.53	535.33	537.73	459.71	478.03	510.45
Lens replicates		6	2	3	2	2	1	1

Table 5.3 Mean measurements (in μm) of E10 lenses cultured for 2 days. a=length of lens, b=height of lens.

			With glucose			Without glucose		
		E12 no culture	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris
E10 + 2days	a	896.02	774.49	841.49	869.76	931.57	812.80	982.74
	b	530.24	516.43	591.20	591.72	570.09	474.83	531.98
Lens replicates		2	1	1	2	2	1	2

Table 5.4 Mean measurements (in μm) of E10 lenses cultured for 4 days. a=length of lens, b=height of lens, c=length of OFZ, d= height of OFZ, e=distance between anterior of the lens and the top of the OFZ and finally f= distance between posterior of the lens and the lowest most point of the OFZ.

			With glucose			Without glucose		
		E14 no culture	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris
E10 + 4days	centre	328.44	0.00	716.28	383.27	458.67	339.29	326.73
	a	758.18	820.06	784.09	864.11	836.06	866.58	810.49
	b	509.34	610.37	522.25	551.18	557.04	582.02	514.41
	c	164.64	0.00	142.13	degraded	204.00	158.00	176.01
	d	150.07	0.00	154.05	250.07	210.04	334.00	231.15
	e	106.84	0.00	228.04	124.00	146.01	108.00	103.08
	f	104.64	0.00	230.14	120.00	146.05	104.00	104.12
Lens replicates		3	2	2	2	1	2	2

Table 5.5 Mean measurements (in μm) of E10 lenses cultured for 6 days. a=length of lens, b=height of lens, c=length of OFZ, d= height of OFZ, e=distance between anterior of the lens and the top of the OFZ and finally f= distance between posterior of the lens and the lowest most point of the OFZ.

			With glucose			Without glucose		
		E16 no culture	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris
E10 +6days	centre	1130.97	342.21	1047.20	467.04	926.77	792.94	542.45
	a	1428.00	716.17	954.21	903.02	920.19	835.53	804.20
	b	864.75	596.59	650.36	527.11	666.58	664.81	522.39
	c	228.32	106.13	113.13	degraded	284.92	239.17	133.97
	d	264.00	103.18	219.00	287.04	243.29	199.35	211.41
	e	360.00	192.76	333.18	88.03	294.36	249.85	175.55
	f	341.32	201.83	333.38	86.02	295.31	251.64	172.26
Lens replicates		2	3	3	2	3	3	2

Table 5.6 Mean measurements (in μm) of E10 lenses cultured for 8 days. a=length of lens, b=height of lens, c=length of OFZ, d= height of OFZ, e=distance between anterior of the lens and the top of the OFZ and finally f= distance between posterior of the lens and the lowest most point of the OFZ.

			With glucose			Without glucose		
		E18 no culture	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris	Lens only	Lens+ Vitreous	Lens, Vitreous +Iris
E10 + 8days	centre	719.42	947.19	848.23	304.73	656.59	511.03	653.45
	a	648.62	825.39	860.87	813.40	794.11	841.38	901.12
	b	398.17	589.57	619.59	729.21	532.65	605.27	477.03
	c	63.45	72.57	97.43	degraded	108.56	140.39	52.26
	d	109.44	213.61	257.76	273.71	222.53	295.09	216.12
	e	227.95	303.15	271.53	130.60	208.73	140.77	209.33
	f	227.27	300.25	269.05	144.50	212.23	140.26	208.78
Lens replicates		4	4	3	2	2	3	2

In all the previous figures (5.3-5.7), the clearest, largest sections showing the centre of the lens were chosen. At all variations, at all time points, a minimum of 3 lenses were sectioned and stained using the same methodology as stated in section 2.5.6. Looking at figure 5.3, the variability between the lenses (i.e. lens directly from embryo and lenses grown under different culture conditions) is already beginning to be

visible after only 4 hours in culture. In each set of cultures carried out (4 lenses per difference in culture) the lenses were obtained from the same batch of eggs, which were incubated at the same time; the lenses were dissected and placed in culture within as short a time frame as possible, the only differences between their treatments being that clarified in the experimental protocol (i.e. the addition or lack of; glucose/ vitreous/ vitreous and iris). After 4 hours, lenses were checked under a darkfield microscope and any imperfections that were observed resulted with the lenses being disposed of. Unfortunately a number of the lenses that were cultured were embedded in the wrong orientation resulting in the lens not being sectioned through its centre. When this occurred the lens was discarded and measurements could not be taken which meant that the required number of replicates (a minimum of 3) were unavailable for statistical analysis of the data though the mean of the measurements that were obtained are shown in tables 5.2-5.6.

In figure 5.3, lenses from all culture variables, after 4 hours in culture, were stained and imaged. From the E10 lens, on the far left, the differentiation of the lens fibre cells can be clearly seen at the equatorial regions with the nuclei all following the same line through the centre of the lens. This line is also found in all the culture variations at the same time point, though in all of these cultured lenses the nuclei are shown to be slightly more dispersed and the nuclei of the fibre cells just beginning to elongate at the equatorial regions are shown to be found closer to the epithelial layer than those at the centre of the lens. This was most noticeable in the lenses cultured with both the iris and vitreous attached. In lenses with iris and vitreous, in both medium with and without glucose, the epithelial layer was found to have pulled away from the fibre mass at the equatorial region shown in the arrows on images of lens with iris and vitreous atached, figure 5.3.

After two days culture (figure 5.4), the morphological variations become more apparent with the nuclei of the newly differentiating fibre cells clearly migrating towards the anterior of the lens where the epithelial layer can be found. The nuclei of the older fibre cells, found at the centre of the lens, have maintained the position of the corresponding cells found in *in ovo* lenses (far left image in figure 5.4) at the same time point. In the lens cultured alone in medium without glucose, the nuclei at the centre of the lens were the most tightly packed in a line progressively losing this order

in younger fibre cells in the cortex; i.e. further from the centre and nearer the equatorial region. Lenses with vitreous attached cultured in both types of medium contained nuclei in central lens fibre cells and these were pycnotic in nature, although the number of these was clearly less than those found in the centre of the uncultured lens (figure 5.4, far left). In all cultures, variations in the morphology of the lens between culture methods, including separation of the epithelial layer from the fibre mass, were visible. This dissociation is most noticeable in the lens cultured without surrounding tissue in medium with glucose and both types of culture medium containing lenses with the iris and vitreous attached. The cultures of the lenses with the iris and vitreous attached in the medium containing glucose has a visibly degraded morphology of the fibre cells at the anterior of the lens (indicated by a star, bottom right, figure 5.4); the epithelial layer has thickened with multiple cell layers spreading towards the fibre cell mass which itself is ruined, the fibre mass is no longer defined, with the ends of the fibre cells closest to the epithelial layer separating and tearing from one another.

Lenses cultured for four days (figure 5.5) were all found to have the nuclei of the newer fibre cells migrating towards the anterior of the eye, towards the epithelial layer, rather than the normal situation in which they migrate along the equator as they differentiate into fibre cells (shown in the normal lens, far left, figure 5.5). In the lenses cultured with both the iris and the vitreous, in either type of medium, the epithelial layer was again found to be separated from the fibre cell mass with the anterior-most parts in the fibre cells degrading and tearing in the lenses cultured in medium containing glucose (figure 5.5, star). At the centre of the lens, not cultured (figure 5.4 far left), the fibre cells at the centre of the lens are empty and OFZ has formed. A corresponding region, though smaller, can be found in all cultured lenses except that of the lens cultured alone in medium containing glucose, of four lenses sectioned and stained for this culture variation only one seemed to contain an OFZ, which was extremely small, and was one of two lenses that were in the wrong orientation and therefore measurements could not be taken.

By six days in culture (figure 5.6) all OFZs had increased in size, with the smallest OFZ being found in lenses cultured alone in medium containing glucose (confirmed by the measurements in table 5.5). The nuclei of the newer fibre cells continue to migrate to the anterior of the eye with the original migrating nuclei, after

the start of culture, having reached two thirds of the way across the anterior of the lens in cultues with and without vitreous in plain medium. The epithelial layer in two out of three lenses cultured with vitreous, in medium containing glucose, were found to have separated from the fibre cells. In lenses cultured with iris and vitreous attached, the epithelial layer was separate from the fibre cell mass and under both medium conditions (plus and minus glucose) the newly differentiating fibre cells were noted to have limited elongation. In lens, with iris and vitreous attached, cultured in medium containing glucose, the limited elongation of differentiating fibre cells is clearly visible with these cells following migration along the posterior of the lens and no longer extending towards the epithelium.

At eight days in culture, more mechanical tears occurred during sectioning, as can be seen in all images of the cultured lenses. In all cultured lenses, after eight days in culture, it was clearly visible that the elongation of the fibre cells at the equatorial region was delayed as these fibre cells continued to migrate towards the posterior of the lens to a certain extent. The least migration towards the posterior of the lens was seen in lenses cultured with vitreous in medium, both with and without glucose, while the most dramatic alteration from normal fibre cell differentiation was observed in lenses cultured with iris and vitreous attached, in medium containing glucose. In these lenses, differentiating fibre cells completely failed to elongate instead continuing to migrate to the posterior of the lens until reaching the counterpart layer of cells from the opposite equator, at this point the fibre cells became more densely packed, folding back on themselves in an apparently random manner.

5.4 Discussion

In previous research on whole chick lens culture, the effects of glucose in the medium were shown to have a role in preventing the formation of cortical cataract due to the developing lens having a high rate of metabolism that was not supported in medium 199 alone (Zhou & Menko, 2002). In figure 5.1 (and table 5.1), this conclusion was supported, to a small extent, in earlier stages of culture with or without glucose in the medium for lenses cultured alone and with vitreous attached. By 8 days in culture

intense opacification was visible in all lenses, in all variations of culture, though to a lesser intensity in lenses with vitreous attached cultured in medium containing glucose.

Though further replications need to be carried out to confirm and clarify the results, the preliminary data suggests that the use of vitreous in culture with glucose helps to maintain the clarity of the lens for a longer period than any other culture method.

The use of tissue from the surrounding eye was shown to have a large affect in the opacification of the lens which had the most extensive opacification at any of the timepoints in comparison to lenses cultured alone or with vitreous attached. It is possible that leaving some of the surrounding tissue attached, i.e. the iris, meant that the nutrients in the medium were not as accessible to the lens therefore causing an increase in opacity.

The use of darkfield microscopy in whole lens culture can give insight into the balance of factors required for maintaining the clarity of the lens through the addition of supplements to the medium, such as glucose as used in this chapter.

Lenses cultured with iris and vitreous attached in medium containing glucose, displayed gross morphological alterations. The signal for the elongation of fibre cells within these cultured lenses, at any time point, was severely limited. This lack of fibre cell elongation towards the anterior of the lens can be seen at the equatorial region, from the earliest time point of only 4 hours in culture. Though elongation did occur to a limited degree, this was only visible when there was open space in the equatorial region of the lens, due to the disassociation of the epithelial layer from the fibre cell mass. During culture, the epithelial cells continued to proliferate and migrate towards the posterior of the lens, due to this the space at the posterior of the lens became condensed. By 8 days of culture, the layer of cells that would normally be elongating fibre cells had condensed as they moved towards the posterior of the lens taking on the morphology of an epithelial cell layer. This layer folded away from the basement membrane when it reached the corresponding cell layer from the opposite equator, it continued to fold into a different direction whenever it encountered an obstruction such as the layer itself or the fibre cell mass. The length of this cell layer suggests that the differentiation of fibre cells, after the start of culture, had ceased and while epithelial cells appeared to be continuing to proliferate and migrate, the signals for the elongation

of fibre cells and the incorporation of these cells into the OFZ seemed to be entirely lost. Though the "differentiating fibre cells" that were initiated after the start of culture seem to have entirely lost their signal to differentiate, the continued development of the OFZ in culture suggests that those cells that had already been induced to differentiate before the lenses were removed from the embryo continue to differentiate resulting in the loss of organelles, denucleation and the cells becoming part of the OFZ at the centre of the lens.

In all cultured lenses, at eight days, mechanical tears were caused during sectioning, though this was attempted to be corrected by softening the tissue before sectioning the tears still occurred. This may suggest an underlying weakening of the the lens tissue. At 8 days in culture it was also clearly visible that the differentiation of the fibre cells at the equatorial region was delayed as the epithelial cells continued to migrate towards the posterior of the lens. From studying the morphology, the most abnormal is found in lens with iris and vitreous cultured in medium with glucose as discussed above. The lenses with iris and vitreous cultured in plain medium were slightly improved but those lenses cultured without iris, in either medium, were significantly more morphologically normal. From histological examination it can be seen that differentiation gets progressively more delayed, with differentiation being initiated gradually more posteriorly as the lens continues to grow in culture. To identify whether the lens epithelial cells are in fact differentiating into fibre cells staining with antibodies to various genes would be useful. An example of a suitable gene to identify fibre cells is βB1-crystallin (Cui *et al.*, 2004).

In transgenic mice Insulin-like growth factor-1 (IGF-1) was over expressed resulting in a delay in the differentiation of the epithelial cells to a more posterior point, a similar morphology to that above, there was also increased proliferation in the epithelial cells. IGF-1, also identified in the chick lens as lentropin, has been shown to increase proliferation and differentiation in lens epithelial cell culture. FGFs, described in section 1.7.5, as well as IGF-1 were first detected in the vitreous humour by Arnold *et al* (1993) and the vitreous has been known to contain factors that affect differentiation since the 1960's (Coulombre *et al.*, 1963). The presence of these factors in the vitreous of the lenses cultured may have contributed to the slower development of opacity in the lenses cultured with vitreous and glucose than the lens cultured alone.

The large morphological alterations between lenses cultured with vitreous and lenses cultured with iris and vitreous attached in glucose tell us it is not possible that the factors such as IGF and FGF from the vitreous themselves caused the large abnormalities in the lenses cultured with iris and virteous. It is reasonable to assume that signals are coming from the iris which either directly or indirectly (through other factors from the vitreous), are stopping differentiation in the lenses cultured with iris and vitreous. It is also possible that proliferation is being separately upregulated through the addition of glucose in the medium or through other factors in the iris. Though addition of IGF or FGF signalling from the iris directly could be resulting in their over expression in the lens which may explain the increased proliferation in the epithelial cells (Shirke *et al.*, 2001).

The cause of the degradation of the anterior parts of the fibre cells in lenses cultured with iris and vitreous attached in medium containing glucose could be due to the disassociation of the epithelial layer from the fibre cell mass which may have severely reduced the signal of factors, such as that of the Wnt's and receptors, into the fibre cells resulting in a lack of elongation and structural maintenance contributing to the degradation seen (Chen *et al.*, 2008b). The reason for the disassociation of the epithelial layer is unknown though it is highly unlikely to be due mechanical insult as all lenses in this culture variation had the same morphology.

The signals from the surrounding ocular tissue have been consistently researched through the years and this methodology, once completely characterised, may provide further insight into the roles of these tissues and the factors released from them. FGFs are prime candidates for the unknown signal that induces epithelial cells to differentiate into fibre cells. The signal is thought to be emitted from the retina and produce a gradient from the posterior to the anterior possibly determining the boundary of the epithelial cells (Lovicu & McAvoy, 2005). From the results of this culture experiment it is not likely that it is a signal from the retina that induces the epithelial cells in culture to begin differentiation initially as the lenses cultured without any surrounding tissue do begin elongating and migrating towards the centre of the lens which are characteristics of differentiation. If this culture experiment was repeated with the retina it is plausible that it could be identified whether the signals from the retina have any role in inducing the terminal differentiation of the new (after culture

had started) fibre cells and also if the signals inducing the gradient suggested to occur above, maintains the nuclei of the differentiating fibre cells to the equator of the cultured lenses.

A similar role to FGF has been proposed for BMPs (Belecky-Adams *et al.*, 2002) described in 1.7.3. Wnt signals described in 1.7.6, are also known to have great involvement in various stages of development and is known to both enhance the FGF signal for lens fibre cell differentiation (Lyu & Joo, 2004) and, very recently, transgenic mice over expressing a wnt signalling antagonist showed attenuated elongation of fibre cells which were also disordered in structure implying a role of Wnt signals in the organisation and structure of the cytoskeleton (Chen *et al.*, 2008b). It is possible that the expression of Wnt's and associated factors present in the lens epithelium, transition zone and iris, such as Wnt7a in the developing murine lens (Ang *et al.*, 2004), may have a role in the abnormal morphology of the older lens cultures grown in glucose with the iris and vitreous still attached.

For definitive results a minimum of one more set of cultures would be required to quantify and statistically analyse the images but this was not possible in this PhD due to the financial and time constrictions. Any attempts to carry out any relevant measurements were found to vary too greatly between replicates to obtain any significant numerical data. For a thorough, quantifiable experiment a larger number of replicates would be preferable, to be able to incorporate the differences in development prior to culture. As a result only a visual comparison was undertaken though the corresponding numerical data was included in the results.

From the morphological study it is hypothesised that despite the abnormal differentiation of new epithelial cells into fibre cells at the equatorial region (during culture), the existing differentiating fibre cells (induced prior to culture) continue to undergo terminal differentiation resulting in the loss of their nuclei as usual. It is known that it takes between 4 and 5 days for a cell that has just begun differentiation to elongate and lose all its organelles before it enters the OFZ during normal lens development (Beebe *et al.*, 2001). If this time (for complete differentiation) is not severely increased during whole lens culture a future experiment of the incorporation of BrdU into whole lens at the start of culture may shed light onto whether the novel

hypothesis that the new (after the start of culture) differentiating fibre cells cannot terminally differentiate is correct.

Chapter 6:
Discussion

6.1 Discussion

During development, epithelial cells at the equator of the lens begin a differentiation process to become secondary fibre cells. The differentiating cells begin to elongate and migrate to the centre of the lens with tips of the cells attached to the epithelial layer and the basement membrane. As the cells reach the centre of the lens, they envelop the fibre cells that have migrated to the centre previously; the tips of the cells meet their counterpart fibre cells from the opposite equator and move down the suture losing contact with the basement membrane and epithelial layer. This migration wraps the cell around the central fibre mass of differentiating fibre cells that have undertaken the process previously. As these cells differentiate and migrate, the intracellular organelles begin to degrade culminating, with complete degradation and denucleation, at the organelle free zone (OFZ) (reviewed in Bassnett, 2002; Wride, 2007; Bassnett, 2008). The OFZ is a transparent tissue that is free from any organelles that would scatter the light entering the eye. Any flaws in the signalling can result in opacities in the lens known as cataract.

The molecular pathways involved in these processes are numerous, have been found to be increasingly complex and many are still to be elucidated (Wride, 2007; Bassnett, 2008). The pathways involved in terminal differentiation of fibre cells have not been confirmed though a number of possibilities have been published. These include the suggestions of;

- an attenuated form of apoptosis; though a large number of apoptosis genes have been shown to be expressed in the developing lens (Dahm, 1999; Wride, 2000; Bassnett, 2002; Wride *et al.*, 2003; Mansergh *et al.*, 2004; Geatrell, 2007b; Geatrell, 2007a), functional studies have not elucidated their roles within this organ; indeed knockout mice of executioner caspases did not identify any defects in the development of the OFZ (Zandy *et al.*, 2005).
- ubiquitin proteasome pathway (UPP); research has shown that during bFGF-induced lens cell proliferation and differentiation in rat lens explants, components of the UPP show differential expression suggesting a role in both lens cell proliferation and differentiation (Guo *et al.*, 2004; Guo *et al.*, 2006). A

study showing nuclear localisation of UPP components supports the theory that they are involved in denucleation (Girao *et al.*, 2005).

- Lipoxygenase; identified to have a role in reticulocyte organelle degradation and identified in the lens epithelial cells and in the fibre cells where organeele degradation occurs (Arora *et al.*, 1996; Grullich *et al.*, 2001).
- Autophagy; is no longer thought to be involved in organelle degradation (Matsui *et al.*, 2006) though lysosomal enzymes such as DNase IIβ have been identified in the lens lysosmes which is released into the cytoplasm as differentiation continues (De Maria & Bassnett, 2007).

In this thesis a number of TNFs and their downstream signalling molecules were identified in the developing chick lens. These include The TRAF family, EDA, EDAR, EDARADD, TRAIL, DR5, TACE and RAIDD. The EDA family have previously been shown to be expressed in the developing mouse and are known to be required in ectodermal organ development such as in mouse tooth and hair development (Pispa *et al.*, 2003), similar expression has been found in the developing chick feather tract though its function is thought to be more of a maintenance role than inducing development (Houghton *et al.*, 2005; Houghton *et al.*, 2007). EDA and EDAR have also been shown in the mouse lens, though the research suggested the staining was non-specific (Pispa *et al.*, 2003). EDA, EDAR and EDARADD are known to be required in ectodermal organ development such as in mouse tooth and hair development and have now also been shown here to be expressed in the chick lens, though their functional roles have not been elucidated. TACE was shown to be expressed in the E12 chick embryo and research targeting deletion of its zinc binding domain resulted in embryological defects similar to those found in TGFα null mice (Luetteke *et al.*, 1993; Kenny & Bissell, 2007). TGFα expression in the lens has been associated with signalling cellular division and differentiation in the equatorial region of the lens (Chen *et al.*, 2001) As well as its role in proteolytically releasing TNFα (Black *et al.*, 1997), which has been proposed to be involved in the degeneration of nuclei within fibre cells (Wride & Sanders, 1998), this shedding of TGFα (Peschon *et al.*, 1998) suggests TACE may have a role in lens differentiation.

RAIDD (receptor-interacting protein [RIP]-associated ICH-1/CED-3-homologous protein with a death domain) was also shown to be expressed in the developing chick lens at E12. RAIDD is fairly well characterised in its role in apoptosis and its interaction with TNFRI but it has recently been shown to be expressed in the equatorial region of the developing mouse lens and is thought to be involved in cell differentiation in a number of organs though its role has not been clarified (Motaln *et al.*, 2005).

During this PhD, DR5 was shown to be expressed in the chick lens and is known to be a pro-apoptotic receptor for TRAIL, though there is no categorised role for either of these genes in the lens, TRAIL has been previously been shown to be expressed in mouse ocular tissue, though the lens was specifically excluded from that research for unknown reasons. TRAIL is thought to be have a role in tumour surveillance (Lee *et al.*, 2002). It was also considered as a prospective gene therapy for PCO, though TRAIL over-expression did not result in apoptosis (Malecaze *et al.*, 2006); here, the expression of TRAIL was found to have no statistically significant difference in expression in the chick lens at different stages in either PCR or Western blotting analysis, though increasing immunohistological staining was found at the equatorial regions of the lens as the lens developed as well as staining in the nuclei of both epithelial and differentiating fibre cells. This staining has not previously been seen in the lens; indeed, the staining in the nuclei has not been identified previously in any tissues and its significance remains undetermined.

Here, all members of the TRAF family have been shown to be expressed in the chick lens using RT-PCR. Of those genes listed above TRAF1, TRAF2, TRAF3 and TRAF4 were shown to be differentially regulated between E6 to E16, where considerable differentiation occurs and around the time point (E12) at which complete organelle degradation begins and the OFZ starts to form (Bassnett & Beebe, 1992). Antibodies were available for TRAF2 and TRAF3 that, after BLAST analysis, were thought may work in protein expression and localisation studies using Western blotting and immunocytochemistry in the developing chick lens.

TRAF2 showed similar RT-PCR and Western blotting expression, though protein expression levels decreased more rapidly as development proceeded.

Immunofluorescence staining revealed novel TRAF2 expression in the ciliary body. Though the ciliary body was only briefly mentioned in the introduction (section 1.5), where the focus was on the lens, it is important for accommodation of the lens, since it contains the ciliary muscle, which attaches to zonules, which in turn attached to the lens capsule. The ciliary body is found just behind the iris and produces the fluid that nourishes the lens, cornea and maintains the pressure in the eye (Coulombre & Coulombre, 1957; To *et al.*, 2002). TRAF2 was also intensely expressed in the iris and the corneal-scleral boundary. The iris is made of two pigmented epithelial layers that extend the entire length of the iris to the papillary margin. Adjacent to this margin the epithelium at the anterior of the eye form epithelial buds whose cells detach and fill the vascular stroma. The muscle cells in the stroma begin to differentiate at 11 days (Ferrari & Koch, 1984) which coincides with the novel expression of TRAF2 found during this research.

The tissue labelled corneal-scleral boundary could not be characterised within this thesis though previous literature was scrutinized. The identity of the tissue could not be confirmed without further histological analysis, such as staining with αSMA which would stain any smooth muscle actin known to be found in the iris and the ciliary muscle as they develop. If staining of this nature were found in the corneal-scleral boundary it would suggest that this region was ciliary muscle as suggested by morphological comparison with figure 1a found in the study on the architecture of the ciliary muscle (Tedesco *et al.*, 2005).

TRAF3 expression levels for both RT-PCR and protein were similar with increasing expression as the lens developed. The subcellular fractionation showed that the larger splice variant was not found in the post mitochondrial supernatant representing the cytosol. This finding suggests that the larger isoform is membrane bound while the shorter isoform is cytosolic, which is implied by the decreased expression in the fractions containing the nucleus and the resuspended debris containing mitochondria and membrane. The cytoplasm would not be totally removed from these fractions, but only found at reduced concentrations. Though 8 splice variant forms of TRAF3 were identified in human lymphoma cell lines with 7, when over expressed, able to induce NF-κB activation in 293 cells, only 3 were active in BJAB cells. The mediation of

TRAF3 functions between cell lines is therefore suggested to involve the differences in isoform (Gamper *et al.*, 2001). It is also suggested that the TRAF3, when full length, is inactive but augments the signalling of the smaller isoforms (van Eyndhoven *et al.*, 1999a).

In much of the published research, the role of TRAF3 as an adaptor in NF-κB signalling mostly through its binding with TNFR family member is highlighted. TRAF3 is known as a cytoplasmic adaptor molecule and has also been shown to interact with p62 nucleoporin which supports the theory that it has a role as an adaptor molecule at the nuclear membrane as well as its known role as an adaptor at the plasma membrane. The previous research supplies tantalising possibilities into the function of TRAF3 in the lens as well as supplying theories into how the signal resulting in the function is undertaken. Future functional research will hopefully provide some relief to the supposition and clarify this novel genes' role in the differentiating (cortical) fibre cells.

During this PhD functional studies investigating the role of TNFs in lens development were planned to be undertaken using whole lens culture. Studying the published research and correspondence with Dr Sue Menko (who has used this technique) highlighted that an assumption had been made during published whole lens culture experiments that the lens developed "relatively normally", though the development of these lenses using the various methods published had never been characterised. A number of the various, published, methods were used and whole lenses were cultured for up to 8 days, it was found that at this time point there was severe opacity under all culture conditions (including with glucose). The lenses were removed from culture on alternate days, fixed, embedded and stained. Preliminary findings have shown that while culturing lens with vitreous attached in glucose produced the most morphologically normal lens, this lens still had morphological abnormalities, namely that the nuclei in the newly diffenetiating fibre cells did not migrate along the equator. Once placed in culture, the nuclei in the newly differentiating fibre cells migrate towards the epithelial layer staying at the anterior of the fibre cells as they move towards the lens centre. The existing fibre cells (prior to culture) continued to differentiate and undergo organelle loss in a normal manner but while the newly differentiating fibre cells elongated and migrated towards the centre of

the lens they were seemingly only able to partially differentiate, leaving the nuclei in evidence, this delayed or incomplete differentiation is likely to be major contributor to the opacities found in the cultured lenses.

The most abnormal cultured lenses were found as those growing in glucose with iris and vitreous attached. In these lenses, though epithelial cells continued to proliferate, the signal for differentiation seemed to be gradually lost as the time in culture increased. At the equatorial region the "differentiating" cells elongated a little, but this did not compare to that found during normal lens development. Whether this was due to the disassociation of the epithelial layer from the fibre cell mass or the loss (or gain) of signal from the iris is not known, but by 8 days in culture the "differentiating" cells had continued to develop in a layer of cells of approximately the same morphological size as epithelial cells and this layer had migrated to the posterior of the lens where it continued to increase in length folding over itself. This strongly suggested that differentiation had discontinued when these lenses had started culture and the size and length of the layer may indicate that proliferation of epithelial cells has been increased.

Though the existing differentiating fibre cells, in the lens alone and lens with vitreous culture options, continued to elongate normally, the migration of the nuclei towards the epithelium raised the question of whether the cells differentiating, after culture began, were entering the OFZ and losing all their organelles like in normal development. To test this hypothesis BrdU incorporation into the whole lens at the start of culture was attempted, it was believed that the labelled, proliferating cells in the germinative zone of the lens would migrate and begin to differentiate during culture. Collecting the lenses at different time points during culture, then fixing, embedding and detecting the BrdU would allow the study of the newly differentiating cells in culture. Within the time and financial constraints, staining of BrdU was not obtained and would be the first task in any future work. However, it was clear from H & E labelling that the existing fibre cells at E10 continued along an intrinsic developmental programme resulting in the loss of their nuclei and organelles. This is the first time that this has been noted in any study to date and suggests that this model system is valid for studying lens fibre cell denucleation and organelle loss instead of or in combination with functional studies manipulating lens development in chick embryos *in ovo*.

In conclusion, this thesis has identified a number of genes previously unknown to be expressed in the developing lens, of these genes TRAF2, TRAF3 and TRAIL were studied further. TRAF2 and TRAF3 had statistically different gene and protein expression in the developing lens, while TRAF2 was also found to be highly expressed in surrounding ocular tissue, such as the developing iris. The novel identification of these genes in the lens has laid a foundation and further studies need to be undertaken to elucidate the role of these novel genes in the developing lens and indeed the development of the eye. The whole lens culture characterisation will aid in the eventual understanding of the role of these and other genes in the differentiation and development of the lens. Ultimately this thesis has unmasked a number of novel genes expressed in the developing lens and characterised a culture system that was ill understood and its potential as a useful tool little known. The continued identification of novel gene expression and understanding their functions in the lens and indeed the surrounding tissue is important and with further refinement could potentially prove to be a therapeutic benefit in a number of ocular disorders.

6.2 Future work

During this PhD, the expressions of a number of TNFs have been shown to be expressed via reverse transcriptase (RT)-PCR. The RT-PCR method is only semi-quantitative and for future studies the use of quantitative PCR would be advisable, especially to assess the expression of those genes that were found in the lens but their expression profiles, for the developmental timepoints studied, were not obtained.

Knock out mice are available for TRAFs1 -6 (reviewed in Bharti & Aggarwal, 2004), but there have been no thorough examinations of eye/lens development in these mice. Examination of the eyes/ lenses of these mice would supply further information suggesting whether these genes had a key role in the development of the eye/lens. If abnormal lenses were found in any of these knockout mice the role of the TRAF could be hypothesised and further researched.

Ideally further characterisation of the whole lens culture method would be carried out using the BrdU method mentioned above to ascertain the level of differentiation of the newly differentiating fibre cells. It may also be possible to improve the method by culturing the lens with other ocular tissues such as the retina,

which may improve the differentiation of the new fibre cells by helping to maintain their nuclei at the equator of the lens and even allowing them continued entry into the OFZ. The tissue or factor involved in maintaining the normal line of nuclei in the fibre cells of lenses in culture was not found during this PhD.

Once satisfactorily characterised, the whole lens culture method could be further used to investigate the effects of a selected recombinant gene, e.g. TRAF3, on cell proliferation and differentiation in whole lens cultures by studying the morphology of the lenses and/or in dissociated lens cell cultures (Wride *et al.*, 1999).

Another technique in chicken embryo research is *in ovo* electroporation. This method is used to create reversible pores in the plasma membrane to allow the introduction of si/ shRNAs (small interfering/small hairpin RNAs) or dominant-negative constructs resulting in the loss of function of selected genes (reviewed in Sauka-Spengler & Barembaum, 2008).

Though it is important to understand the roles, within the lens, of the genes identified in this research there are also wider implications. Understanding the varied functions of these genes within one tissue may not categorically imply the same functions is undertaken in another tissue. Gaining knowledge of the physiological roles of these TNF related genes in multiple tissues can only help in the development of therapies that may use gene manipulation in treatments of diseases from ocular tumours (Gregory *et al.*, 2005) to psoriasis (Tan *et al.*, 2007).

Bibliography

(1991). Risk factors for age-related cortical, nuclear, and posterior subcapsular cataracts. The Italian-American Cataract Study Group. *American Journal of Epidemiology* **133,** 541-553.

Abdalla, S. A., Horiuchi, H., Furusawa, S. & Matsuda, H. (2004). Molecular study on chicken tumor necrosis factor receptor-II and tumor necrosis factor receptor-associated factor-5. *Veterinary Immunology and Immunopathology* **98,** 31-41.

Abell, A. N. & Johnson, G. L. (2005). MEKK4 is an effector of the embryonic TRAF4 for JNK activation. *Journal of Biological Chemistry* **280,** 35793-35796.

Alizadeh, A., Clark, J., Seeberger, T., Hess, J., Blankenship, T. & FitzGerald, P. G. (2003). Targeted deletion of the lens fiber cell-specific intermediate filament protein filensin. *Investigative Ophthalmology & Visual Science* **44,** 5252-5258.

Alizadeh, A., Clark, J., Seeberger, T., Hess, J., Blankenship, T. & Fitzgerald, P. G. (2004). Characterization of a mutation in the lens-specific CP49 in the 129 strain of mouse. *Investigative Ophthalmology & Visual Science* **45,** 884-891.

Andley, U. P., Song, Z., Wawrousek, E. F. & Bassnett, S. (1998). The molecular chaperone alphaA-crystallin enhances lens epithelial cell growth and resistance to UVA stress. *Journal of Biological Chemistry* **273,** 31252-31261.

Andley, U. P., Song, Z., Wawrousek, E. F., Brady, J. P., Bassnett, S. & Fleming, T. P. (2001). Lens epithelial cells derived from alphaB-crystallin knockout mice demonstrate hyperproliferation and genomic instability. *The FASEB Journal* **15,** 221-229.

Ang, S. J., Stump, R. J., Lovicu, F. J. & McAvoy, J. W. (2004). Spatial and temporal expression of Wnt and Dickkopf genes during murine lens development. *Gene Expression Patterns* **4,** 289-295.

Apple, D. J., Solomon, K. D., Tetz, M. R., Assia, E. I., Holland, E. Y., Legler, U. F., Tsai, J. C., Castaneda, V. E., Hoggatt, J. P. & Kostick, A. M. (1992). Posterior capsule opacification. *Survey of Opthalmology* **37,** 73-116.

Arch, R. H. & Thompson, C. B. (1998). 4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappa B. *Molecular and Cellular Biology* **18,** 558-565.

Arnold, D. R., Moshayedi, P., Schoen, T. J., Jones, B. E., Chader, G. J. & Waldbillig, R. J. (1993). Distribution of IGF-I and -II, IGF binding proteins (IGFBPs) and IGFBP mRNA in ocular fluids and tissues: potential sites of synthesis of IGFBPs in aqueous and vitreous. *Experimental Eye Research* **56,** 555-565.

Arora, J. K., Lysz, T. W. & Zelenka, P. S. (1996). A role for 12(S)-HETE in the response of human lens epithelial cells to epidermal growth factor and insulin. *Investigative Ophthalmology & Visual Science* **37,** 1411-1418.

Asbell, P. A., Dualan, L., Mindel, J., Brocks, D., Ahmad, M. & Epstein, S. (2005). Age-related cataract. *Lancet* **365,** 599-609.

Azuma, M., Fukiage, C., Higashine, M., Nakajima, T., Ma, H. & Shearer, T. R. (2000). Identification and characterization of a retina-specific calpain (Rt88) from rat. *Current Eye research* **21,** 710-720.

Bailey, A. P., Bhattacharyya, S., Bronner-Fraser, M. & Streit, A. (2006). Lens specification is the ground state of all sensory placodes, from which FGF promotes olfactory identity. *Developmental Cell* **11,** 505-517.

Baker, S. J. & Reddy, E. P. (1998). Modulation of life and death by the TNF receptor superfamily. *Oncogene* **17,** 3261-3270.

Bassnett, S. (1992). Mitochondrial dynamics in differentiating fiber cells of the mammalian lens. *Current Eye research* **11,** 1227-1232.

Bassnett, S. (1995). The fate of the Golgi apparatus and the endoplasmic reticulum during lens fiber cell differentiation. *Investigative Ophthalmology & Visual Science* **36,** 1793-1803.

Bassnett, S. (2002). Lens organelle degradation. *Experimental Eye Research* **74,** 1-6.

Bassnett, S. (2008). On the mechanism of organelle degradation in the vertebrate lens. *Experimental Eye Research.*

Bassnett, S. & Beebe, D. C. (1992). Coincident loss of mitochondria and nuclei during lens fiber cell differentiation. *Developmental Dynamics* **194,** 85-93.

Bassnett, S. & Mataic, D. (1997). Chromatin degradation in differentiating fiber cells of the eye lens. *Journal of Cell Biology* **137,** 37-49.

Bassnett, S., Missey, H. & Vucemilo, I. (1999). Molecular architecture of the lens fiber cell basal membrane complex. *Journal of Cell Biology* **112 (Pt 13),** 2155-2165.

Batterbury, M. & Bowling, B. (2001). *Opthalmology: An illlustrated colour text.* Harcourt Publishers Ltd.

Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y. & Karin, M. (1999). Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes and Development* **13,** 1297-1308.

Bavik, C., Ward, S. J. & Chambon, P. (1996). Developmental abnormalities in cultured mouse embryos deprived of retinoic by inhibition of yolk-sac retinol binding protein synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 3110-3114.

Beebe, D. C. (2003). The Lens. In *Alder's Physiology of the eye.* ed. Kaufmann Pl & Alm A, pp. 117-159. Mosby, Inc.

Beebe, D. C., Vasiliev, O., Guo, J. L., Shui, Y. B. & Bassnett, S. (2001). Changes in adhesion complexes define stages in the differentiation of lens fiber cells. *Investigative Ophthalmology & Visual Science* **42,** 727-734.

Belecky-Adams, T. L., Adler, R. & Beebe, D. C. (2002). Bone morphogenetic protein signaling and the initiation of lens fiber cell differentiation. *Development* **129,** 3795-3802.

Benedetti, E. L., Dunia, I., Bentzel, C. J., Vermorken, A. J., Kibbelaar, M. & Bloemendal, H. (1976). A portrait of plasma membrane specializations in eye lens epithelium and fibers. *Biochimica et Biophysica Acta* **457,** 353-384.

Bharti, A. C. & Aggarwal, B. B. (2004). Ranking the role of RANK ligand in apoptosis. *Apoptosis* **9,** 677-690.

Bhat, S. P. (2001). The ocular lens epithelium. *Bioscience Reports* **21,** 537-563.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J. & Cerretti, D. P. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385,** 729-733.

Blixt, A., Mahlapuu, M., Aitola, M., Pelto-Huikko, M., Enerback, S. & Carlsson, P. (2000). A forkhead gene, FoxE3, is essential for lens epithelial proliferation and closure of the lens vesicle. *Genes and Development* **14,** 245-254.

Bloemendal, H. (1977). The vertebrate eye lens. *Science* **197,** 127-138.

Boatright, K. M. & Salvesen, G. S. (2003). Mechanisms of caspase activation. *Current Opinion in Cell Biology* **15,** 725-731.

Bodmer, J. L., Schneider, P. & Tschopp, J. (2002). The molecular architecture of the TNF superfamily. *Trends in Biochemical Sciences* **27,** 19-26.

Bonizzi, G., Bebien, M., Otero, D. C., Johnson-Vroom, K. E., Cao, Y., Vu, D., Jegga, A. G., Aronow, B. J., Ghosh, G., Rickert, R. C. & Karin, M. (2004). Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *Embo Journal* **23,** 4202-4210.

Bourdon, J. C. (2007). p53 Family isoforms. *Current Pharmaceutical Biotechnology* **8,** 332-336.

Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P. O., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A. M., Schirle, M., Schlegl, J., Schwab, M., Stein, M. A., Bauer, A., Casari, G., Drewes, G., Gavin, A. C., Jackson, D. B., Joberty, G., Neubauer, G., Rick, J., Kuster, B. & Superti-Furga, G. (2004). A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. *Nature cell biology* **6,** 97-105.

Boyle, D. L., Takemoto, L., Brady, J. P. & Wawrousek, E. F. (2003). Morphological characterization of the Alpha A- and Alpha B-crystallin double knockout mouse lens. *BMC Ophthalmology* **3,** 3.

Brink, R. & Lodish, H. F. (1998). Tumor necrosis factor receptor (TNFR)-associated factor 2A (TRAF2A), a TRAF2 splice variant with an extended RING finger domain that inhibits TNFR2-mediated NF-kappaB activation. *Journal of Biological Chemistry* **273,** 4129-4134.

Brown, S. A., Richards, C. M., Hanscom, H. N., Feng, S. L. & Winkles, J. A. (2003). The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-kappaB activation. *Biochemical Journal* **371,** 395-403.

Cain, S., Martinez, G., Kokkinos, M. I., Turner, K., Richardson, R. J., Abud, H. E., Huelsken, J., Robinson, M. L. & de Iongh, R. U. (2008). Differential requirement for beta-catenin in epithelial and fiber cells during lens development. *Developmental Biology* **321,** 420-433.

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. & Goeddel, D. V. (1996). TRAF6 is a signal transducer for interleukin-1. *Nature* **383,** 443-446.

Chauhan, B. K., Reed, N. A., Yang, Y., Cermak, L., Reneker, L., Duncan, M. K. & Cvekl, A. (2002a). A comparative cDNA microarray analysis reveals a spectrum of genes regulated by Pax6 in mouse lens. *Genes to Cells* **7,** 1267-1283.

Chauhan, B. K., Reed, N. A., Zhang, W., Duncan, M. K., Kilimann, M. W. & Cvekl, A. (2002b). Identification of genes downstream of Pax6 in the mouse lens using cDNA microarrays. *Journal of Biological Chemistry* **277,** 11539-11548.

Chauhan, B. K., Zhang, W., Cveklova, K., Kantorow, M. & Cvekl, A. (2002c). Identification of differentially expressed genes in mouse Pax6 heterozygous lenses. *Investigative Ophthalmology & Visual Science* **43,** 1884-1890.

Chen, F., Mrock, L. K. & Ireland, M. E. (2001). A role for endogenous TGFalpha and associated signaling pathways in the differentiation of lens fiber cells. *Journal of Cellular Physiology* **186,** 288-297.

Chen, X., Taube, J. R., Simirskii, V. I., Patel, T. P. & Duncan, M. K. (2008a). Dual roles for Prox1 in the regulation of the chicken betaB1-crystallin promoter. *Invest Ophthalmol Vis Sci* **49,** 1542-1552.

Chen, Y., Stump, R. J., Lovicu, F. J. & McAvoy, J. W. (2004). Expression of Frizzleds and secreted frizzled-related proteins (Sfrps) during mammalian lens development. *International Journal of Developmental Biology* **48,** 867-877.

Chen, Y., Stump, R. J., Lovicu, F. J. & McAvoy, J. W. (2006). A role for Wnt/planar cell polarity signaling during lens fiber cell differentiation? *Seminars in Cell & Developmental Biology* **17,** 712-725.

Chen, Y., Stump, R. J., Lovicu, F. J., Shimono, A. & McAvoy, J. W. (2008b). Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. *Developmental Biology.*

Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S. & Baltimore, D. (1995). Involvement of CRAF1, a relative of TRAF, in CD40 signaling. *Science* **267,** 1494-1498.

Chow, R. L. & Lang, R. A. (2001). Early eye development in vertebrates. *Annual Review of Cell and Developmental Biology* **17,** 255-296.

Chung, J. Y., Park, Y. C., Ye, H. & Wu, H. (2002). All TRAFs are not created equal: common and distinct molecular mechanisms of TRAF-mediated signal transduction. *Journal of Cell Science* **115,** 679-688.

Columbano, A. (1995). Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. *Journal of Cellular Biochemistry* **58,** 181-190.

Coulombre, A. J. & Coulombre, J. L. (1957). The role of intraocular pressure in the development of the chick eye: III. Ciliary body. *American Journal of Opthalmology* **44,** 85-93.

Coulombre, A. J. & Coulombre, J. L. (1964). Lens Development. I. Role of the Lens in Eye Growth. *Journal of Experimental Zoology* **156,** 39-47.

Coulombre, A. J., Steinberg, S. N. & Coulombre, J. L. (1963). The role of intraocular pressure in the development of the chick eye. V. Pigmented epithelium. *Investigative Ophthalmology* **2,** 83-89.

Coulombre, J. L. & Coulombre, A. J. (1969). Lens development. IV. Size, shape, and orientation. *Investigative Ophthalmology* **8,** 251-257.

Coulombre, J. L. & Coulombre, A. J. (1971). Lens development. V. Histological analysis of the mechanism of lens reconstitution from implants of lens epithelium. *Journal of Experimental Zoology* **176,** 15-24.

Counis, M. F., Chaudun, E., Arruti, C., Oliver, L., Sanwal, M., Courtois, Y. & Torriglia, A. (1998). Analysis of nuclear degradation during lens cell differentiation. *Cell Death and Differentiation* **5,** 251-261.

Counis, M. F. & Torriglia, A. (2000). DNases and apoptosis. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire* **78,** 405-414.

Creuzet, S., Vincent, C. & Couly, G. (2005). Neural crest derivatives in ocular and periocular structures. *International Journal of Developmental Biology* **49,** 161-171.

Cui, W. W., Tomarev, S. I., Piatigorsky, J., Chepelinsky, A. B. & Duncan, M. K. (2004). Mafs, Prox1, and Pax6 can regulate chicken beta B1-crystallin gene expression. *Journal of Biological Chemistry* **279,** 11088-11095.

Cvekl, A. & Duncan, M. K. (2007). Genetic and epigenetic mechanisms of gene regulation during lens development. *Progress in Retinal and Eye Research* **26,** 555-597.

Cvekl, A. & Piatigorsky, J. (1996). Lens development and crystallin gene expression: many roles for Pax-6. *Bioessays* **18,** 621-630.

Dahm, R. (1999). Lens fibre cell differentiation - A link with apoptosis? *Ophthalmic Research* **31,** 163-183.

Dahm, R. (2004). Dying to see. *Scientific American* **291,** 82-89.

Dahm, R., Bramke, S., Dawczynski, J., Nagaraj, R. H. & Kasper, M. (2003). Developmental aspects of galectin-3 expression in the lens. *Histochemistry and Cell Biology* **119,** 219-226.

Dahm, R., Procter, J. E., Ireland, M. E., Lo, W. K., Mogensen, M. M., Quinlan, R. A. & Prescott, A. R. (2007a). Reorganization of centrosomal marker proteins coincides with epithelial cell differentiation in the vertebrate lens. *Experimental Eye Research* **85,** 696-713.

Dahm, R., Schonthaler, H. B., Soehn, A. S., van Marle, J. & Vrensen, G. F. (2007b). Development and adult morphology of the eye lens in the zebrafish. *Experimental Eye Research* **85,** 74-89.

Dawson, C. W., Tramountanis, G., Eliopoulos, A. G. & Young, L. S. (2003). Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to promote cell survival and induce actin filament remodeling. *Journal of Biological Chemistry* **278,** 3694-3704.

De Maria, A. & Arruti, C. (2004). DNase I and fragmented chromatin during nuclear degradation in adult bovine lens fibers. *Molecular Vision* **10,** 74-82.

De Maria, A. & Bassnett, S. (2007). DNase IIbeta distribution and activity in the mouse lens. *Investigative Ophthalmology & Visual Science* **48,** 5638-5646.

Dempsey, P. W., Doyle, S. E., He, J. Q. & Cheng, G. H. (2003). The signaling adaptors and pathways activated by TNF superfamily. *Cytokine & Growth Factor Reviews* **14,** 193-209.

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C. & Chen, Z. J. (2000). Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **103,** 351-361.

Dewulf, N., Verschueren, K., Lonnoy, O., Moren, A., Grimsby, S., Vande Spiegle, K., Miyazono, K., Huylebroeck, D. & Ten Dijke, P. (1995). Distinct spatial and temporal expression patterns of two type I receptors for bone morphogenetic proteins during mouse embryogenesis. *Endocrinology* **136,** 2652-2663.

Duckett, C. S. & Thompson, C. B. (1997). CD30-dependent degradation of TRAF2: implications for negative regulation of TRAF signaling and the control of cell survival. *Genes and Development* **11,** 2810-2821.

Dudley, A. T. & Robertson, E. J. (1997). Overlapping expression domains of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. *Developmental Dynamics* **208,** 349-362.

Duncan, M. K., Cui, W., Oh, D. J. & Tomarev, S. I. (2002). Prox1 is differentially localized during lens development. *Mechanisms of Development* **112,** 195-198.

Duncan, M. K., Haynes, J. I., 2nd, Cvekl, A. & Piatigorsky, J. (1998). Dual roles for Pax-6: a transcriptional repressor of lens fiber cell-specific beta-crystallin genes. *Molecular and Cellular Biology* **18,** 5579-5586.

Duncan, M. K., Xie, L. K., David, L. L., Robinson, M. L., Taube, J. R., Cui, W. W. & Reneker, L. W. (2004). Ectopic Pax6 expression disturbs lens fiber cell differentiation. *Investigative Ophthalmology & Visual Science* **45,** 3589-3598.

Enwright, J. F., 3rd & Grainger, R. M. (2000). Altered retinoid signaling in the heads of small eye mouse embryos. *Developmental Biology* **221,** 10-22.

Evan, G. (2003). Microsoft Encarta, Ecyclopedia Standard Edition,. Microsoft.

Fadok, V. A., Bratton, D. L., Frasch, S. C., Warner, M. L. & Henson, P. M. (1998). The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death and Differentiation* **5,** 551-562.

Faulkner-Jones, B., Zandy, A. J. & Bassnett, S. (2003). RNA stability in terminally differentiating fibre cells of the ocular lens. *Experimental Eye Research* **77,** 463-476.

Ferrari, P. A. & Koch, W. E. (1984). Development of the iris in the chicken embryo. I. A study of growth and histodifferentiation utilizing immunocytochemistry for muscle differentiation. *J Embryol Exp Morphol* **81,** 153-167.

Ferreira-Cornwell, M. C., Veneziale, R. W., Grunwald, G. B. & Menko, A. S. (2000). N-cadherin function is required for differentiation-dependent cytoskeletal reorganization in lens cells in vitro. *Experimental Cell Research* **256,** 237-247.

Fisher, M. & Grainger, R. M. (2004). Lens induction and determination. In *Development of the Ocular Lens*. ed. Lovicu Fj, Robinson, M.L. Cambridge Univ. Press., New York.

Flaye, D. E., Sullivan, K. N., Cullinan, T. R., Silver, J. H. & Whitelocke, R. A. (1989). Cataracts and cigarette smoking. The City Eye Study. *Eye* **3 (Pt 4),** 379-384.

Fokina, V. M. & Frolova, E. I. (2006). Expression patterns of Wnt genes during development of an anterior part of the chicken eye. *Developmental Dynamics* **235,** 496-505.

Force, W. R., Cheung, T. C. & Ware, C. F. (1997). Dominant negative mutants of TRAF3 reveal an important role for the coiled coil domains in cell death signaling by the lymphotoxin-beta receptor. *Journal of Biological Chemistry* **272,** 30835-30840.

Francis, P. J., Berry, V., Moore, A. T. & Bhattacharya, S. (1999). Lens biology - development and human cataractogenesis. *Trends in Genetics* **15,** 191-196.

Francis, P. J. & Moore, A. T. (2004). Genetics of childhood cataract. *Current Opinion in Ophthalmology* **15,** 10-15.

Frenzel, E. M. & Johnson, R. G. (1996). Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin. *Developmental Biology* **179,** 1-16.

Furuta, Y. & Hogan, B. L. M. (1998). BMP4 is essential for lens induction in the mouse embryo. *Genes & Development* **12,** 3764-3775.

Gabella, G. & Clarke, E. (1983). Embryonic development of the smooth and striated musculatures of the chicken iris. *Cell Tissue Res* **229,** 37-59.

Gamper, C., Omene, C. O., van Eyndhoven, W. G., Glassman, G. D. & Lederman, S. (2001). Expression and function of TRAF-3 splice-variant isoforms in human lymphoma cell lines. *Human Immunology* **62,** 1167-1177.

Gamper, C., van Eyndhoven, W. G., Schweiger, E., Mossbacher, M., Koo, B. & Lederman, S. (2000). TRAF-3 interacts with p62 nucleoporin, a component of the nuclear pore central plug that binds classical NLS-containing import complexes. *Molecular Immunology* **37,** 73-84.

Geatrell, J. (2007a). Apoptosis gene expression profiling in lens development. In *School of Optometry and Vision Sciences*, vol. Doctor of Philosophy, pp. 171. Cardiff University, Cardiff.

Geatrell, J. (2007b). Apoptosis Gene Expression Profiling in Lens Development. In *School of Optometry and Vision Sciences*, vol. Doctor of Philosophy, pp. 1-172. Caardiff University, Cardiff.

Gehring, W. J. (1996). The master control gene for morphogenesis and evolution of the eye. *Genes to Cells* **1,** 11-15.

Ghosh, M. P. & Zigler, J. S., Jr. (2005). Lack of fiber cell induction stops normal growth of rat lenses in organ culture. *Molecular Vision* **11,** 901-908.

Gilbert, S. P. (2000). *Developmental Biology*. Sinauer Associates Inc., Massechusetts.

Girao, H., Pereira, P., Taylor, A. & Shang, F. (2005). Subcellular redistribution of components of the ubiquitin-proteasome pathway during lens differentiation and maturation. *Investigative Ophthalmology & Visual Science* **46,** 1386-1392.

Gong, X., Cheng, C. & Xia, C. H. (2007). Connexins in lens development and cataractogenesis. *Journal of Membrane Biology* **218,** 9-12.

Gong, X., Li, E., Klier, G., Huang, Q., Wu, Y., Lei, H., Kumar, N. M., Horwitz, J. & Gilula, N. B. (1997). Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice. *Cell* **91,** 833-843.

Graw, J. (1999). Cataract mutations and lens development. *Progress in Retinal and Eye Research* **18,** 235-267.

Graw, J. (2003). The genetic and molecular basis of congenital eye defects. *Nature Reviews Genetics* **4,** 876-888.

Graw, J., Klopp, N., Neuhauser-Klaus, A., Favor, J. & Loster, A. (2002). Crygf(Rop): The first mutation in the Crygf gene causing a unique radial lens opacity. *Investigative Ophthalmology & Visual Science* **43,** 2998-3002.

Graw, J. & Loster, J. (2003). Developmental genetics in ophthalmology. *Ophthalmic Genetics* **24,** 1-33.

Gregory, M. S., Koh, S., Huang, E., Saff, R. R., Marshak-Rothstein, A., Mukai, S. & Ksander, B. R. (2005). A novel treatment for ocular tumors using membrane FasL vesicles to activate innate immunity and terminate immune privilege. *Investigative Ophthalmology & Visual Science* **46,** 2495-2502.

Grindley, J. C., Davidson, D. R. & Hill, R. E. (1995). The role of Pax-6 in eye and nasal development. *Development* **121,** 1433-1442.

Grullich, C., Duvoisin, R. M., Wiedmann, M. & van Leyen, K. (2001). Inhibition of 15-lipoxygenase leads to delayed organelle degradation in the reticulocyte. *Febs Letters* **489,** 51-54.

Guo, W., Shang, F., Liu, Q., Urim, L., West-Mays, J. & Taylor, A. (2004). Differential regulation of components of the ubiquitin-proteasome pathway during lens cell differentiation. *Investigative Ophthalmology & Visual Science* **45,** 1194-1201.

Guo, W., Shang, F., Liu, Q., Urim, L., Zhang, M. & Taylor, A. (2006). Ubiquitin-proteasome pathway function is required for lens cell proliferation and differentiation. *Investigative Ophthalmology & Visual Science* **47,** 2569-2575.

Hamburger, V. & Hamilton, H. L. (1992). A Series of Normal Stages in the Development of the Chick-Embryo, (Reprinted from Journal of Morphology, Vol 88, 1951). *Developmental Dynamics* **195,** 231-&.

Harada, H. & Grant, S. (2003). Apoptosis regulators. *Review of Clinical Experimental Hematology* **7,** 117-138.

Hawse, J. R., DeAmicis-Tress, C., Cowell, T. L. & Kantorow, M. (2005). Identification of global gene expression differences between human lens epithelial and cortical fiber cells reveals specific genes and their associated pathways important for specialized lens cell functions. *Molecular Vision* **11,** 274-283.

Hayden, M. S. & Ghosh, S. (2008). Shared principles in NF-kappaB signaling. *Cell* **132,** 344-362.

He, J. Q., Saha, S. K., Kang, J. R., Zarnegar, B. & Cheng, G. (2007). Specificity of TRAF3 in its negative regulation of the noncanonical NF-kappa B pathway. *Journal of Biological Chemistry* **282,** 3688-3694.

Heiba, I. M., Elston, R. C., Klein, B. E. & Klein, R. (1995). Evidence for a major gene for cortical cataract. *Investigative Ophthalmology & Visual Science* **36,** 227-235.

Hershko, A., Ciechanover, A. & Varshavsky, A. (2000). Basic Medical Research Award. The ubiquitin system. *Nature Medicine* **6,** 1073-1081.

Hetz, C., Bernasconi, P., Fisher, J., Lee, A. H., Bassik, M. C., Antonsson, B., Brandt, G. S., Iwakoshi, N. N., Schinzel, A., Glimcher, L. H. & Korsmeyer, S. J. (2006). Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. *Science* **312,** 572-576.

Hightower, K. R. & McCready, J. (1991). Effect of thiol reagents on Ca-ATPase in rabbit lens epithelium. *Current Eye research* **10,** 299-303.

Hirono, I., Nam, B. H., Kurobe, T. & Aoki, T. (2000). Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder Paralychthys olivaceus. *Journal of Immunology* **165,** 4423-4427.

Hirosumi, J., Tuncman, G., Chang, L., Gorgun, C. Z., Uysal, K. T., Maeda, K., Karin, M. & Hotamisligil, G. S. (2002). A central role for JNK in obesity and insulin resistance. *Nature* **420,** 333-336.

Hoover, K. B. & Bryant, P. J. (2000). The genetics of the protein 4.1 family: organizers of the membrane and cytoskeleton. *Current Opinion in Cell Biology* **12,** 229-234.

Horie, R., Watanabe, T., Ito, K., Morisita, Y., Watanabe, M., Ishida, T., Higashihara, M., Kadin, M. & Watanabe, T. (2002). Cytoplasmic aggregation of TRAF2 and TRAF5 proteins in the Hodgkin-Reed-Sternberg cells. *American Journal of Pathology* **160,** 1647-1654.

Hostager, B. S. & Bishop, G. A. (1999). Cutting edge: contrasting roles of TNF receptor-associated factor 2 (TRAF2) and TRAF3 in CD40-activated B lymphocyte differentiation. *Journal of Immunology* **162,** 6307-6311.

Houghton, L., Lindon, C. & Morgan, B. A. (2005). The ectodysplasin pathway in feather tract development. *Development* **132,** 863-872.

Houghton, L., Lindon, C. M., Freeman, A. & Morgan, B. A. (2007). Abortive placode formation in the feather tract of the scaleless chicken embryo. *Developmental Dynamics* **236,** 3020-3030.

Hu, H. M., O'Rourke, K., Boguski, M. S. & Dixit, V. M. (1994). A novel RING finger protein interacts with the cytoplasmic domain of CD40. *Journal of Biological Chemistry* **269,** 30069-30072.

Hurt, E. C. (1993). The nuclear pore complex. *Febs Letters* **325,** 76-80.

Irmler, M., Steiner, V., Ruegg, C., Wajant, H. & Tschopp, J. (2000). Caspase-induced inactivation of the anti-apoptotic TRAF1 during Fas ligand-mediated apoptosis. *Febs Letters* **468,** 129-133.

Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mosialos, G., Kieff, E., Yamamoto, T. & Inoue, J. (1996a). Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. *Journal of Biological Chemistry* **271,** 28745-28748.

Ishida, T., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T. & Inoue, J. (1996b). TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 9437-9442.

Ishizaki, Y., Jacobson, M. D. & Raff, M. C. (1998). A role for caspases in lens fiber differentiation. *Journal of Cell Biology* **140,** 153-158.

Jabara, H., Laouini, D., Tsitsikov, E., Mizoguchi, E., Bhan, A., Castigli, E., Dedeoglu, F., Pivniouk, V., Brodeur, S. & Geha, R. (2002). The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. *Immunity* **17,** 265-276.

Jahngen, J. H., Haas, A. L., Ciechanover, A., Blondin, J., Eisenhauer, D. & Taylor, A. (1986). The eye lens has an active ubiquitin-protein conjugation system. *Journal of Biological Chemistry* **261,** 13760-13767.

Jia, J., Lin, M., Zhang, L., York, J. P. & Zhang, P. (2007). The Notch signaling pathway controls the size of the ocular lens by directly suppressing p57Kip2 expression. *Molecular and Cellular Biology* **27,** 7236-7247.

Jiang, H., Jin, Y., Bu, L., Zhang, W., Liu, J., Cui, B., Kong, X. & Hu, L. (2003). A novel mutation in GJA3 (connexin46) for autosomal dominant congenital nuclear pulverulent cataract. *Molecular Vision* **9,** 579-583.

Jiang, J. X. & Goodenough, D. A. (1998). Phosphorylation of lens-fiber connexins in lens organ cultures. *European journal of Biochemistry* **255,** 37-44.

Jin, Z. & El-Deiry, W. S. (2005). Overview of cell death signaling pathways. *Cancer Biology & Therapy* **4,** 139-163.

Johnston, M. C., Noden, D. M., Hazelton, R. D., Coulombre, J. L. & Coulombre, A. J. (1979). Origins of avian ocular and periocular tissues. *Experimental Eye Research* **29,** 27-43.

Jordan, J. F., Kociok, N., Grisanti, S., Jacobi, P. C., Esser, J. M., Luther, T. T., Krieglstein, G. K. & Esser, P. (2001). Specific features of apoptosis in human lens epithelial cells induced by mitomycin C in vitro. *Graefes Archive for Clinical and Experimental Ophthalmology* **239,** 613-618.

Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R. & Kondoh, H. (1998). Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. *Development* **125,** 2521-2532.

Kamachi, Y., Uchikawa, M., Tanouchi, A., Sekido, R. & Kondoh, H. (2001). Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. *Genes & Development* **15,** 1272-1286.

Kamradt, M. C., Lu, M. L., Werner, M. E., Kwan, T., Chen, F., Strohecker, A., Oshita, S., Wilkinson, J. C., Yu, C. J., Oliver, P. G., Duckett, C. S., Buchsbaum, D. J., LoBuglio, A. F., Jordan, V. C. & Cryns, V. L. (2005). The small heat shock protein alpha B-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. *Journal of Biological Chemistry* **280,** 11059-11066.

Kastner, P., Mark, M. & Chambon, P. (1995). Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* **83,** 859-869.

Kedinger, V., Alpy, F., Tomasetto, C., Thisse, C., Thisse, B. & Rio, M. C. (2005). Spatial and temporal distribution of the traf4 genes during zebrafish development. *Gene Expression Patterns* **5,** 545-552.

Kenny, P. A. & Bissell, M. J. (2007). Targeting TACE-dependent EGFR ligand shedding in breast cancer. *The Journal of clinical investigation* **117,** 337-345.

Kerr, J. F. (1971). Shrinkage necrosis: a distinct mode of cellular death. *Journal of Pathology* **105,** 13-20.

Kerr, J. F. (2002). History of the events leading to the formulation of the apoptosis concept. *Toxicology* **181-182,** 471-474.

Kerr, J. F. & Searle, J. (1972). The digestion of cellular fragments within phagolysosomes in carcinoma cells. *Journal of Pathology* **108,** 55-58.

Kidd, V. J., Lathi, J. M. & Teitz, T. (2000). Proteolytic regulation of apoptosis. *Seminars in Cell & Developmental Biology* **11,** 191-201.

Kimberley, F. C. & Screaton, G. R. (2004). Following a TRAIL: Update on a ligand and its five receptors. *Cell Research* **14,** 359-372.

Kondoh, H. (1999). Transcription factors for lens development assessed in vivo. *Current Opinion in Genetics & Development* **9,** 301-308.

Koster, R. W., Kuhnlein, R. P. & Wittbrodt, J. (2000). Ectopic Sox3 activity elicits sensory placode formation. *Mechanisms of Development* **95,** 175-187.

Krag, S. & Andreassen, T. T. (2003). Mechanical properties of the human lens capsule. *Progress in Retinal and Eye Research* **22,** 749-767.

Krishna, M. & Narang, H. (2008). The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cellular and Molecular Life Sciences.*

Krishnamurthy, R. & Vanderveen, D. K. (2008). Infantile cataracts. *International Ophthalmology Clinics* **48,** 175-192.

Kuai, J., Nickbarg, E., Wooters, J., Qiu, Y., Wang, J. & Lin, L. L. (2003a). Endogenous association of TRAF2, TRAF3, cIAP1, and Smac with lymphotoxin beta receptor reveals a novel mechanism of apoptosis. *J Biol Chem* **278,** 14363-14369.

Kuai, J., Nickbarg, E., Wooters, J., Qiu, Y. C., Wang, J. & Lin, L. L. (2003b). Endogenous association of TRAF2, TRAF3, cIAP1, and Smac with lymphotoxin beta receptor reveals a novel mechanism of apoptosis. *Journal of Biological Chemistry* **278,** 14363-14369.

Kuszak, J. R., Mazurkiewicz, M. & Zoltoski, R. (2006). Computer modelling of secondary fiber development and growth: I. Nonprimate lenses. *Molecular Vision* **12,** 251-270.

Laine, A. & Ronai, Z. (2005). Ubiquitin chains in the ladder of MAPK signaling. *Science STKE: signal transduction knowledge environment* **2005,** re5.

Land, M. F. (2005). The optical structures of animal eyes. *Current Biology* **15,** R319-323.

Landgren, H., Blixt, A. & Carlsson, P. (2008). Persistent FoxE3 expression blocks cytoskeletal remodeling and organelle degradation during lens fiber differentiation. *Investigative Ophthalmology & Visual Science.*

LeBlanc, H. N. & Ashkenazi, A. (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death and Differentiation* **10,** 66-75.

Lee, H. O., Herndon, J. M., Barreiro, R., Griffith, T. S. & Ferguson, T. A. (2002). TRAIL: A mechanism of tumor surveillance in an immune privileged site. *Journal of Immunology* **169,** 4739-4744.

Lee, N. K. & Lee, S. Y. (2002). Modulation of life and death by the tumor necrosis factor receptor-associated factors (TRAFs). *Journal of Biochemistry and Molecular Biology* **35,** 61-66.

Lee, S. Y., Kaufman, D. R., Mora, A. L., Santana, A., Boothby, M. & Choi, Y. (1998). Stimulus-dependent synergism of the antiapoptotic tumor necrosis factor receptor-associated factor 2 (TRAF2) and nuclear factor kappaB pathways. *Journal of Experimental Medicine* **188,** 1381-1384.

Lee, S. Y., Reichlin, A., Santana, A., Sokol, K. A., Nussenzweig, M. C. & Choi, Y. (1997). TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity* **7,** 703-713.

Leo, E., Welsh, K., Matsuzawa, S., Zapata, J. M., Kitada, S., Mitchell, R. S., Ely, K. R. & Reed, J. C. (1999). Differential requirements for tumor necrosis factor receptor-associated factor family proteins in CD40 mediated induction of NF-kappa B and Jun N-terminal kinase activation. *Journal of Biological Chemistry* **274,** 22414-22422.

Liao, G. X., Zhang, M. Y., Harhaj, E. W. & Sun, S. C. (2004). Regulation of the NF-kappa B-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *Journal of Biological Chemistry* **279,** 26243-26250.

Lin, J. H., Li, H., Yasumura, D., Cohen, H. R., Zhang, C., Panning, B., Shokat, K. M., Lavail, M. M. & Walter, P. (2007). IRE1 signaling affects cell fate during the unfolded protein response. *Science* **318,** 944-949.

Ling, L. & Goeddel, D. V. (2000). MIP-T3, a novel protein linking tumor necrosis factor receptor-associated factor 3 to the microtubule network. *Journal of Biological Chemistry* **275,** 23852-23860.

Link, B. A. & Nishi, R. (1998). Development of the avian iris and ciliary body: mechanisms of cellular differentiation during the smooth-to-striated muscle transition. *Developmental Biology* **203,** 163-176.

Litsiou, A., Hanson, S. & Streit, A. (2005). A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. *Development* **132,** 4051-4062.

Lo, W. K., Wen, X. J. & Zhou, C. J. (2003). Microtubule configuration and membranous vesicle transport in elongating fiber cells of the rat lens. *Experimental Eye Research* **77,** 615-626.

Lockshin, R. A. & Zakeri, Z. (2004). Caspase-independent cell death? *Oncogene* **23,** 2766-2773.

Locksley, R. M., Killeen, N. & Lenardo, M. J. (2001). The TNF and TNF receptor superfamilies: Integrating mammalian biology. *Cell* **104,** 487-501.

Lovicu, F. J. & McAvoy, J. W. (2001). FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling. *Development* **128,** 5075-5084.

Lovicu, F. J. & McAvoy, J. W. (2005). Growth factor regulation of lens development. *Developmental Biology* **280,** 1-14.

Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O. & Lee, D. C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73,** 263-278.

Lyu, J. & Joo, C. K. (2004). Wnt signaling enhances FGF2-triggered lens fiber cell differentiation. *Development* **131,** 1813-1824.

MacEwan, D. J. (2002). TNF ligands and receptors - a matter of life and death. *British Journal of Pharmacology* **135,** 855-875.

MacFarlane, M. (2003). TRAIL-induced signalling and apoptosis. *Toxicol Lett* **139,** 89-97.

MacLachlan, T. K. & Giordano, A. (1998). TRAF2 expression in differentiated muscle. *Journal of Cellular Biochemistry* **71,** 461-466.

Malecaze, F., Decha, A., Serre, B., Penary, M., Duboue, M., Berg, D., Levade, T., Lubsen, N. H., Kremer, E. J. & Couderc, B. (2006). Prevention of posterior capsule opacification by the induction of therapeutic apoptosis of residual lens cells. *Gene Therapy.*

Mansergh, F., Wride, M., Walker, V., Adams, S., Hunter, S. & Evans, M. (2004). Gene expression changes during cataract progression in Sparc null mice: Differential regulation of mouse globins in the lens. *Molecular Vision* **10,** 490-511.

Masson, R., Regnier, C. H., Chenard, M. P., Wendling, C., Mattei, M. G., Tomasetto, C. & Rio, M. C. (1998). Tumor necrosis factor receptor associated factor 4 (TRAF4) expression pattern during mouse development. *Mechanisms of Development* **71,** 187-191.

Mathers, P. H., Grinberg, A., Mahon, K. A. & Jamrich, M. (1997). The Rx homeobox gene is essential for vertebrate eye development. *Nature* **387,** 603-607.

Mathias, R. T., Rae, J. L. & Baldo, G. J. (1997). Physiological properties of the normal lens. *Physiological Reviews* **77,** 21-50.

Matsui, M., Yamamoto, A., Kuma, A., Ohsumi, Y. & Mizushima, N. (2006). Organelle degradation during the lens and erythroid differentiation is independent of autophagy. *Biochemical and Biophysical Research Communications* **339,** 485-489.

McAvoy, J. W. & Chamberlain, C. G. (1989). Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. *Development* **107,** 221-228.

McCarty, C. A. & Taylor, H. R. (1996). Recent developments in vision research: light damage in cataract. *Investigative Ophthalmology & Visual Science* **37,** 1720-1723.

Menko, A. S., Klukas, K. A. & Johnson, R. G. (1984). Chicken embryo lens cultures mimic differentiation in the lens. *Developmental Biology* **103,** 129-141.

Menko, S. A. (2002). Lens epithelial cell differentiation. *Experimental Eye Research* **75,** 485-490.

Mic, F. A., Molotkov, A., Molotkova, N. & Duester, G. (2004). Raldh2 expression in optic vesicle generates a retinoic acid signal needed for invagination of retina during optic cup formation. *Developmental Dynamics* **231,** 270-277.

Modak, S. P. & Perdue, S. W. (1970). Terminal lens cell differentiation. I. Histological and microspectrophotometric analysis of nuclear degeneration. *Experimental Cell Research* **59,** 43-56.

Morita, Y., Kanei-Ishii, C., Nomura, T. & Ishii, S. (2005). TRAF7 Sequesters c-Myb to the Cytoplasm by Stimulating Its Sumoylation. *Molecular Biology of the Cell.*

Morozov, V. & Wawrousek, E. F. (2005). Alpha-crystallin prevents cellular disintegration during the apoptosis-like program of lens fiber cell maturation. *Investigative Ophthalmology & Visual Science* **46,** -.

Morozov, V. & Wawrousek, E. F. (2006). Caspase-dependent secondary lens fiber cell disintegration in alpha A-/alpha B-crystallin double-knockout mice. *Development* **133,** 813-821.

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C. & Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* **80,** 389-399.

Motaln, H., McWhir, J. & Horvat, S. (2005). In situ analysis of Raidd-beta-galactosidase fusion gene expression in transgenic mouse midgestation embryos. *Transgenic Research* **14,** 27-40.

Munoz, B., Tajchman, U., Bochow, T. & West, S. (1993). Alcohol use and risk of posterior subcapsular opacities. *Archives of Ophthalmology* **111,** 110-112.

Muta, M., Kamachi, Y., Yoshimoto, A., Higashi, Y. & Kondoh, H. (2002). Distinct roles of SOX2, Pax6 and Maf transcription factors in the regulation of lens-specific delta1-crystallin enhancer. *Genes to Cells* **7,** 791-805.

Nagata, S. (2005). DNA Degradation in Development and Programmed Cell Death. *Annual Review of Immunology* **23,** 853-875.

Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T. & Inoue, J. (1999). Severe osteopetrosis, defective interleukin-1 signalling and lymph node organogenesis in TRAF6-deficient mice. *Genes to Cells* **4,** 353-362.

Nakahara, M., Nagasaka, A., Koike, M., Uchida, K., Kawane, K., Uchiyama, Y. & Nagata, S. (2007). Degradation of nuclear DNA by DNase II-like acid DNase in cortical fiber cells of mouse eye lens. *Febs Journal* **274,** 3055-3064.

Nakamura, I., Kadono, Y., Takayanagi, H., Jimi, E., Miyazaki, T., Oda, H., Nakamura, K., Tanaka, S., Rodan, G. A. & Duong le, T. (2002). IL-1 regulates cytoskeletal organization in osteoclasts via TNF receptor-associated factor 6/c-Src complex. *Journal of Immunology* **168,** 5103-5109.

Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C. F., Yagita, H. & Okumura, K. (1996). TRAF5, an activator of NF-kappaB and putative signal transducer for the lymphotoxin-beta receptor. *Journal of Biological Chemistry* **271,** 14661-14664.

Nakano, H., Sakon, S., Koseki, H., Takemori, T., Tada, K., Matsumoto, M., Munechika, E., Sakai, T., Shirasawa, T., Akiba, H., Kobata, T., Santee, S. M., Ware, C. F., Rennert, P. D., Taniguchi, M., Yagita, H. & Okumura, K. (1999). Targeted disruption of Traf5 gene causes defects in CD40- and CD27-mediated lymphocyte activation. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 9803-9808.

Nguyen, L. T., Duncan, G. S., Mirtsos, C., Ng, M., Speiser, D. E., Shahinian, A., Marino, M. W., Mak, T. W., Ohashi, P. S. & Yeh, W. C. (1999). TRAF2 deficiency results in hyperactivity of certain TNFR1 signals and impairment of CD40-mediated responses. *Immunity* **11,** 379-389.

Nishimoto, S., Kawane, K., Watanabe-Fukunaga, R., Fukuyama, H., Ohsawa, Y., Uchiyama, Y., Hashida, N., Ohguro, N., Tano, Y., Morimoto, T., Fukuda, Y. & Nagata, S. (2003). Nuclear cataract caused by a lack of DNA degradation in the mouse eye lens. *Nature* **424,** 1071-1074.

Nonaka, T., Nishiura, M. & Ohkuma, M. (1976). Gap junctions of lens fiber cells in freeze-fracture replicas. *Journal of Electron Microscopy (Tokyo)* **25,** 35-36.

Norton, T. T. (1999). Animal Models of Myopia: Learning How Vision Controls the Size of the Eye. *Institute for Laboratory Animal Research Journal* **40,** 59-77.

Ofri, R. (2002). Intraocular pressure and glaucoma. *Vetinary Clinics of North America. Exotic Animal Practice* **5,** 391-406, vii-viii.

Ogino, H. & Yasuda, K. (1998). Induction of lens differentiation by activation of a bZIP transcription factor, L-maf. *Science* **280,** 115-118.

Ogino, H. & Yasuda, K. (2000). Sequential activation of transcription factors in lens induction. *Development Growth & Differentiation* **42,** 437-448.

Ohuchi, H., Tomonari, S., Itoh, H., Mikawa, T. & Noji, S. (1999). Identification of chick rax/rx genes with overlapping patterns of expression during early eye and brain development. *Mechanisms of Development* **85,** 193-195.

Oliver, G. & Gruss, P. (1997). Current views on eye development. *Trends in Neurosciences* **20,** 415-421.

Ozeki, H., Ogura, Y., Hirabayashi, Y. & Shimada, S. (2001). Suppression of lens stalk cell apoptosis by hyaluronic acid leads to faulty separation of the lens vesicle. *Experimental Eye Research* **72,** 63-70.

Patek, C. E. & Clayton, R. M. (1990). Age-related changes in the response of chick lens cells during long-term culture to insulin, cyclic AMP, retinoic acid and a bovine retinal extract. *Experimental Eye Research* **50,** 345-354.

Patthey, C., Gunhaga, L. & Edlund, T. (2008). Early development of the central and peripheral nervous systems is coordinated by Wnt and BMP signals. *PLoS ONE* **3,** e1625.

Perng, M. D., Zhang, Q. & Quinlan, R. A. (2007). Insights into the beaded filament of the eye lens. *Experimental Cell Research* **313,** 2180-2188.

Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlosky, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J. & Black, R. A. (1998).

An essential role for ectodomain shedding in mammalian development. *Science* **282,** 1281-1284.

Piatigorsky, J. (1981a). Lens differentiation in vertebrates. A review of cellular and molecular features. *Differentiation* **19,** 134-153.

Piatigorsky, J. (1981b). Structural and functional similarities of delta-crystallin messenger ribonucleic acids from duck and chicken lenses. *Biochemistry* **20,** 6427-6431.

Pispa, J., Mikkola, M. L., Mustonen, T. & Thesleff, I. (2003). Ectodysplasin, Edar and TNFRSF19 are expressed in complementary and overlapping patterns during mouse embryogenesis. *Gene Expression Patterns* **3,** 675-679.

Plaza, S., Dozier, C. & Saule, S. (1993). Quail Pax-6 (Pax-QNR) encodes a transcription factor able to bind and trans-activate its own promoter. *Cell growth & differentiation* **4,** 1041-1050.

Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J. & Kehry, M. R. (1999). High-affinity interactions of tumor necrosis factor receptor-associated factors (TRAFs) and CD40 require TRAF trimerization and CD40 multimerization. *Biochemistry* **38,** 10168-10177.

Pullen, S. S., Miller, H. G., Everdeen, D. S., Dang, T. T., Crute, J. J. & Kehry, M. R. (1998). CD40-tumor necrosis factor receptor-associated factor (TRAF) interactions: regulation of CD40 signaling through multiple TRAF binding sites and TRAF hetero-oligomerization. *Biochemistry* **37,** 11836-11845.

Ranta, P., Tommila, P. & Kivela, T. (2004). Retinal breaks and detachment after neodymium: YAG laser posterior capsulotomy: five-year incidence in a prospective cohort. *Journal of Cataract and Refractive Surgery* **30,** 58-66.

Rao, P. V. & Maddala, R. (2006). The role of the lens actin cytoskeleton in fiber cell elongation and differentiation. *Seminars in Cell & Developmental Biology* **17,** 698-711.

Reddy, M. A., Francis, P. J., Berry, V., Bhattacharya, S. S. & Moore, A. T. (2004). Molecular genetic basis of inherited cataract and associated phenotypes. *Survey of Opthalmology* **49,** 300-315.

Regnier, C. H., Masson, R., Kedinger, V., Textoris, J., Stoll, I., Chenard, M. P., Dierich, A., Tomasetto, C. & Rio, M. C. (2002). Impaired neural tube closure, axial skeleton malformations, and tracheal ring disruption in TRAF4-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 5585-5590.

Regnier, C. H., Tomasetto, C., Moog-Lutz, C., Chenard, M. P., Wendling, C., Basset, P. & Rio, M. C. (1995). Presence of a new conserved domain in CART1, a novel member of the tumor necrosis factor receptor-associated protein family, which is expressed in breast carcinoma. *Journal of Biological Chemistry* **270,** 25715-25721.

Reza, H. M., Ogino, H. & Yasuda, K. (2002). L-Maf, a downstream target of Pax6, is essential for chick lens development. *Mechanisms of Development* **116,** 61-73.

Reza, H. M. & Yasuda, K. (2004). Roles of Maf family proteins in lens development. *Developmental Dynamics* **229,** 440-448.

Ricci, M. S., Jin, Z., Dews, M., Yu, D., Thomas-Tikhonenko, A., Dicker, D. T. & El-Deiry, W. S. (2004). Direct repression of FLIP expression by c-myc is a major determinant of TRAIL sensitivity. *Molecular and Cellular Biology* **24,** 8541-8555.

Ring, B. Z., Cordes, S. P., Overbeek, P. A. & Barsh, G. S. (2000). Regulation of mouse lens fiber cell development and differentiation by the Maf gene. *Development* **127,** 307-317.

Robman, L. & Taylor, H. (2005). External factors in the development of cataract. *Eye* **19,** 1074-1082.

Rothe, M., Sarma, V., Dixit, V. M. & Goeddel, D. V. (1995). TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* **269,** 1424-1427.

Rothe, M., Wong, S. C., Henzel, W. J. & Goeddel, D. V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* **78,** 681-692.

Rubinfeld, H. & Seger, R. (2005). The ERK cascade: a prototype of MAPK signaling. *Molecular Biotechnology* **31,** 151-174.

Saha, S. K., Pietras, E. M., He, J. Q., Kang, J. R., Liu, S. Y., Oganesyan, G., Shahangian, A., Zarnegar, B., Shiba, T. L., Wang, Y. & Cheng, G. (2006). Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *Embo Journal* **25,** 3257-3263.

Salvesen, G. S. & Riedl, S. J. (2008). Caspase mechanisms. *Advances in Experimental Medical Biology* **615,** 13-23.

Sandberg, M., Hammerschmidt, W. & Sugden, B. (1997). Characterization of LMP-1's association with TRAF1, TRAF2, and TRAF3. *Journal of Virology* **71,** 4649-4656.

Sanders, E. J. & Parker, E. (2003). Retroviral overexpression of bcl-2 in the embryonic chick lens influences denucleation in differentiating lens fiber cells. *Differentiation* **71,** 425-433.

Sanders, E. J. & Wride, M. A. (1997). Roles for growth and differentiation factors in avian embryonic development. *Poultry Science* **76,** 111-117.

Sandilands, A., Prescott, A. R., Wegener, A., Zoltoski, R. K., Hutcheson, A. M., Masaki, S., Kuszak, J. R. & Quinlan, R. A. (2003). Knockout of the intermediate filament protein CP49 destabilises the lens fibre cell cytoskeleton and decreases lens optical quality, but does not induce cataract. *Experimental Eye Research* **76,** 385-391.

Sato, T., Irie, S. & Reed, J. C. (1995). A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. *Febs Letters* **358,** 113-118.

Sauka-Spengler, T. & Barembaum, M. (2008). Gain- and loss-of-function approaches in the chick embryo. *Methods in Cell Biology* **87,** 237-256.

Sayers, T. J. & Murphy, W. J. (2006). Combining proteasome inhibition with TNF-related apoptosis-inducing ligand (Apo2L/TRAIL) for cancer therapy. *Cancer immunology, immunotherapy : CII* **55,** 76-84.

Semina, E. V., Murray, J. C., Reiter, R., Hrstka, R. F. & Graw, J. (2000). Deletion in the promoter region and altered expression of Pitx3 homeobox gene in aphakia mice. *Human Molecular Genetics* **9,** 1575-1585.

Semina, E. V., Reiter, R. S. & Murray, J. C. (1997). Isolation of a new homeobox gene belonging to the Pitx/Rieg family: expression during lens development and mapping to the aphakia region on mouse chromosome 19. *Human Molecular Genetics* **6,** 2109-2116.

Shang, F., Gong, X., McAvoy, J. W., Chamberlain, C., Nowell, T. R., Jr. & Taylor, A. (1999). Ubiquitin-dependent pathway is up-regulated in differentiating lens cells. *Experimental Eye Research* **68,** 179-192.

Shestopalov, V. I. & Bassnett, S. (2000). Expression of autofluorescent proteins reveals a novel protein permeable pathway between cells in the lens core. *Journal of Cell Science* **113 (Pt 11),** 1913-1921.

Shestopalov, V. I. & Bassnett, S. (2002). Development of the lens core syncytium. *Investigative Ophthalmology & Visual Science* **43,** 4631.

Shestopalov, V. I. & Bassnett, S. (2003). Development of a macromolecular diffusion pathway in the lens. *Journal of Cell Science* **116,** 4191-4199.

Shiels, A., Mackay, D., Ionides, A., Berry, V., Moore, A. & Bhattacharya, S. (1998). A missense mutation in the human connexin50 gene (GJA8) underlies autosomal dominant "zonular pulverulent" cataract, on chromosome 1q. *American Journal of Human Genetics* **62,** 526-532.

Shimada, N., Aya-Murata, T., Reza, H. M. & Yasuda, K. (2003). Cooperative action between L-Maf and Sox2 on delta-crystallin gene expression during chick lens development. *Mechanisms of Development* **120,** 455-465.

Shirke, S., Faber, S. C., Hallem, E., Makarenkova, H. P., Robinson, M. L., Overbeek, P. A. & Lang, R. A. (2001). Misexpression of IGF-I in the mouse lens expands the transitional zone and perturbs lens polarization. *Mechanisms of Development* **101,** 167-174.

Sjodal, M., Edlund, T. & Gunhaga, L. (2007). Time of exposure to BMP signals plays a key role in the specification of the olfactory and lens placodes ex vivo. *Developmental Cell* **13,** 141-149.

Slee, E. A., Adrain, C. & Martin, S. J. (2001). Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *Journal of Biological Chemistry* **276,** 7320-7326.

Smith, R. S., Hawes, N. L., Chang, B., Roderick, T. H., Akeson, E. C., Heckenlively, J. R., Gong, X., Wang, X. & Davisson, M. T. (2000). Lop12, a mutation in mouse Crygd causing lens opacity similar to human Coppock cataract. *Genomics* **63,** 314-320.

So, T., Salek-Ardakani, S., Nakano, H., Ware, C. F. & Croft, M. (2004). TNF receptor-associated factor 5 limits the induction of Th2 immune responses. *J Immunol* **172,** 4292-4297.

Song, H. Y., Regnier, C. H., Kirschning, C. J., Goeddel, D. V. & Rothe, M. (1997). Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proceedings of the National Academy of Sciences of the United States of America* **94,** 9792-9796.

Song, Y. J., Jen, K. Y., Soni, V., Kieff, E. & Cahir-McFarland, E. (2006). IL-1 receptor-associated kinase 1 is critical for latent membrane protein 1-induced p65/RelA serine 536 phosphorylation and NF-kappaB activation. *Proceedings of the National Academy of Sciences of the United States of America* **103,** 2689-2694.

Speiser, D. E., Lee, S. Y., Wong, B., Arron, J., Santana, A., Kong, Y. Y., Ohashi, P. S. & Choi, Y. (1997). A regulatory role for TRAF1 in antigen-induced apoptosis of T cells. *Journal of Experimental Medicine* **185,** 1777-1783.

Stradner, A., Foffi, G., Dorsaz, N., Thurston, G. & Schurtenberger, P. (2007). New insight into cataract formation: enhanced stability through mutual attraction. *Physical Review Letters* **99,** 198103.

Stump, R. J., Ang, S., Chen, Y., von Bahr, T., Lovicu, F. J., Pinson, K., de Iongh, R. U., Yamaguchi, T. P., Sassoon, D. A. & McAvoy, J. W. (2003). A role for Wnt/beta-catenin signaling in lens epithelial differentiation. *Developmental Biology* **259,** 48-61.

Szalay, K., Razga, Z. & Duda, E. (1997). TNF inhibits myogenesis and downregulates the expression of myogenic regulatory factors myoD and myogenin. *European Journal of Cell Biology* **74,** 391-398.

Tan, J. K., Aphale, A., Malaviya, R., Sun, Y. & Gottlieb, A. B. (2007). Mechanisms of action of etanercept in psoriasis. *The Journal of Investigative Dermatology* **12,** 38-45.

Tan, Y., Rouse, J., Zhang, A., Cariati, S., Cohen, P. & Comb, M. J. (1996). FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *European Molecular Biology Organization (EMBO) Journal* **15,** 4629-4642.

Taylor-Papadimitriou, J., Shearer, M. & Stoker, M. G. (1977). Growth requirements of human mammary epithelial cells in culture. *International Journal of Cancer* **20,** 903-908.

Taylor-Papadimitriou, J., Shearer, M. & Watling, D. (1978). Growth requirements of calf lens epithelium in culture. *Journal of Cellular Physiology* **95,** 95-103.

Tedesco, R. C., Calabrese, K. S. & Smith, R. L. (2005). Architecture of the ciliary muscle of Gallus domesticus. *Anat Rec A Discov Mol Cell Evol Biol* **284,** 544-549.

Thain, M. & Hickman, M. (2000). *Penguin Dictionary of Biology*. Penguin Group, London.

Thornberry, N. A. (1997). The caspase family of cysteine proteases. *British Medical Bulletin* **53,** 478-490.

Tini, M., Otulakowski, G., Breitman, M. L., Tsui, L. C. & Giguere, V. (1993). An everted repeat mediates retinoic acid induction of the gamma F-crystallin gene: evidence of a direct role for retinoids in lens development. *Genes & Development* **7,** 295-307.

To, C. H., Kong, C. W., Chan, C. Y., Shahidullah, M. & Do, C. W. (2002). The mechanism of aqueous humour formation. *Clinical and Experimental Optometry* **85,** 335-349.

Tomarev, S. I., Sundin, O., Banerjee-Basu, S., Duncan, M. K., Yang, J. M. & Piatigorsky, J. (1996). Chicken homeobox gene Prox 1 related to Drosophila prospero is expressed in the developing lens and retina. *Developmental Dynamics* **206,** 354-367.

Treisman, J. E. (2004). How to make an eye. *Development* **131,** 3823-3827.

Trousse, F., Esteve, P. & Bovolenta, P. (2001). BMP4 mediates apoptotic cell death in the developing chick eye. *Journal of Neuroscience* **21,** 1292-1301.

Truneh, A., Sharma, S., Silverman, C., Khandekar, S., Reddy, M. P., Deen, K. C., McLaughlin, M. M., Srinivasula, S. M., Livi, G. P., Marshall, L. A., Alnemri, E. S., Williams, W. V. & Doyle, M. L. (2000). Temperature-sensitive differential affinity of TRAIL for its receptors - DR5 is the highest affinity receptor. *Journal of Biological Chemistry* **275,** 23319-23325.

Tsitsikov, E. N., Laouini, D., Dunn, I. F., Sannikova, T. Y., Davidson, L., Alt, F. W. & Geha, R. S. (2001). TRAF1 is a negative regulator of TNF signaling. enhanced TNF signaling in TRAF1-deficient mice. *Immunity* **15,** 647-657.

Uhlik, M. T., Abell, A. N., Cuevas, B. D., Nakamura, K. & Johnson, G. L. (2004). Wiring diagrams of MAPK regulation by MEKK1, 2, and 3. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire* **82,** 658-663.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P. & Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* **287,** 664-666.

van Eyndhoven, W. G., Cho, E. & Lederman, S. (1999a). Splice-deletion variants of TRAF-3 encode putative isoforms that induce NF-kappa B activation. *The Federation of American Societies for Experimental Biology Journal* **13,** A988-A988.

van Eyndhoven, W. G., Gamper, C. J., Cho, E., Mackus, W. J. & Lederman, S. (1999b). TRAF-3 mRNA splice-deletion variants encode isoforms that induce NF-kappaB activation. *Molecular Immunology* **36,** 647-658.

van Leyen, K. (1998). Pictures in cell biology. Un-LOXing the door to organelle degradation. *Trends in Cell Biology* **8,** 436.

Vijaya, R., Gupta, R., Panda, G., Ravishankar, K. & Kumaramanickavel, G. (1997). Genetic analysis of adult-onset cataract in a city-based ophthalmic hospital. *Clinical Genetics* **52,** 427-431.

Vijayvergiya, C., De Angelis, D., Walther, M., Kuhn, H., Duvoisin, R. M., Smith, D. H. & Wiedmann, M. (2004). High-level expression of rabbit 15-lipoxygenase induces collapse of the mitochondrial pH gradient in cell culture. *Biochemistry* **43,** 15296-15302.

Vogel-Hopker, A., Momose, T., Rohrer, H., Yasuda, K., Ishihara, L. & Rapaport, D. H. (2000). Multiple functions of fibroblast growth factor-8 (FGF-8) in chick eye development. *Mechanisms of Development* **94,** 25-36.

Vrensen, G. F., Graw, J. & De Wolf, A. (1991). Nuclear breakdown during terminal differentiation of primary lens fibres in mice: a transmission electron microscopic study. *Experimental Eye Research* **52,** 647-659.

Wajant, H., Pfizenmaier, K. & Scheurich, P. (2003). Tumor necrosis factor signaling. *Cell Death Differentiation* **10,** 45-65.

Wakely, J. (1977). Scanning electron microscope study of the extracellular matrix between presumptive lens and presumptive retina of the chick embryo. *Anatomy and Embryology* **150,** 163-170.

Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V. & Baldwin, A. S., Jr. (1998). NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281,** 1680-1683.

Wang, S. H., Mezosi, E., Wolf, J. M., Cao, Z., Utsugi, S., Gauger, P. G., Doherty, G. M. & Baker, J. R., Jr. (2004). IFNgamma sensitization to TRAIL-induced apoptosis in human thyroid carcinoma cells by upregulating Bak expression. *Oncogene* **23,** 928-935.

Wawersik, S. & Maas, R. L. (2000). Vertebrate eye development as modeled in Drosophila. *Human Molecular Genetics* **9,** 917-925.

Wawersik, S., Purcell, P. & Maas, R. L. (2000). Pax6 and the genetic control of early eye development. *Results and Problems in Cell Differentiation* **31,** 15-36.

Weber, G. F. & Menko, A. S. (2004). Actin filaments promote survival of differentiating lens cells. *Molecular Biology of the Cell* **15,** 2539-2542.

Weber, G. F. & Menko, A. S. (2005). The canonical intrinsic mitochondrial death pathway has a non-apoptotic role in signaling lens cell differentiation. *Journal of Biological Chemistry.*

Weber, G. F. & Menko, A. S. (2006). Phosphatidylinositol 3-kinase is necessary for lens fiber cell differentiation and survival. *Investigative Ophthalmology and Visual Science* **47,** 4490-4499.

Wertz, I. E. & Dixit, V. M. (2008). Ubiquitin-mediated regulation of TNFR1 signaling. *Cytokine Growth Factor Reviews* **19,** 313-324.

West, S. K. & Valmadrid, C. T. (1995). Epidemiology of risk factors for age-related cataract. *Survey of Opthalmology* **39,** 323-334.

White, T. W., Goodenough, D. A. & Paul, D. L. (1998). Targeted ablation of connexin50 in mice results in microphthalmia and zonular pulverulent cataracts. *Journal of Cell Biology* **143,** 815-825.

Wigle, J. T., Chowdhury, K., Gruss, P. & Oliver, G. (1999). Prox1 function is crucial for mouse lens-fibre elongation. *Nature Genetics* **21,** 318-322.

Wistow, G. J. & Piatigorsky, J. (1988). Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. *Annual Review of Biochemistry* **57,** 479-504.

Wride, M. A. (1996). Cellular and molecular features of lens differentiation: A review of recent advances. *Differentiation* **61,** 77-93.

Wride, M. A. (2000). Minireview: Apoptosis as seen through a lens. *Apoptosis* **5,** 203-209.

Wride, M. A. (2007). Role of proteases in the development and diseases of the lens *Expert review of ophthalmology* **2,** 833-844.

Wride, M. A., Geatrell, J. & Guggenheim, J. A. (2006). Proteases in eye development and disease. *Birth Defects Res C Embryo Today* **78,** 90-105.

Wride, M. A., Mansergh, F. C., Adams, S., Everitt, R., Minnema, S. E., Rancourt, D. E. & Evans, M. J. (2003). Expression profiling and gene discovery in the mouse lens. *Molecular Vision* **9,** 360-396.

Wride, M. A., Parker, E. & Sanders, E. J. (1999). Members of the bcl-2 and caspase families regulate nuclear degeneration during chick lens fibre differentiation. *Developmental Biology* **213,** 142-156.

Wride, M. A. & Sanders, E. J. (1995). Potential Roles for Tumor-Necrosis-Factor-Alpha During Embryonic-Development. *Anatomy and Embryology* **191,** 1-10.

Wride, M. A. & Sanders, E. J. (1998). Nuclear degeneration in the developing lens and its regulation by TNF alpha. *Experimental Eye Research* **66,** 371-383.

Wu, C. J., Conze, D. B., Li, X., Ying, S. X., Hanover, J. A. & Ashwell, J. D. (2005). TNF-alpha induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination. *European Molecular Biology Organization (EMBO) Journal.*

Xi, J. H., Bai, F. & Andley, U. P. (2003). Reduced survival of lens epithelial cells in the alphaA-crystallin-knockout mouse. *Journal of Cell Science* **116,** 1073-1085.

Xia, C. H., Cheng, C., Huang, Q., Cheung, D., Li, L., Dunia, I., Benedetti, L. E., Horwitz, J. & Gong, X. (2006a). Absence of alpha3 (Cx46) and alpha8 (Cx50) connexins leads to cataracts by affecting lens inner fiber cells. *Experimental Eye Research* **83,** 688-696.

Xia, C. H., Liu, H., Cheung, D., Cheng, C., Wang, E., Du, X., Beutler, B., Lo, W. K. & Gong, X. (2006b). Diverse gap junctions modulate distinct mechanisms for fiber cell formation during lens development and cataractogenesis. *Development* **133,** 2033-2040.

Xu, L. G., Li, L. Y. & Shu, H. B. (2004). TRAF7 potentiates MEKK3-induced AP1 and CHOP activation and induces apoptosis. *Journal of Biological Chemistry* **279,** 17278-17282.

Xu, Y., Cheng, G. H. & Baltimore, D. (1996). Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. *Immunity* **5,** 407-415.

Yang, P., Wiser, J. L., Peairs, J. J., Ebright, J. N., Zavodni, Z. J., Rickman, C. B. & Jaffe, G. J. (2005). Human RPE Expression of Cell Survival Factors. *Investigative Ophthalmology & Visual Science* **46,** 1755-1764.

Yao, K., Wang, K., Xu, W., Sun, Z., Shentu, X. & Qiu, P. (2003). Caspase-3 and its inhibitor Ac-DEVD-CHO in rat lens epithelial cell apoptosis induced by hydrogen in vitro. *Chinese Medical Journal (English)* **116,** 1034-1038.

Yeh, W. C., Shahinian, A., Speiser, D., Kraunus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rothe, M., Goeddel, D. V. & Mak, T. W. (1997). Early lethality, functional NF-kappaB activation, and increased sensitivity to TNF-induced cell death in TRAF2-deficient mice. *Immunity* **7,** 715-725.

Yoshida, H., Jono, H., Kai, H. & Li, J. D. (2005). The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. *Journal of Biological Chemistry* **280,** 41111-41121.

Yujiri, T., Sather, S., Fanger, G. R. & Johnson, G. L. (1998). Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. *Science* **282,** 1911-1914.

Zandy, A. J., Lakhani, S., Zheng, T., Flavell, R. A. & Bassnett, S. (2005). Role of the executioner caspases during lens development. *Journal of Biological Chemistry* **280,** 30263-30272.

Zapata, J. M. & Reed, J. C. (2002). TRAF1: lord without a RING. *Science STKE: signal transduction knowledge environment* **2002,** PE27.

Zelenka, P. S. (2004). Regulation of cell adhesion and migration in lens development. *International Journal of Developmental Biology* **48,** 857-865.

Zhang, G. (2004). Tumor necrosis factor family ligand-receptor binding. *Current Opinion in Structural Biology* **14,** 154-160.

Zhang, L., Mathers, P. H. & Jamrich, M. (2000). Function of Rx, but not Pax6, is essential for the formation of retinal progenitor cells in mice. *Genesis* **28,** 135-142.

Zhou, J. & Menko, A. S. (2002). The role of Src family kinases in cortical cataract formation. *Investigative Ophthalmology & Visual Science* **43,** 2293-2300.

Zhou, J. & Menko, A. S. (2004). Coordinate signaling by Src and p38 kinases in the induction of cortical cataracts. *Investigative Ophthalmology & Visual Science* **45,** 2314-2323.

Appendices

Appendix 1- Solution concentrations

10x PBS (Phosphate Buffered Saline)

800mls of *dd* (double distilled) water added to a sterile flask. Add magnetic stirrer and mix (not so vigorously that oxygen is introduced). For 10x PBS add 80g of Na_2Cl, 2g of KCl, 14.4g of Na_2HPO_4 and 2.5g of KH_2PO_4. Dissolve for 5 minutes or until fully dissolved. To adjust the pH, calibrate pH meter, reduce speed of mixing then pH the solution to 7.4. Put the solution into a sterile graduated cylinder and add *d.d.* water up to 1 litre. Put solution into a litre bottle and then autoclave.

10x MOPS (morpholinepropanesulfonic acid)

To make up one litre, add 41.85g 4-morpholinepropanesulphonic acid (MOPS-acid free) and 6.8g sodium acetate-$3H_2O$ to 800 mls of double autoclaved H_20 and stir until completely dissolved. Add 20mls 0.5M Na_2EDTA solution and adjust pH to 7.0 with 10x NaOH. Adjust volume to 1 litre with double autoclaved water. Store in fridge at 4°C wrapped in foil

Composition of RNA gel

To produce a gel volume of 150mls, 2.75g of agarose was added to 108mls *da* (double autoclaved) water, giving a final 1.5% agarose concentration. The agarose solution was brought to boil until all the agarose had dissolved then 27mls Formaldehyde and 15mls 10xMOPS was added under a fume hood, poored into a casting gel and left to set for 1 hour.

RNA loading buffer recipe

50% Glycerol (so it sinks quicker into the gel wells), 10mM EDTA, 0.25% Bromothenol Blue, and 0.25% Xylene.

4% PFA (Paraformaldehyde)

Dissolve 4g Paraformaldehyde (using face mask when weighing PFA) in 100mls – heat until dissolved- store at 4°C.

10% SDS

100mls SDS into 900mls H_20.

10% APS

1g of Ammonium persulphate in 10mls distilled water. store at 4°C

0.5M Tris-HCl buffer pH 6.8

6g Tris base, 60mls d water, pH to 6.8 with HCl. Make up to 100mls with d water, Store at 4°C.

1.5M Tris buffer pH8.8

27.23g Tris base, 80mls d water, pH to 8.8 with HCl. Make up to 150mls with d water.

DNA ladder.

Ladder 1µl
Loading Buffer 3µl
H_2O 11µl

Fractionation Buffer

0.32 M sucrose (11g in 100ml buffer)
2 mM Hepes (200µl of 1M stock)
dH2O to 100ml
Stored frozen in 10ml aliquots.

Laemmli Buffer (2x stock)

4x stock 500µl
β-mercaptoethanol 100µl or 15.4 mg DTT
d.H2O 400µl

1M Hepes

238mg / ml, alter pH to 7.2 – 7.5 with NaOH.

2x Loading Buffer

2x sample loading buffer	(to make 10ml)
4% SDS	4ml of 10% SDS
20% glycerol	2ml
120mM Tris	2.4ml 0.5M Tris pH 6.8
Water	1.6ml

Bromophenol blue 0.01% (w/v)
Aliquot and freeze at -20oC.

Western blotting running buffer

100ml 10x Tris/glycine/SDS buffer (Bio-Rad, UK) to 900ml distilled water

Transfer buffer
200ml methanol (Fisher Scientific, UK), 100ml Tris/glycine buffer (Bio-Rad, UK) and 700ml water

10 xTBS
10mM Tris, 10mM NaCl, pH 7.6. Make up to 1l with water

TBS/Tween.
100mls 10x TBS in 900mls d water, add 1ml Tween20 (Sigma, UK)

Stripping buffer
15mls glycine, 1g SDS, 10ml Tween20, pH to 2.2. Make up to 1l with da water.

VIP peroxidase
5mls PBS + 3 drops of reagents 1, 2 and 3, mix well + 3 drops of Hydrogen peroxide.

Appendix 2- PCRs, primers, densitometry and statistics

Table A.2.1 Primers designed showing primer sequences, band size, optimised temperature and cycle number. This table shows the sequence of the primers designed from the accession numbers listed and using Primer3 as described in chap 2.2.6. Also listed are the expected PCR product sizes, the optimised annealing temperatures and cycle numbers used. Primers highlighted in yellow indicate those whose expression was studied in triplicate. GAPDH, highlighted in green, is the housekeeping gene. The primers not highlighted were shown to be expressed in the lens at E12, but a thorough temporal expression analysis was not undertaken due to time and financial constraints. Table 7.2.22, below, shows expression of these genes.

Primers	Band size	Annealing Temp.	Cycles	Forward primers	µl used	Reverse primers	µl used	Accession Number
GAPDH	169	61	18	GGAGAAACCAGCCAAGTATGATG	1	AAAGGTGGAAGAATGGCTGTCA	1	PMID:12957145
TRAF1	183	60	30	GAATGGAGATGGGATGGGAAA	1	TGAAAGGAAGCAGAAGCCAAG	1	XM 415406
TRAF2	260	62	32	CTTCCTGCCATTCCCTCATC	2	GCACCACACACCTTGTTACTC	1	XM 415561
TRAF3	658	62	28	TCAGGGAACAAACCAACAAA	1	AAGGTAAACTCTGGCACACATC	1	XM 421378
TRAF4	204	62	27	AGGAGAGCACCAAGGCACAC	1	TGTAGAAAGGAGGGCTGAAGAA	1	XM 415823
TRAF5	300	Multiple bands		ACAGATGGCAAACCAGCAAC	1	CTGAAGATGGAGAGGACACGA	1	NM 204219.2
TRAF6	172	62	32	GCTGCTTTCCTGCTCTTCTGT	1	GTTCTCAACTGCTGCTTCTGG	2	XM 421089.2
TRAF7	228	62	28	AACGGGACAAGAATGGAAACA	1	GCAATGAACGGACGGAGATAG	1	NM 001012528.1
TRAIL	146	62	26	GGAACGAATAAAGAATCCCAAG	1	CGCCCTGGTAGACGGAATAAA	2	NM 204379
TNFR1	154	62	30	CACTGAACTCCCCATCTCTACCTT	1	CCCACTTGCTTCCACTTCTTG	1	XM 414067
TACE	172	62	34	GTCCAGTATCCAGCAGCACTC	3	CTTCCCTTCACCATCCACAA	1	NM 001008682.1
DR5	102	64	32	TTGTGCCCCGTTCTACTGCT	1	GAGGTCTGGCTTCTGGAGGT	1	NM 204115
CD40L	237	60	38	GCTGAAGTGGATGACGACGAG	1	TCACAGAGAGCCGTGGAGGT	2	NM 204733.1
EDA	228	62	38	GGTGCTCGCTTTGATAGTGGT	1	AGAGGTGGTGGTGAGGTGATG	1	XM 420158.1
EDAR	245	Multiple bands		TTTCTGGTGGTTTCCTTGGTG	1	AATCCCTCGCAGTCCTTGTG	1	NM 001012611.1
EDARADD	108	62	36	GTCCAGCAGCCAGAAGACAAA	1	ACGGAATAAGCACAAGGAGCA	1	NM 001012405.2

Densitometry Results

Table A.2.2 Densitometry results of gene expression found via semi-quantitative PCR. PCRs were carried out in triplicate and densitometry analysis of the resulting bands was quantified using Scion Image software (Scion Corporation, freeware). The results were normalised from the GAPDH intensities then the mean intensity and standard error of the mean (SEM) were calculated.

		Embryonic day					
		6	8	10	12	14	16
TRAIL	set1	273.38	230.78	156.6	158.74	169.34	40.24
	set2	232.85	313.26	258.87	311.8	185.14	190.43
	set3	153.35	349.15	227.94	308.35	382.52	213.14
	Mean	219.86	297.73	214.47	259.63	245.67	147.94
	SEM	35.25	35.04	30.28	50.45	68.58	54.25
TRAF1	set1	31.41	134.81	26.19	17.16	34.04	6.37
	set2	22.12	168.37	36.55	36.52	23.23	9.29
	set3	45.39	176.54	44.22	13.03	22.05	4.91
	Mean	32.97	159.91	35.65	22.24	26.44	6.86
	SEM	6.76	12.77	5.22	7.24	3.82	1.29
TRAF2	set1	66.44	57.24	45.35	44.98	47.28	31.45
	set2	105.75	51.75	41.4	88.42	34.88	27.42
	set3	85.04	75.54	62.69	63.56	51.06	17.54
	Mean	85.74	61.51	49.81	65.65	44.41	25.47
	SEM	11.35	7.19	6.54	12.58	4.89	4.13
TRAF3	set1	4.28	2.48	37.22	49.09	99.87	88.16
	set2	7.02	2.74	18.85	33.16	82.65	92.64
	set3	5.65	1.14	24.44	32.13	91.81	85.63
	Mean	5.65	2.12	26.84	38.13	91.45	88.81
	SEM	0.79	0.49	5.44	5.49	4.97	2.05
TRAF4	set1	36.77	32.2	43.59	61.9	34.87	71.23
	set2	17.62	11.2	36.31	31.46	44.3	39.28
	set3	38.1	36.59	33.33	39.96	40.81	63.04
	Mean	30.83	26.66	37.74	44.44	39.99	57.85
	SEM	6.62	7.83	3.05	9.07	2.75	9.58
TRAF7	Set1	224.77	237.41	223.99	235.29	229.28	225.06
	Set2	231.8	221.16	236.72	218.17	211.54	210.51
	Set3	206.01	234.46	230.16	228.92	211.04	211.09
	Mean	220.86	231.01	230.29	227.46	217.29	215.55
	SEM	7.7	5	3.68	5	6	4.76

Statistical Analysis

One way ANOVA was carried out on the normalised densitometry results and this was followed by Turkey's post-hoc test. Statistical analysis was carried out to identify any significant changes in expression. In each case, the test for homogeneity of variance was not significant showing that the variability within the sample groups was similar, thereby allowing these statistical tests to be undertaken.

TRAIL

Table A.2.3 TRAIL Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximu
					Lower Bound	Upper Bound		
1.00	3	219.8	61.06	35.25	68.18	371.54	153.35	273.
2.00	3	297.7	60.69	35.04	146.96	448.50	230.78	349.
3.00	3	214.5	52.45	30.28	84.18	344.76	156.60	258.
4.00	3	259.6	87.39	50.45	42.54	476.72	158.74	311.
5.00	3	245.7	118.78	68.58	-49.40	540.74	169.34	382.
6.00	3	147.9	93.96	54.24	-85.46	381.34	40.24	213.
Total	18	230.9	83.96	19.79	189.13	272.63	40.24	382.

Table A.2.4 TRAIL ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38353.37	5	7670.67	1.130	.396
Within Groups	81473.89	12	6789.49		
Total	119827.25	17			

Table A.2.5 TRAIL Multiple Comparisons

Dependent Variable: Tukey HSD

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-77.87	67.27	0.848	-303.85	148.11
	3	5.39	67.27	1	-220.59	231.37
	4	-39.77	67.27	0.99	-265.75	186.21
	5	-25.8	67.27	0.999	-251.78	200.17
	6	71.92	67.27	0.884	-154.05	297.9
2	1	77.87	67.27	0.848	-148.11	303.85
	3	83.26	67.27	0.811	-142.72	309.24
	4	38.1	67.27	0.992	-187.88	264.08
	5	52.06	67.27	0.967	-173.91	278.04
	6	149.79	67.27	0.294	-76.18	375.77
3	1	-5.39	67.27	1	-231.37	220.59
	2	-83.26	67.27	0.811	-309.24	142.72
	4	-45.16	67.27	0.982	-271.14	180.82
	5	-31.2	67.27	0.997	-257.17	194.78
	6	66.53	67.28	0.913	-159.45	292.51
4	1	39.77	67.28	0.99	-186.21	265.75
	2	-38.1	67.28	0.992	-264.08	187.88
	3	45.16	67.28	0.982	-180.82	271.14
	5	13.96	67.28	1	-212.02	239.94
	6	111.69	67.28	0.579	-114.29	337.67
5	1	25.81	67.28	0.999	-200.17	251.79
	2	-52.06	67.28	0.967	-278.04	173.92
	3	31.2	67.28	0.997	-194.78	257.18
	4	-13.96	67.28	1	-239.94	212.02
	6	97.73	67.28	0.698	-128.25	323.71
6	1	-71.92	67.28	0.884	-297.9	154.06
	2	-149.79	67.28	0.294	-375.77	76.19
	3	-66.53	67.28	0.913	-292.51	159.45
	4	-111.69	67.28	0.579	-337.67	114.29
	5	-97.73	67.28	0.698	-323.71	128.25

TRAF1

Table A.2.6 TRAF1 Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3.00	32.97	11.71	6.76	3.88	62.07	22.12	45.39
2.00	3.00	159.91	22.11	12.77	104.97	214.84	134.81	176.54
3.00	3.00	35.65	9.05	5.22	13.18	58.13	26.19	44.22
4.00	3.00	22.24	12.54	7.24	-8.92	53.39	13.03	36.52
5.00	3.00	26.44	6.61	3.82	10.02	42.86	22.05	34.04
6.00	3.00	6.86	2.23	1.29	1.32	12.40	4.91	9.29
Total	18.00	47.34	53.68	12.65	20.65	74.04	4.91	176.54

Table A.2.7 TRAF1 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47160.36	5	9432.07	61.91	.000
Within Groups	1828.13	12	152.34		
Total	48988.49	17			

Table A.2.8 TRAF1 Multiple Comparisons

Dependent Variable: Tukey HSD

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-126.93(*)	10.08	0.00	-160.78	-93.08
	3.00	-2.68	10.08	1.00	-36.53	31.17
	4.00	10.74	10.08	0.89	-23.11	44.59
	5.00	6.53	10.08	0.99	-27.32	40.38
	6.00	26.12	10.08	0.17	-7.73	59.97
2.00	1.00	126.93(*)	10.08	0.00	93.08	160.78
	3.00	124.25(*)	10.08	0.00	90.40	158.10
	4.00	137.67(*)	10.08	0.00	103.82	171.52
	5.00	133.47(*)	10.08	0.00	99.62	167.32
	6.00	153.05(*)	10.08	0.00	119.20	186.90
3.00	1.00	2.68	10.08	1.00	-31.17	36.53
	2.00	-124.25(*)	10.08	0.00	-158.10	-90.40
	4.00	13.42	10.08	0.76	-20.43	47.27
	5.00	9.21	10.08	0.94	-24.64	43.06
	6.00	28.80	10.08	0.11	-5.05	62.65
4.00	1.00	-10.74	10.08	0.89	-44.59	23.11
	2.00	-137.67(*)	10.08	0.00	-171.52	-103.82
	3.00	-13.42	10.08	0.76	-47.27	20.43
	5.00	-4.20	10.08	1.00	-38.05	29.65
	6.00	15.38	10.08	0.66	-18.47	49.23
5.00	1.00	-6.53	10.08	0.99	-40.38	27.32
	2.00	-133.47(*)	10.08	0.00	-167.32	-99.62
	3.00	-9.21	10.08	0.94	-43.06	24.64
	4.00	4.20	10.08	1.00	-29.65	38.05
	6.00	19.58	10.08	0.42	-14.27	53.43
6.00	1.00	-26.12	10.08	0.17	-59.97	7.73
	2.00	-153.05(*)	10.08	0.00	-186.90	-119.20
	3.00	-28.80	10.08	0.11	-62.65	5.05
	4.00	-15.38	10.08	0.66	-49.23	18.47
	5.00	-19.58	10.08	0.42	-53.43	14.27

* The mean difference is significant at the .05 level.

TRAF2

Table A.2.9 TRAF2 Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	85.74	19.66	11.35	36.89	134.59	66.44	105.75
2	3	61.51	12.46	7.19	30.57	92.45	51.75	75.54
3	3	49.81	11.33	6.54	21.68	77.95	41.4	62.69
4	3	65.65	21.80	12.58	11.51	119.80	44.98	88.42
5	3	44.41	8.46	4.89	23.38	65.43	34.88	51.06
6	3	25.47	7.16	4.13	7.69	43.25	17.54	31.45
Total	18	55.43	22.84	5.38	44.07	66.79	17.54	105.75

Table A.2.10 TRAF2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6333.13	5	1266.63	5.99	.005
Within Groups	2536.04	12	211.34		
Total	8869.17	17			

Table A.2.11 TRAF2 Multiple Comparisons

Dependent Variable: Tukey HSD

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	24.23	11.87	0.38	-15.64	64.10
	3.00	35.93	11.87	0.09	-3.94	75.80
	4.00	20.09	11.87	0.56	-19.78	59.96
	5.00	41.34(*)	11.87	0.04	1.47	81.21
	6.00	60.27(*)	11.87	0.00	20.40	100.14
2.00	1.00	-24.23	11.87	0.38	-64.10	15.64
	3.00	11.70	11.87	0.91	-28.17	51.57
	4.00	-4.14	11.87	1.00	-44.01	35.73
	5.00	17.10	11.87	0.70	-22.77	56.97
	6.00	36.04	11.87	0.09	-3.83	75.91
3.00	1.00	-35.93	11.87	0.09	-75.80	3.94
	2.00	-11.70	11.87	0.91	-51.57	28.17
	4.00	-15.84	11.87	0.76	-55.71	24.03
	5.00	5.41	11.87	1.00	-34.46	45.28
	6.00	24.34	11.87	0.37	-15.53	64.21
4.00	1.00	-20.09	11.87	0.56	-59.96	19.78
	2.00	4.14	11.87	1.00	-35.73	44.01
	3.00	15.84	11.87	0.76	-24.03	55.71
	5.00	21.25	11.87	0.51	-18.62	61.12
	6.00	40.18(*)	11.87	0.05	0.31	80.05
5.00	1.00	-41.34(*)	11.87	0.04	-81.21	-1.47
	2.00	-17.10	11.87	0.70	-56.97	22.77
	3.00	-5.41	11.87	1.00	-45.28	34.46
	4.00	-21.25	11.87	0.51	-61.12	18.62
	6.00	18.94	11.87	0.62	-20.93	58.81
6.00	1.00	-60.27(*)	11.87	0.00	-100.14	-20.40
	2.00	-36.04	11.87	0.09	-75.91	3.83
	3.00	-24.34	11.87	0.37	-64.21	15.53
	4.00	-40.18(*)	11.87	0.05	-80.05	-0.31
	5.00	-18.94	11.87	0.62	-58.81	20.93

* The mean difference is significant at the .05 level

TRAF3

Table A.2.12 TRAF3 Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	5.65	1.37	0.79	2.25	9.05	4.28	7.02
2	3	2.12	0.86	0.50	-0.01	4.25	1.14	2.74
3	3	26.84	9.42	5.44	3.44	50.23	18.85	37.22
4	3	38.13	9.51	5.49	14.51	61.75	32.13	49.09
5	3	91.44	8.62	4.97	70.04	112.85	82.65	99.87
6	3	88.81	3.55	2.05	79.99	97.63	85.63	92.64
Total	18	42.16	37.51	8.84	23.51	60.82	1.14	99.87

Table A.2.13 TRAF3 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23376.97	5	4675.394	104.465	.000
Within Groups	537.07	12	44.755		
Total	23914.03	17			

Table A.2.14 TRAF3 Multiple Comparisons

Dependent Variable: Tukey HSD

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	3.53	5.46	0.99	-14.82	21.88
	3.00	-21.19(*)	5.46	0.02	-39.53	-2.84
	4.00	-32.48*)	5.46	0.00	-50.82	-14.13
	5.00	-85.79(*)	5.46	0.00	-104.14	-67.45
	6.00	-83.16(*)	5.46	0.00	-101.51	-64.81
2.00	1.00	-3.53	5.46	0.99	-21.88	14.82
	3.00	-24.72(*)	5.46	0.01	-43.06	-6.37
	4.00	-36.01(*)	5.46	0.00	-54.35	-17.66
	5.00	-89.32(*)	5.46	0.00	-107.67	-70.98
	6.00	-86.69(*)	5.46	0.00	-105.04	-68.34
3.00	1.00	21.19(*)	5.46	0.02	2.84	39.53
	2.00	24.72(*)	5.46	0.01	6.37	43.06
	4.00	-11.29	5.46	0.36	-29.64	7.06
	5.00	-64.61(*)	5.46	0.00	-82.95	-46.26
	6.00	-61.97(*)	5.46	0.00	-80.32	-43.63
4.00	1.00	32.48(*)	5.46	0.00	14.13	50.82
	2.00	36.01(*)	5.46	0.00	17.66	54.35
	3.00	11.29	5.46	0.36	-7.06	29.64
	5.00	-53.32(*)	5.46	0.00	-71.66	-34.97
	6.00	-50.68(*)	5.46	0.00	-69.03	-32.34
5.00	1.00	85.79(*)	5.46	0.00	67.45	104.14
	2.00	89.32*)	5.46	0.00	70.98	107.67
	3.00	64.61(*)	5.46	0.00	46.26	82.95
	4.00	53.32(*)	5.46	0.00	34.97	71.66
	6.00	2.63	5.46	1.00	-15.71	20.98
6.00	1.00	83.16(*)	5.46	0.00	64.81	101.51
	2.00	86.69(*)	5.46	0.00	68.34	105.04
	3.00	61.97(*)	5.46	0.00	43.63	80.32
	4.00	50.68(*)	5.46	0.00	32.34	69.03
	5.00	-2.63	5.46	1.00	-20.98	15.71

* The mean difference is significant at the .05 level.

TRAF4

Table A.2.15 TRAF4 Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	30.83	11.46	6.62	2.36	59.30	17.62	38.1
2	3	26.66	13.57	7.83	-7.05	60.37	11.2	36.59
3	3	37.74	5.28	3.05	24.63	50.85	33.33	43.59
4	3	44.44	15.71	9.07	5.42	83.46	31.46	61.9
5	3	39.99	4.77	2.75	28.15	51.84	34.87	44.3
6	3	57.85	16.60	9.58	16.63	99.07	39.28	71.23
Total	18	39.59	14.53	3.42	32.36	46.81	11.2	71.23

Table A.2.16 TRAF4 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
1.949	5	12	.159

Table A.2.17 TRAF4 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1813.07	5	362.62	2.450	.095
Within Groups	1776.33	12	148.02		
Total	3589.41	17			

Table A.2.18 TRAF4 Multiple Comparisons

Dependent Variable: Tukey HSD

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	4.17	9.93	1.00	-29.20	37.53
	3	-6.91	9.93	0.98	-40.28	26.45
	4	-13.61	9.93	0.74	-46.98	19.76
	5	-9.16	9.93	0.93	-42.53	24.20
	6	-27.02	9.93	0.14	-60.39	6.35
2	1	-4.17	9.93	1.00	-37.53	29.20
	3	-11.08	9.93	0.87	-44.45	22.29
	4	-17.78	9.93	0.51	-51.14	15.59
	5	-13.33	9.93	0.76	-46.70	20.04
	6	-31.19	9.93	0.07	-64.55	2.18
3	1	6.91	9.93	0.98	-26.45	40.28
	2	11.08	9.93	0.87	-22.29	44.45
	4	-6.70	9.93	0.98	-40.06	26.67
	5	-2.25	9.93	1.00	-35.62	31.12
	6	-20.11	9.93	0.38	-53.47	13.26
4	1	13.61	9.93	0.74	-19.76	46.98
	2	17.78	9.93	0.51	-15.59	51.14
	3	6.70	9.93	0.98	-26.67	40.06
	5	4.45	9.93	1.00	-28.92	37.81
	6	-13.41	9.93	0.75	-46.78	19.96
5	1	9.16	9.93	0.93	-24.20	42.53
	2	13.33	9.93	0.76	-20.04	46.70
	3	2.25	9.93	1.00	-31.12	35.62
	4	-4.45	9.93	1.00	-37.81	28.92
	6	-17.86	9.93	0.50	-51.22	15.51
6	1	27.02	9.93	0.14	-6.35	60.39
	2	31.19	9.93	0.07	-2.18	64.55
	3	20.11	9.93	0.38	-13.26	53.47
	4	13.41	9.93	0.75	-19.96	46.78
	5	17.86	9.93	0.50	-15.51	51.22

TRAF7

Table A.2.19 TRAF7 Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	3	220.86	13.33	7.70	187.74	253.98	206.01	231.80
2	3	231.01	8.66	5.00	209.51	252.52	221.16	237.41
3	3	230.29	6.37	3.68	214.48	246.10	223.99	236.72
4	3	227.46	8.65	5.00	205.97	248.95	218.17	235.29
5	3	217.29	10.39	6.00	191.48	243.10	211.04	229.28
6	3	215.55	8.24	4.76	195.09	236.02	210.51	225.06
Total	18	223.74	10.20	2.40	218.67	228.81	206.01	237.41

Table A.2.20 TRAF7 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
.711	5	12	.627

Table A.2.21 TRAF7 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	679.67	5	135.93	1.50	.26
Within Groups	1087.79	12	90.65		
Total	1767.46	17			

Table A.2.22 TRAF7 Multiple Comparisons

Dependent Variable: Tukey HSD

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-10.15	7.77	0.78	-36.26	15.96
	3	-9.43	7.77	0.82	-35.54	16.68
	4	-6.60	7.77	0.95	-32.71	19.51
	5	3.57	7.77	1.00	-22.54	29.69
	6	5.31	7.77	0.98	-20.81	31.42
2	1	10.15	7.77	0.78	-15.96	36.26
	3	0.72	7.77	1.00	-25.39	26.83
	4	3.55	7.77	1.00	-22.56	29.66
	5	13.72	7.77	0.52	-12.39	39.84
	6	15.46	7.77	0.40	-10.66	41.57
3	1	9.43	7.77	0.82	-16.68	35.54
	2	-0.72	7.77	1.00	-26.83	25.39
	4	2.83	7.77	1.00	-23.28	28.94
	5	13.00	7.77	0.57	-13.11	39.12
	6	14.74	7.77	0.45	-11.38	40.85
4	1	6.60	7.77	0.95	-19.51	32.71
	2	-3.55	7.77	1.00	-29.66	22.56
	3	-2.83	7.77	1.00	-28.94	23.28
	5	10.17	7.77	0.78	-15.94	36.29
	6	11.91	7.77	0.65	-14.21	38.02
5	1	-3.57	7.77	1.00	-29.69	22.54
	2	-13.72	7.77	0.52	-39.84	12.39
	3	-13.00	7.77	0.57	-39.12	13.11
	4	-10.17	7.77	0.78	-36.29	15.94
	6	1.73	7.77	1.00	-24.38	27.85
6	1	-5.31	7.77	0.98	-31.42	20.81
	2	-15.46	7.77	0.40	-41.57	10.66
	3	-14.74	7.77	0.45	-40.85	11.38
	4	-11.91	7.77	0.65	-38.02	14.21
	5	-1.73	7.77	1.00	-27.85	24.38

Table A.2.23 Genes shown to be expressed at E12, without further studies undertaken. Table showing expression of genes expressed at E12 with optimised temperature and cycle number. Temporal expression was not undertaken on these genes due to limited time and finances.

Primers	Band size	Temperature	Cycle number	E12
δ-Crystallin	100	64	20	
TRAF5	300	60	28	Multiple bands
TRAF6	172	62	32	
TNFR1	154	62	30	
TACE	172	62	28	
DR5	102	62	32	
CD40L	237	60	34	
RAIDD	186	60	28	
EDA	228	62	34	
EDARADD	108	62	34	
EDAR	245	60	32	

Appendix 3- Western Blotting analysis

TRAIL western blotting analysis

Table A3.1.1 TRAIL densitometry results

TRAIL	6	8	10	12	14	16
set1	97.78	124.81	179.84	278.74	161.25	295.75
set2	36.21	43.89	40.90	25.54	29.33	42.49
set3	29.04	120.38	108.82	34.64	107.16	219.25

Table A3.1.2 TRAIL Descriptives from SPSS analysis

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
6	3	54.34	37.79	21.82	-39.52	148.21	29.04	97.78
8	3	96.36	45.50	26.27	-16.66	209.38	43.89	124.81
10	3	109.85	69.47	40.11	-62.73	282.44	40.90	179.84
12	3	112.97	143.63	82.93	-243.83	469.78	25.54	278.74
14	3	99.25	66.32	38.29	-65.49	263.98	29.33	161.25
16	3	185.83	129.89	74.99	-136.84	508.50	42.49	295.75
Total	18	109.77	86.75	20.45	66.63	152.91	25.54	295.75

Table A3.1.3 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2.41	5	12	.098

Table A3.1.4 TRAIL ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	27474.03	5	5494.81	.66	.66
Within Groups	100448.33	12	8370.69		
Total	127922.36	17			

Table A3.1.5 TRAIL Multiple Comparisons using Tukey's post hoc test

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6	8	-42.01	74.70	0.99	-292.93	208.91
	10	-55.51	74.70	0.97	-306.43	195.41
	12	-58.63	74.70	0.97	-309.55	192.29
	14	-44.90	74.70	0.99	-295.82	206.02
	16	-131.49	74.70	0.52	-382.41	119.43
8	6	42.01	74.70	0.99	-208.91	292.93
	10	-13.49	74.70	1.00	-264.41	237.42
	12	-16.62	74.70	1.00	-267.54	234.30
	14	-2.89	74.70	1.00	-253.81	248.03
	16	-89.47	74.70	0.83	-340.39	161.45
10	6	55.51	74.70	0.97	-195.41	306.43
	8	13.49	74.70	1.00	-237.42	264.41
	12	-3.12	74.70	1.00	-254.04	247.80
	14	10.61	74.70	1.00	-240.31	261.53
	16	-75.98	74.70	0.90	-326.90	174.94
12	6	58.63	74.70	0.97	-192.29	309.55
	8	16.62	74.70	1.00	-234.30	267.54
	10	3.12	74.70	1.00	-247.80	254.04
	14	13.73	74.70	1.00	-237.19	264.65
	16	-72.86	74.70	0.92	-323.78	178.06
14	6	44.90	74.70	0.99	-206.02	295.82
	8	2.89	74.70	1.00	-248.03	253.81
	10	-10.61	74.70	1.00	-261.53	240.31
	12	-13.73	74.70	1.00	-264.65	237.19
	16	-86.58	74.70	0.85	-337.50	164.34
16	6	131.49	74.70	0.52	-119.43	382.41
	8	89.47	74.70	0.83	-161.45	340.39
	10	75.98	74.70	0.90	-174.94	326.90
	12	72.86	74.70	0.92	-178.06	323.78
	14	86.58	74.70	0.85	-164.34	337.50

Table A3.1.6 TRAIL Tukey's Post Hoc

Timepoint	N	Subset for alpha = .05
		1.00
6.00	3.00	54.34
8.00	3.00	96.36
14.00	3.00	99.25
10.00	3.00	109.85
12.00	3.00	112.97
16.00	3.00	185.83
Sig.		0.52

Means for groups in homogeneous subsets are displayed. Uses Harmonic Mean Sample Size = 3.000.

TRAF3 (higher band) western blotting analysis

Table A3.2.1 TRAF3 (higher band) densitometry results

TRAF3		6	8	10	12	14	16
set1	Higher band	73.31	41.92	74.12	73.43	47.14	33.05
set2	Higher band	34.25	58.17	109.63	94.54	86.66	156.81
set3	Higher band	92.68	90.58	114.46	42.47	22.29	80.35

Table A3.2.2 TRAF3 (higher band) Descriptives from SPSS analysis

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
6.00	3.00	66.75	29.76	17.18	-7.19	140.69	34.25	92.68
8.00	3.00	63.55	24.78	14.30	2.01	125.10	41.92	90.58
10.00	3.00	99.40	22.03	12.72	44.68	154.13	74.12	114.46
12.00	3.00	70.15	26.19	15.12	5.09	135.21	42.47	94.54
14.00	3.00	45.36	22.24	12.84	-9.89	100.61	22.29	66.66
16.00	3.00	64.07	26.88	15.52	-2.70	130.84	33.05	80.35
Total	18.00	68.21	27.00	6.36	54.79	81.64	22.29	114.46

Table A3.2.3 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
.123	5	12	.98

Table A3.2.4 TRAF3 (higher band) ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4619.32	5.00	923.86	1.43	0.28
Within Groups	7775.94	12.00	648.00		
Total	12395.26	17.00			

Table A3.2.5 TRAF3 (higher band) Multiple Comparisons using Tukey's post hoc test

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6.00	8.00	3.19	20.78	1.00	-66.62	73.01
	10.00	-32.66	20.78	0.63	-102.47	37.16
	12.00	-3.40	20.78	1.00	-73.21	66.41
	14.00	21.38	20.78	0.90	-48.43	91.20
	16.00	2.68	20.78	1.00	-67.14	72.49
8.00	6.00	-3.19	20.78	1.00	-73.01	66.62
	10.00	-35.85	20.78	0.54	-105.66	33.96
	12.00	-6.59	20.78	1.00	-76.41	63.22
	14.00	18.19	20.78	0.95	-51.62	88.00
	16.00	-0.52	20.78	1.00	-70.33	69.30
10.00	6.00	32.66	20.78	0.63	-37.16	102.47
	8.00	35.85	20.78	0.54	-33.96	105.66
	12.00	29.26	20.78	0.72	-40.56	99.07
	14.00	54.04	20.78	0.17	-15.77	123.85
	16.00	35.33	20.78	0.56	-34.48	105.15
12.00	6.00	3.40	20.78	1.00	-66.41	73.21
	8.00	6.59	20.78	1.00	-63.22	76.41
	10.00	-29.26	20.78	0.72	-99.07	40.56
	14.00	24.78	20.78	0.83	-45.03	94.60
	16.00	6.08	20.78	1.00	-63.74	75.89
14.00	6.00	-21.38	20.78	0.90	-91.20	48.43
	8.00	-18.19	20.78	0.95	-88.00	51.62
	10.00	-54.04	20.78	0.17	-123.85	15.77
	12.00	-24.78	20.78	0.83	-94.60	45.03
	16.00	-18.71	20.78	0.94	-88.52	51.11
16.00	6.00	-2.68	20.78	1.00	-72.49	67.14
	8.00	0.52	20.78	1.00	-69.30	70.33
	10.00	-35.33	20.78	0.56	-105.15	34.48
	12.00	-6.08	20.78	1.00	-75.89	63.74
	14.00	18.71	20.78	0.94	-51.11	88.52

Table A3.2.6 TRAF3 (higher band) Tukey's Post Hoc

Timepoint	N	Subset for alpha = .05
		1
14	3	45.36
8	3	63.55
16	3	64.07
6	3	66.75
12	3	70.15
10	3	99.40
Sig.		0.17

Means for groups in homogeneous subsets are displayed. Uses Harmonic Mean Sample Size = 3.000.

TRAF3 (lower band) western blotting analysis

Table A3.3.1 TRAF3 (lower band) densitometry results

TRAF3		6	8	10	12	14	16
set1	Lower	3.80	8.05	9.09	7.52	9.53	127.99
set2	Lower	3.13	9.32	28.24	39.46	58.59	150.30
set3	Lower	1.87	10.67	32.64	113.75	140.69	221.27

Table A3.3.2 TRAF3 (lower band) Descriptives from SPSS analysis

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
6.00	3	2.93	0.98	0.57	0.50	5.36	1.87	3.80
8.00	3	9.35	1.31	0.76	6.10	12.60	8.05	10.67
10.00	3	23.32	12.52	7.23	-7.78	54.43	9.09	32.64
12.00	3	76.15	37.15	21.45	-16.14	168.44	39.46	113.75
14.00	3	98.19	41.13	23.75	-3.98	200.36	58.59	140.69
16.00	3	183.19	35.77	20.65	94.33	272.04	150.30	221.27
Total	18	65.52	68.88	16.24	31.27	99.78	1.87	221.27

Table A3.3.3 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2.346	5	12	.105

Table A3.3.4 TRAF3 (lower band) ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	71637.07	5	14327.42	19.06	0
Within Groups	9021.28	12	751.77		
Total	80658.35	17			

Table A3.3.5 TRAF3 (lower band) Multiple Comparisons using Tukey's post hoc test

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6.00	8.00	-6.42	22.39	1.00	-81.62	68.78
	10.00	-20.39	22.39	0.94	-95.59	54.80
	12.00	-73.22	22.39	0.06	-148.42	1.98
	14.00	-95.26(*)	22.39	0.01	-170.46	-20.06
	16.00	-180.26(*)	22.39	0.00	-255.45	-105.06
8.00	6.00	6.42	22.39	1.00	-68.78	81.62
	10.00	-13.98	22.39	0.99	-89.17	61.22
	12.00	-66.80	22.39	0.09	-142.00	8.39
	14.00	-88.84(*)	22.39	0.02	-164.04	-13.64
	16.00	-173.84(*)	22.39	0.00	-249.03	-98.64
10.00	6.00	20.39	22.39	0.94	-54.80	95.59
	8.00	13.98	22.39	0.99	-61.22	89.17
	12.00	-52.83	22.39	0.24	-128.02	22.37
	14.00	-74.86	22.39	0.05	-150.06	0.33
	16.00	-159.86(*)	22.39	0.00	-235.06	-84.67
12.00	6.00	73.22	22.39	0.06	-1.98	148.42
	8.00	66.80	22.39	0.09	-8.39	142.00
	10.00	52.83	22.39	0.24	-22.37	128.02
	14.00	-22.04	22.39	0.91	-97.23	53.16
	16.00	-107.04(*)	22.39	0.01	-182.23	-31.84
14.00	6.00	95.26(*)	22.39	0.01	20.06	170.46
	8.00	88.84(*)	22.39	0.02	13.64	164.04
	10.00	74.86	22.39	0.05	-0.33	150.06
	12.00	22.04	22.39	0.91	-53.16	97.23
	16.00	-85(*)	22.39	0.02	-160.19	-9.80
16.00	6.00	180.26(*)	22.39	0.00	105.06	255.45
	8.00	173.84(*)	22.39	0.00	98.64	249.03
	10.00	159.86(*)	22.39	0.00	84.67	235.06
	12.00	107.04(*)	22.39	0.01	31.84	182.23
	14.00	85(*)	22.39	0.02	9.80	160.19

* The mean difference is significant at the .05 level.

Table A3.3.6 TRAF3 (lower band) Tukey's Post Hoc

Timepoint	N	Subset for alpha = .05		
		1	2	3
6.00	3	2.9296		
8.00	3	9.3481		
10.00	3	23.3239	23.3239	
12.00	3	76.1507	76.1507	
14.00	3		98.1889	
16.00	3			183.1865
Sig.		.058	.051	1.000

Means for groups in homogeneous subsets are displayed. Uses Harmonic Mean Sample Size = 3.000.

TRAF2 western blotting analysis

Table A3.4.1 TRAF2 densitometry results

TRAF2	6.00	8.00	10.00	12.00	14.00	16.00
Set1	196.84	130.06	200.19	147.95	72.07	29.13
Set2	181.52	66.54	248.66	127.66	30.91	19.53
Set3	220.52	215.38	224.22	73.34	23.64	29.92

Table A3.4.2 TRAF2 Descriptives from SPSS analysis

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
6.00	3	199.63	19.65	11.35	150.80	248.45	181.52	220.52
8.00	3	137.33	74.69	43.12	-48.21	322.86	66.54	215.38
10.00	3	224.36	24.23	13.99	164.15	284.56	200.19	248.66
12.00	3	116.32	38.57	22.27	20.49	212.14	73.34	147.95
14.00	3	42.21	26.12	15.08	-22.67	107.09	23.64	72.07
16.00	3	26.20	5.78	3.34	11.82	40.57	19.53	29.92
Total	18	124.34	82.02	19.33	83.55	165.12	19.53	248.66

Table A3.4.3 Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2.27	5	12	.114

Table A3.4.4 TRAF2 ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	96845.32	5	19369.06	13.27	.000
Within Groups	17511.20	12	1459.27		
Total	114356.52	17			

Table A3.4.5 TRAF2 Multiple Comparisons using Tukey's post hoc test

(I) Timepoint	(J) Timepoint	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
6	8	62.3	31.19	0.4	-42.47	167.07
	10	-24.73	31.19	0.96	-129.5	80.04
	12	83.31	31.19	0.15	-21.46	188.08
	14	157.42(*)	31.19	0	52.65	262.18
	16	173.43(*)	31.19	0	68.67	278.2
8	6	-62.3	31.19	0.4	-167.07	42.47
	10	-87.03	31.19	0.13	-191.8	17.74
	12	21.01	31.19	0.98	-83.76	125.78
	14	95.12	31.19	0.08	-9.65	199.88
	16	111.13(*)	31.19	0.04	6.36	215.9
10	6	24.73	31.19	0.96	-80.04	129.5
	8	87.03	31.19	0.13	-17.74	191.8
	12	108.04(*)	31.19	0.04	3.27	212.8
	14	182.14(*)	31.19	0	77.38	286.91
	16	198.16(*)	31.19	0	93.39	302.93
12	6	-83.31	31.19	0.15	-188.08	21.46
	8	-21.01	31.19	0.98	-125.78	83.76
	10	-108.04(*)	31.19	0.04	-212.8	-3.27
	14	74.11	31.19	0.24	-30.66	178.87
	16	90.12	31.19	0.11	-14.64	194.89
14	6	-157.42(*)	31.19	0	-262.18	-52.65
	8	-95.12	31.19	0.08	-199.88	9.65
	10	-182.14(*)	31.19	0	-286.91	-77.38
	12	-74.11	31.19	0.24	-178.87	30.66
	16	16.02	31.19	1	-88.75	120.78
16	6	-173.43(*)	31.19	0	-278.2	-68.67
	8	-111.13(*)	31.19	0.04	-215.9	-6.36
	10	-198.16(*)	31.19	0	-302.93	-93.39
	12	-90.12	31.19	0.11	-194.89	14.64
	14	-16.02	31.19	1	-120.78	88.75

* The mean difference is significant at the .05 level.

Table A3.4.6 TRAF2 Tukey's Post Hoc

Timepoint	N	Subset for alpha = .05			
		1	2	3	4
16	3	26.20			
14	3	42.21	42.21		
12	3	116.32	116.32	116.32	
8	3		137.33	137.33	137.33
6	3			199.63	199.63
10	3				224.36
Sig.		0.11	0.08	0.15	0.13

Means for groups in homogeneous subsets are displayed. Uses Harmonic Mean Sample Size = 3.000.

Appendix 4- Lens culture

Lens opacity following whole lens culture

Table A4.1 Densitometry results of opacities in cultured lens

	With glucose			Without glucose		
	Lens only	Lens+ Vitreous	Lens, Vitreous+Iris	Lens only	Lens+ Vitreous	Lens, Vitreous+Iris
E10 + 2	65.26	44.78	105.26	37.83	47.32	84.65
E10 + 4	42.41	48.98	116.14	84.39	38.47	70.46
E10 + 6	77.82	71.04	135.54	74.75	61.11	148.03
E10 + 8	149.18	146.51	177.24	139.03	194.92	189.74