The Use of Human Embryonic Stem Cells to Investigate Human Lens and Cataract Development

Thesis submitted in fulfilment of the requirements for the degree
Doctor of Philosophy
By
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Dedication

To my family, Mum, Dad, Daniel and Chad you are my world.

All my love, Trish.
STATEMENT OF AUTHENTICATION

This is a signed statement to the effect that the work has not been submitted for a higher degree at any other institution and an undertaking that the work is original and as a result of the candidates own research endeavour.

…………………………………………
Patricia A Murphy

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Abstracts:

1. Optimising the differentiation of human pluripotent stem cells to lens epithelial cells. **PA Murphy**, MD O’Connor. 4th annual ASSCR meeting, Leura October 2011.

2. Development of an Excel-based tool and associated pipeline facilitates identification of novel molecular biology within the lens and gut. MD O’Connor1,2, **PA Murphy**1-Presented at the Sino-Australian Cell Therapy Summit, July 2013 (Gansu province, China).

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**Provisional patent:**

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Inventors: **PA Murphy** and **MD O’Connor**.

PCT submission occurred 29/1/15 (PCT/AU2015/000046)

**Abstracts and Papers from PhD not published within thesis:**

An additional experimental chapter was obtained during this thesis, although using similar methods it was centered on the gut. For continuity it has been left out.


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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>- RT</td>
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<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>+ RT</td>
<td>With reverse transcriptase</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
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<td>Microlitres</td>
</tr>
<tr>
<td>µM</td>
<td>Micro molar</td>
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<td>Absent</td>
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<td>Activin A receptor, type I</td>
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<td>angiotensinogen</td>
</tr>
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<td>AP-1</td>
<td>transcription factor AP-1</td>
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<tr>
<td>AP2</td>
<td>transcription factor AP-2 alpha</td>
</tr>
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<td>Age-related nuclear</td>
</tr>
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<td>BTB and CNC homology 1, basic leucine zipper transcription factor 2</td>
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<td>Bone morphogenic proteins</td>
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<td>BMP7</td>
<td>Bone morphogenetic protein 7</td>
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<td>Base pairs</td>
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<td>Brn3b POU domain transcription factor</td>
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<td>Fucosyltransferase 4</td>
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<td>CD44 molecule</td>
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<td>-------------</td>
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<tr>
<td>CDH4</td>
<td>Cadherin 4, type 1, R-cadherin</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cMET</td>
<td>Met proto-oncogene (hepatocyte growth factor receptor)</td>
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<td>CO2</td>
<td>Carbon dioxide</td>
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<td>CRYA</td>
<td>Crystallin alpha</td>
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<td>CRYAA</td>
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<td>Crystallin alpha B</td>
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<td>CRYG</td>
<td>Gamma crystallin</td>
</tr>
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<td>Ct</td>
<td>Cycle threshold</td>
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<td>Dickkopf</td>
</tr>
<tr>
<td>DKK1</td>
<td>Dickkopf WNT signaling pathway inhibitor 1</td>
</tr>
<tr>
<td>DKK3</td>
<td>Dickkopf WNT signaling pathway inhibitor 3</td>
</tr>
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<td>DKK4</td>
<td>Dickkopf WNT signaling pathway inhibitor 4</td>
</tr>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMEM:F12</td>
<td>Dulbecco’s Modified Eagle's Medium and Ham's F-12 Nutrient Mixture</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>Dnase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide Triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGR1</td>
<td>Early growth response 1</td>
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</table>
EGTA    Ethylene glycol tetraacetic acid
ELK1    ELK1, member of ETS oncogene family
ERK     Elk-related tyrosine kinase
ES      Embryonic stem
ETS     E-twenty-six
FBS     Foetal bovine serum
FGF     Fibroblast growth factor
FGF1    Fibroblast growth factor 1
FGF2    Basic fibroblast growth factor
FGFR1   Fibroblast growth factor receptor 1
FHL 124 Foetal human lens cell line
FOS     FBJ murine osteosarcoma viral oncogene homolog
g      G-force
GAPDH   Glyceraldehyde 3-phosphate dehydrogenase
GAS1    Growth arrest-specific 1
GEO     Gene Expression Omnibus
GF      growth factors
GO      Gene ontology
GO-Phase G zero phase
GSH     Gluathione
GSK3    Glycogen synthase kinase 3
h      Hours
H2O    Water
HGFR    Met proto-oncogene (hepatocyte growth factor receptor)
HNK-1   Beta-1,3-glucuronyltransferase 1
<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ID</td>
<td>Identification numbers</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IOL</td>
<td>Intraocular lens</td>
</tr>
<tr>
<td>IQGAP1</td>
<td>IQ motif containing GTPase activating protein 1</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton, unit of mass</td>
</tr>
<tr>
<td>KOSR</td>
<td>Knockout serum replacement</td>
</tr>
<tr>
<td>LBR</td>
<td>Lysis buffer R</td>
</tr>
<tr>
<td>LEC</td>
<td>Lens epithelial cell</td>
</tr>
<tr>
<td>LFC</td>
<td>Lens fibre cell</td>
</tr>
<tr>
<td>Lrp6</td>
<td>Low density lipoprotein receptor-related protein 6</td>
</tr>
<tr>
<td>M199</td>
<td>Medium 199</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamps</td>
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<tr>
<td>MAF</td>
<td>v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated kinase-like protein</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>mg</td>
<td>Milligrams</td>
</tr>
<tr>
<td>MgCl2</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MIP</td>
<td>Major intrinsic protein</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetres</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Message ribonucleic acid</td>
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</table>
Nd:YAG  Neodymium-doped yttrium aluminum garnet
ng  Nanogram
nM  Nanometre
NTC  Non-template control
P  Present
p75  Nerve growth factor receptor
PASTAA  Predicting associated transcription factors from annotated affinities
PAX  Paired box
PAX5  Paired box 5
PAX6  Paired box 6
PBS  Phosphate buffered saline
PCO  Posterior capsule opacification
PCR  Polymerase chain reaction
PDGF  Platelet-derived growth factor beta
pH  Measure of acidity or basicity of an aqueous solution
PI  Protease Inhibitor
PI3K  Phosphoinositide 3-kinase
PPR  Pre-placodal region
PVDF  Polyvinylidene difluoride
PWM  Position weight matrix
Q  Quantification
RNA  Ribonucleic acid
Rnase  Ribonuclease
ROCK  Rho-associated protein kinase
SDS  Sodium dodecyl sulfate
SFRP             Secreted frizzled related protein
SFRP1            Secreted frizzled-related protein 1
SFRP2            Secreted frizzled-related protein 2
SFRP3            Secreted frizzled-related protein 3
SFRP4            Secreted frizzled-related protein 4
SMAD             SMAD family member
SMAD3            SMAD family member 3
SMAD4            SMAD family member 4
SP1              Sp3 transcription factor
SP3              Sp1 transcription factor
TF               Transcription Factors
TGFβ             Transforming growth factor beta
U                Unit
UV               Ultra violet
V                Volts
VEGF             Vascular endothelial growth factor
W                Watts
w/v              Weight per volume
Wnt              Wingless-type
WNT3A            Wingless-type MMTV integration site family, member 3A
WNT7A            Wingless-related MMTV integration site 7A
THESIS ABSTRACT

The aim of this thesis was to improve the use of human pluripotent stem cells to provide a refined cell source, for lens and cataract research. Chapter 1 provides a general background to the impact of cataract and the complications associated with current treatment strategies, in order to highlight the need to develop novel anti- cataract treatments and the need for new lens models to aid future anti-cataract drug discovery. Chapter 2 was focused on optimising a published, though imperfect, differentiation system to generate a pure population of human embryonic stem cell derived lens epithelial cells that can be used in downstream anti-cataract drug screens. From the results of this chapter (i.e. the partial improvement in lens epithelial cell production) it was clear that greater knowledge of the regulation involved in the lens was needed to improve future differentiation methods. For this reason a simple bioinformatics tool was created and used in Chapter 3 to identify candidate new regulators of lens growth factor signalling pathways. The success obtained through Chapter 3 led to use of the bioinformatics tool in Chapter 4 to develop a method for purifying lens epithelial cells (via specific surface antigens) from mixed populations of lens fibre cells and non-lens cell types. Subsequent use of these purified human lens cells demonstrated a proof of principle for anti-cataract drug screening. Chapter 5 is a general discussion of the outcomes and benefits of this research and future initiatives for improvement.

This thesis has been submitted in a composite format largely centred on the Investigative Ophthalmology and Visual Science (IOVS) manuscript format, however, section headers and numbering was added to aid reviewers. The Introduction of this thesis is an invited review manuscript, and the 3 included
experimental chapters have been prepared for submission to IOVS (with delayed submission due to preparation and submission of the Australian Provisional Patent Application # 2014900269: A method for the purification of eye lens cells).

The title of these manuscripts are as follows:


ii) CHAPTER 2 (page 26 to 65): Murphy PA and O'Connor MD. Growth factor optimisation for production of lens epithelial cells from human embryonic stem cells.

iii) CHAPTER 3 (page 66 to 99): Murphy PA and O'Connor MD. Identification and expression profile of a novel lens transcription factor.

iv) CHAPTER 4 (page 100 to 155): Murphy PA and O'Connor MD. Large-scale production of purified human lens epithelial cells for anti-cataract drug screening.
CHAPTER ONE: INTRODUCTION

Stem cells and the ocular lens: Implications for cataract research and therapy
1.1 ABSTRACT

The ocular lens is a transparent, biconvex tissue whose function is to focus light onto the retina. Age-related cataracts result in an increased light scattering within the ocular lens and account for 51% of global blindness. To date the mechanisms of cataract formation are unclear. Whilst surgical lens replacement is the only means of restoring sight, the cost of treatment places a large financial burden on medical systems worldwide. For third-world countries, inadequate access to surgery leaves many patients blind and even with treatment complications can arise (e.g. secondary cataracts). Thus, attitudes towards developing new cataract treatments are becoming more favourable. An important step towards this goal is the development of improved human lens models that identify new molecular mechanisms of lens development and maintenance. As stem cell based technologies develop overtime the improvements may lead the way to identify and engineer these sought after new therapies.
1.2 INTRODUCTION

The crystalline lens has a distinctive polarised structure, namely an anteriorly positioned monolayer of lens epithelial cells (LECs) surrounding a mass of lens fibre cells (LFCs). Lens growth occurs throughout life as equatorial epithelial cells coordinately differentiate into successive layers of LFCs that elongate to surround the underlying central fibre cell mass\(^1,2\). Lens transparency is required for clear sight and facilitates light to travel directly through the lens to the retina (Figure 1). Together, the correct arrangement and protein composition of lens cells as well as maintenance of its true shape is required to conserve lens transparency.

The oldest cells of the lens reside within its cortex, these cells and their proteins are exposed to both environmental and light induced insults for the longest period of an individual’s life. Whilst embedded mechanisms such as UV filters and glutathione-based free radical scavenging are central to protecting all lens cells from oxidative stress or UV related insults, these protective systems become less effective with age\(^3\). As a result, age related lens diseases such as cataract are becoming more prevalent worldwide, a trend associated with the continuing increase in the average human lifespan. For sufferers of cataract, existing treatments are available but are not free from complication. Complications such as the formation of posterior capsule opacification (PCO), loss of accommodation, and visual disturbances including glare and halos additionally impede upon quality of life.

Stem cell research utilises the successfully developed isolation and culturing techniques for both human induced pluripotent stem cells and human embryonic stem (ES) cells\(^4,5\). Induced human pluripotent stem cells are derived directly from
Figure 1. Schematic diagram of the lens within the eye.

The lens is situated between the cornea and the retina. The anterior portion of the lens is bathed by the aqueous fluid while the posterior section is bathed by the vitreous fluid. The fully formed lens consists of an anterior epithelium overlying a tightly packed mass of elongating fibre cells. Growth of the lens occurs as a result of proliferating LECs, primarily within the region close to the germinative zone, posterior migration of these cells and their subsequent differentiation into LFCs. During this process the differentiating fibre cells elongate with their apical and basal ends reaching the anterior and posterior poles of the lens respectively resulting in the formation of lens sutures. It is this process that results in the addition of concentric layers of tightly packed, parallel aligned cells to the fibre cell mass.
adult somatic cells that are reprogramed to gain pluripotency. Human ES cells are derived from surplus pre-implantation blastocysts, donated for research purposes after informed consent. Since these two cell types have similar properties, for example, the ability to self-renew or to differentiate into all cells within the body, they are collectively known as human pluripotent stem cells. For the lens, our inability to access sufficient amounts of primary human tissues for research purposes has slowed the development of our present understanding of the complicated events that take place during early human lens development and cataract formation. This limitation has further negatively impacted on the identification and development of novel anti-cataract drugs. However, human ES cells offer an opportunity to overcome these issues by providing a means of generating scalable, functional, lens cell types for a multitude of downstream applications such as investigating human lens and cataract development. Thus, the use of human ES cells holds significant promise for understanding human developmental biology, drug discovery and toxicity screening.

### 1.2.1 Cataract and its global impact

Opacities that occur within the ocular lens, commonly known as cataract, reduce lens transparency resulting in increased light scattering and an inadequate amount of light reaching the retina. If left untreated, disease progression ultimately leads to blindness. According to recent global estimates, cataract is responsible for low vision in over 80 million people and blindness in 19.9 million people.

For children, although visual impairment and blindness is not as prevalent as seen for adults, the potential lifespan of a child means that the resulting lifelong impact is
large. Childhood cataract is defined by the age of onset, where congenital cataract is present from birth and paediatric cataract occurs within 16 years post birth\textsuperscript{14}. Congenital cataract is the leading cause of surgically treatable blindness in children in many resource-poor settings occurring in part as a result of rubella and consanguinity\textsuperscript{14, 15}, with reports of 200,000 blind children worldwide\textsuperscript{16}. Congenital cataract has been associated with mutations in genes associated with the lens, for example the structural protein CRYA\textsuperscript{17}, and can affect one (unilateral) or both (bilateral) eyes. Unilateral congenital cataract is generally not associated with systemic diseases and in the majority of cases is idiopathic, however, for bilateral forms they generally occur in association with a multitude of birth defects\textsuperscript{14, 18}. Current treatments are documented to have significant complications that can result in life-long learning delays and disadvantages, thereby affecting life-long quality of life, earning power and ongoing medical costs\textsuperscript{19}. Additionally, for congenital cataract this demands urgent intervention, within 3 months, as affected children become blind if left untreated\textsuperscript{20}. 

The incidence of cataract greatly escalates with increasing age and is primarily associated with adults over 50 years of age. Estimates suggest that between the years 2001 to 2021 the number of Australians over the age of 50 affected by cataract will increase by 63\textsuperscript{\%}\textsuperscript{21}. Therefore as our population ages, the incidence of cataract will increase. This trend has also been documented for a number of other countries including America, India, Africa and China \textsuperscript{22}. There are three main forms of age-related cataract; nuclear cataract, referred to as age-related nuclear (ARN) cataract, cortical cataract and posterior sub-capsular cataract. Of these ARN cataract is the most common\textsuperscript{23}, accounting for 50\% of total cataract cases\textsuperscript{24} and resulting in both
full and partial opacification of the lens nucleus. The second most common type, cortical cataract, occurs in the periphery of the lens i.e. between the cortex and inner nucleus. Cortical cataract forms when the order of fibres in the lens cortex is disturbed, creating space between cells which fills with water and debris and contributes to light scattering and/or absorption\textsuperscript{25}. Although posterior sub-capsular cataract is not a common age associated type, it arises from the uncontrolled proliferation of LECs across the posterior or anterior sides of the lens beneath the lens capsule\textsuperscript{26-28}. It is also important to note that all these morphologies of age-related cataract can occur singly or in combination.

### 1.2.2 Causes of age-related cataracts

For age-related cataracts the underlying cause(s) are less defined but are of particular interest due to their widespread global effects. For ARN cataract, the molecular mechanisms responsible are not completely defined, however, potential risk factors have been identified. These include age, gender, genetics, smoking, dehydration, weight, diabetes, UV light, heat, oxidation and level of education\textsuperscript{3, 29-34}. One working theory for the mechanism surrounding ARN cataract is that in the aged the formation of a lens barrier inhibits homogenous diffusion of antioxidants including glutathione (GSH) and ascorbic acid, catalysts of the GSH redox cycle and other nutrients in the lens ultimately leading to abnormal protein conformations, insolubility and consequent light scatter\textsuperscript{3}. Studies using donated human lenses support this theory by exhibiting a significant decline in GSH levels in both the aging and cataractous lens\textsuperscript{3, 24, 35}. However, it is not clear as to how protein changes within the lens are linked to the onset of this barrier.
Over the years scientists have examined ARN cataracts and defined characteristic features thought to contribute to the arising light scatter. Some of these include post-translational protein modifications within the aged lens (glycation, deamination and oxidation of crystallin proteins) and ultrastructural assessment revealing the presence of light scattering multilamella bodies\textsuperscript{36-38}. Many studies have further demonstrated that when ARN cataracts occur, proteins of the lens nucleus degenerate and darken (brunescence) first to yellow then red or brown, causing light to scatter\textsuperscript{3,39-41}. Despite all these milestones, many questions still remain unanswered. For example, it is unclear what chromophores are responsible for the colour changes seen in ARN cataract.

\subsubsection*{1.2.3 Limitations of current cataract treatments}

If left untreated, disruption of lens transparency by cataract leads to blindness. Surgical lens replacement is currently the only means of returning sight to cataract patients, however, the annual number of operations and cost of treatment places a large financial burden on medical systems worldwide. In Australia alone, 180,000 cataract operations are performed annually costing over $326 million\textsuperscript{42}, but in North America these figures are approximately 4 fold higher and with an aging population, the cataract burden is only expected to worsen\textsuperscript{43}.

Presently cataract surgery involves the removal of the cataractous lens contents whilst leaving the lens capsule in place (extracapsular cataract extraction). However, it is common practice in third world countries to remove the entire lens (intracapsular cataract extraction) thereby reducing the risk of remigration of residual LECs resulting in PCO\textsuperscript{44-46}. Once removed, lens function is almost always restored through
the implantation of an inflexible non-accommodating intraocular lens (IOLs), or alternatively through the use of external contact lenses or glasses.\textsuperscript{44-46} This surgical approach results in immediate loss of the native accommodative ability of the normal lens\textsuperscript{47}. Although some commercially available accommodating IOLs are available they are imperfect, often causing glare and halos\textsuperscript{48-51}. Novel lens replacement strategies are being pursued such as the use of injectable polymers that mimic the refractive index of the lens. As yet his method has not reached clinical application, due to complications associated with filling the lens capsule after cataract removal and formation of PCO\textsuperscript{52,53}.

For third-world countries, inadequate access to surgery leaves many patients blind and when surgery is available various complications can arise, including PCO\textsuperscript{54}. The ability of the lens epithelium to consistently grow throughout life and regenerate itself post injury is advantageous to normal lens function. However, it is these traits which cause post-operative complications after primary lens surgery. Thus after extracapsular cataract extraction and implantation of an IOL there is potential for the residual LECs to migrate posteriorly to undergo aberrant fibre differentiation and wrinkle the posterior capsule. The continually dividing cells can then cover the posterior capsule and promote light scatter\textsuperscript{27}.

Treatment for PCO is achieved through the removal of the posterior lens capsule via neodymium:YAG laser\textsuperscript{54} and this additional requirement for surgery adds to the financial and social burden of cataract. The use of sharp edged IOL’s has reported to both delay and reduce the occurrence of PCO, however, the requirement for treatment via neodymium:YAG laser is still high. For example, a 10 year survey of
Nd:YAG laser use to treat PCO in Sweden identified approximately 37% of adults younger than 65 years of age and 20% of those over 65 years of age required treatment\textsuperscript{55}, similar findings have been found in Austrian based studies\textsuperscript{56}. PCO additionally affects paediatric cataract patients where onset occurs on average 6.1 months after primary cataract surgery. Despite the use of foldable IOL’s, younger children develop PCO more often than older children, for example, approximately 70% of children under the age of 1 versus 6% in children over the age of 7 were documented to develop PCO\textsuperscript{57}.

Laser treatment of PCO has been estimated to be the second most commonly performed ophthalmic procedure behind primary cataract surgery. In 1993 this treatment was estimated to cost $250 million annually in the US alone\textsuperscript{58}. Laser treatment for PCO is not free of its own complications such as retinal detachment reported to occur at a rate of ~0.5 to 4\% \textsuperscript{59,60}. The high rate of PCO and the potential for severe complications from Nd:YAG laser capsulotomy demonstrate the ineffective treatment systems in place today. Scientists and surgeons are therefore keen to develop new treatments that avoid cataract surgery (e.g. anti-cataract drugs).

\subsection*{1.2.4 Mammalian embryonic lens development}

The presumptive human lens develops as early as Carnegie stage 14-15 and has been widely studied as a model of embryonic induction\textsuperscript{61}. The correct induction of the lens is a multistep process controlled by a variety of signals responsible for first establishing its primitive architecture as well as maintaining lens cell differentiation throughout life. To truly value the complexities scientists and surgeons face when
designing alternative cataract therapies it is important to understand lens development at both the molecular and structural level.

1.2.5 Embryonic and postnatal lens growth

Mammalian eye development is initiated during late gastrulation when the ectoderm is divided into four domains; the neural plate, neural crest, pre-placodal region (PPR), and epidermis. The PPR contains multipotent cells that give rise to all sensory and neurogenic placodes, including the lens placode. The lens develops from the lens placode, a dish-shaped thickening of the head surface ectoderm that lies on either side of the head and closely opposes the anterior surface of the optic vesicles\(^6^1\).

In most vertebrates the lens placode invaginates to form the lens pit which then separates from the surface epithelium to form a spherical monolayer of epithelial cells termed the lens vesicle. As a result of factors within the vitreous fluid cells of the posterior half of the lens vesicle initiate their fibre differentiation program\(^1\), \(^2\), \(^6^2\), \(^6^3\), elongating toward the anterior epithelial layer to form primary LFCs. This process results in the filling of the lens vesicle lumen by tightly packed elongated primary fibre cells with an anterior monolayer of epithelial cells overlying them. As lens fibres terminally differentiate all organelles within them including the endoplasmic reticulum and golgi, as well as the mitochondria and nucleus are degraded\(^6^4\) ensuring the removal of potential light scattering objects\(^6^5\).

During the process of lens development lens cells express specialised cytoplasmic crystallin proteins including CRYA, CRYB and CRYG that are responsible for increasing the refractive index of the lens. CRYA are expressed by both LECs and
LFCs. During embryonic development CRYAB expression commences in the lens placode\textsuperscript{66, 67} and CRYAA is initiated later in the cells of the invaginating lens placode/lens pit. CRYB expression increases substantially as LECs elongate to form the LFCs present in the lens nucleus, and CRYG is expressed in terminally differentiated LFCs\textsuperscript{68-70}. Additionally, the lens is avascular as blood vessels would interfere in light transmission and result in light scatter, therefore proteins such as connexions and aquaporins are thought to facilitate the balance of ions, metabolites and fluid between lens cells\textsuperscript{65}.

During the process of lens vesicle production the lens cells become enveloped in a thick basement membrane called the lens capsule\textsuperscript{1, 2}. This transparent, smooth membrane contributes to the moulding of the lens shape and consists of collagen type IV, laminin, entactin, heparin sulfate proteoglycan, and fibronectin synthesised by the lens epithelium\textsuperscript{71}. Once the basic lens structure is achieved the lens continues to grow whilst maintaining its polarised shape. Coordinated differentiation of the cells along the equatorial edges of the anterior LECs monolayer leads to development of the secondary LFCs. These cells elongate along the anterior and posterior surfaces of the primary LFCs, wrapping the primary LFCs in layers of secondary LFCs. During an individual’s life new secondary LFCs continue to develop resulting in increased density of these fibres within the centre of the lens. As the cells differentiate, their apical ends extend toward the anterior pole of the lens and their basal ends extend toward the posterior pole. Once the apical and basal ends of the fibres reach their respective poles lens sutures are formed\textsuperscript{1, 2} and together these developmental aspects allow concentric layers of tightly packed, parallel aligned cells to be added to the mass of fibres.
1.2.6 Lens cell characteristics determine lens function

The role of the ocular lens is to focus incoming light onto the retina to enable clear sight. It provides 30% of the eye’s focusing power, an ability that is aided by maintenance of its transparency, refractive index and accommodative ability. To maintain lens transparency the fibre cells adopt a flattened hexagonal profile which facilitates their packing over a lifetime into an ordered array where the spaces between each neighboring cell are smaller than the wavelength of light.

The majority of crystallin proteins within the lens are believed not to turn over and thus must remain stable for a lifetime in order to preserve lens transparency\textsuperscript{72}. CRYA account for nearly 50% of the protein mass within the lens and possess the function of a chaperone\textsuperscript{73}, interacting with many unfolded substrates in the lens to prevent their irreversible aggregation and insolubility\textsuperscript{74} to therefore conserve lens transparency. Mutations in CRYA have been shown to cause cataracts, with single point mutations sufficient to alter CRYA structure or function resulting in protein insolubility, the accumulation of unstable proteins and loss of protein homeostasis\textsuperscript{75,76}. For the CRYAB knockout mice, while they initially possess relatively similar phenotypes to wild type mice they also possess an increased tendency for hyper-proliferation in culture and genomic instability\textsuperscript{77}. Furthermore, CRYAA knockout mouse models have enforced their importance to maintaining the solubility of the other crystallins within the lens, demonstrated through CRYAA knockout mice that developed an accumulation of CRYAB inclusion bodies in the central lens fibre cells\textsuperscript{78}.
As previously mentioned crystallins mainly function as structural proteins, accumulating to high concentrations in the lens fibre cells, thereby generating the high refractive index and transparency needed to focus while limiting light scatter. The refractive index of the lens increases gradually from the periphery of the lens to the centre, referred to as a radial gradient. This is primarily due to high concentration of crystallins at the centre compared to the periphery. This feature allows the eye to image with good resolution and low aberration at both short and long distances\textsuperscript{79}.

1.2.7 Lens stem cells and lens regeneration

Adult somatic stem cells are a rare population of cells that have the capacity for unlimited or prolonged self-renewal, differentiating to produce one or more cells types of the organs they occupy\textsuperscript{80}. Since the lens has the ability to grow throughout life, differentiating and maintaining lens cell types whilst maintaining its ability to function suggests the presence of a lens stem cell population.

1.2.7.1 Evidence for lens stem cells

Although the thought of a population of lens stem cells has not widely been discussed alternative organ systems stem cells have been discovered and differences in their proliferative ability documented, as exemplified within hematopoietic and gut systems. Stem cells of the hematopoietic system are multipotential and generate an intermediate population of committed progenitors referred to as transient amplifying cells, with lower proliferative potential and a more restricted potential for differentiation than stem cells\textsuperscript{81-83}. These transient amplifying cells amplify the effect of each stem cell division. Thereby reducing the number of stem cell divisions made to maintain the production of a large number of terminally differentiated cells\textsuperscript{84}. This
premise allows hematopoietic stem cells to exist predominantly in state of quiescence, resting at a non-dividing $G_0$-phase of the cell cycle whilst maintaining a high differentiation potential under certain conditions such as blood loss$^{84-86}$. Conversely, stem cells of the small intestine are constantly cycling. These stem cells additionally have transit amplifying cell progenitors however, to accommodate the rapid and consistent cell renewal occurring daily in the crypts of the small intestine these cells exist in a fast cycling state$^{87-89}$.

The few studies that have addressed the concept of lens stem cells have applied DNA labelling techniques by using DNA precursors such as thymidine and/or bromodeoxyuridine in rat, chick and mouse lenses, identifying areas of lens cell growth, areas of DNA replication and therefore areas where lens stem cells may reside. These studies report that most proliferation occurs in the peripheral region of the anterior lens known as the germinative zone (Figure 1), with LECs in the central anterior region considered to be mostly mitotically quiescent$^{90-93}$. Longer-term DNA-labelling experiments in mouse have assessed the rate of growth of these label retaining cells over a period of 18.5 weeks and found that central LECs show slow cycling properties described by their ability to retain DNA label longer than cells in the lens germinative zone$^{93}$. Additionally, these slow cycling cells were also induced to proliferate upon wounding. By contrast, cells with the lowest amount of retained label due to greater number of cell divisions were found in both central and germinative zones, with a select number in the germinative zone proliferating in response to wounding$^{93}$. Using similar DNA staining techniques and detection of proliferating cell nuclear antigen, mouse LECs were shown to actively cycle in the germinative zone while in the anterior region LECs have a more dormant
proliferating activity\textsuperscript{94}. DNA labelling studies suggest that the slow cycling relatively quiescent lens stem cells exist in the central lens epithelium and that can be stimulated to proliferate in response to injury. Furthermore, more active and/or transit amplifying stem cells appear to reside within the germinative zone of the lens.

\textbf{1.2.8 PCO and lens stem cells}

The capacity for remigration of residual LECs in vivo in mammalian lens after lens removal has been documented for almost two centuries\textsuperscript{95}. Whilst only a few studies have openly attempted to locate and characterise stem cells within the lens, the idea that residual lens stem cells are responsible for PCO has not yet been covered.

For human lenses, re-migration after primary cataract surgery is observed in the form of Soemmering’s ring and Elschnig perls. Soemmering’s ring results from the fusion of both anterior and posterior capsules which trap proliferating and degenerating LECs between them\textsuperscript{96-101}. Elschnig perls are transparent, globular masses of randomly mixed epithelial and fibre cells that appear after primary cataract surgery\textsuperscript{100}. Interestingly, documented ultrastructural analysis of Sommerings ring’s in human has revealed their lens-like cellular organisation, namely a monolayer of epithelial cells lining the remnant anterior capsule, a mixture of fibre cells in the interior ring and a region of fibre cells resembling the bow region of the normal lens\textsuperscript{99, 100}. Understanding what stimulates or inhibits the proliferation of these lens stem cells i.e., the growth factors and transcriptional targets they act upon, is of great importance especially when their aberrant proliferation results in these forms of PCO.
1.2.9 In vitro cataract models from lens stem cells

Seminal work by Coulombre and Coulombre in embryonic chick lenses identified that factors required for lens polarity and the generation and maintenance of LECs and LFCs were supplied by the ocular fluids. By microsurgical inversion of embryonic lenses around anterior-posterior axis their work showed the re-establishment of lens polarity and demonstrated that, (i) all embryonic lens cells are capable of differentiating into fibres with the right stimuli, (ii) the conditions necessary for the initiation and maintenance of lens fibre differentiation are provided by the vitreous fluid, and (iii) the conditions necessary for the growth and maintenance of an epithelial monolayer are provided by the aqueous fluid. These novel perspectives further stimulated investigation to identify the controlling factors present within the aqueous and vitreous fluid, and mammalian rat lens explant systems adapted from early chick were pivotal to this. In a co-culture setting LECs were first shown to proliferate and differentiate as a result of exposure to the neural retina and later in retina-conditioned media. The inducing factor generated by the retina was then identified as a member of the fibroblast growth factor (FGF) family and that a range of FGF members including FGF1 and FGF2 and their receptors were expressed in the lens.

Rat lens explants have further demonstrated that FGF was central to promoting the LEC changes characteristic of LFC differentiation in vivo. These changes, distinctively, included cell elongation, expression of fibre specific proteins and accumulation of complex membrane specialisations. Most significantly these studies had shown the dose dependent effects of FGF on cell growth, where low doses (5 ng/ml) encouraged LEC proliferation and high dose (100 ng/ml)
stimulated LEC migration and fibre cell differentiation\textsuperscript{108, 109, 117}. This idea was later strengthened when the concentration of FGF was found to be higher in vitreous than aqueous\textsuperscript{122}. FGF knockout work in transgenic mice has further supported a role for FGF in lens differentiation and development, where overexpression of a truncated FGF receptor designed to act in a dominant-negative manner led to the inhibition of fibre differentiation in vivo, therefore establishing a supported mechanism of FGF induced lens fibre cell differentiation\textsuperscript{123}.

Despite the involvement of FGF signalling in lens development, replacement of the vitreous fluid for FGFs within these rat lens culture systems did not support true lens regeneration. Even though paired rat lens cell explants treated with FGF1 or FGF2 displayed features characteristic of LFC differentiation, the use of these individual FGFs resulted in the formation of non-transparent and incorrectly organised degenerating cell aggregates\textsuperscript{124}. Therefore, while FGF signalling appears to be a key requirement for lens development and growth, it appears that input from other growth factor pathways is also required. The complexity of lens signalling has also been extended to potentially include regulation form noggin, TGFβ/BMP, Wnt, PDGF, EGF, and hedgehog pathways, whether as yet unknown factors are required, remains to be determined\textsuperscript{125-127}.

\textbf{1.2.10 Human pluripotent stem cells for lens research}

The era of pluripotent stem cells, has triggered many expectations that differentiation programs can be used to provide a renewable source of functional human cells in place of less specific animal model systems. As animal models will only progress our understanding of human lens development and disease to a certain
point, human ES cell-derived lens cells offer an opportunity to move beyond this point to study the molecular mechanisms of human lens and cataract development in vitro. This development holds significant promise for understanding human developmental biology, for drug discovery and toxicity screening\(^8\). By combining what is already known of the signalling pathways involved in the lens and trial and error testing of alternative growth factor pathways it may be possible to establish efficient and defined protocols that allow the generation of scalable, functional normal or diseased lens cell types for a multitude of downstream applications.

### 1.2.11 Lens differentiation methods for human pluripotent cells

To date, three protocols have been published that differentiate both mouse and human ES cells into LECs: the first utilised co-culture with mouse PA6 stromal cells\(^{128}\); the second involved sequential addition of recombinant growth factors\(^{127}\) and third the use of chemically defined, serum-free medium, insulintransferrin-selenium\(^{129}\).

In 2003 Hirano et al showed that mouse ES cells cultured on a monolayer of PA6 stromal cells supported the induction of eye like structures\(^{128}\). These structures contained cells expressing specific phenotypic markers representative of lens cells (CRYAB, CYRAA, CRYB) and pigmented retinal cells (Brn3b, syntaxin). Assessment of gross morphology of these structures showed that most of the ocular cell types except retinal pigment epithelium were mixed within the multilayered cell masses with no organised structure. This study demonstrated the ability of ES stem cells to produces lens cell types in culture, however, the method used produced
heterogeneous populations of both lens and non-lens cells a feature that is not suited to downstream lens applications.

Seven years later a 3-stage growth factor treatment was first reported, that differentiated human ES cells into lens progenitor-like cells\textsuperscript{127}. This published lens differentiation program began by treatment with noggin, a growth factor involved in the inhibition of bone morphogenetic protein (BMP) signalling\textsuperscript{130} implemented to trigger differentiation of human ES cells toward neuroectoderm lineage\textsuperscript{127}. To transition neuroectoderm toward lens progenitor lineage BMP activation and activation of FGF signalling was then stimulated via BMP4, BMP7 and FGF2 treatment. Finally, treatment with FGF2 and Wnt-3a was used to promote the proliferation and differentiation of LECs and LFCs resulting in the formation of lens like structures termed “lentoids”\textsuperscript{127}. This study successfully generated lens like cells, however, much of these lens cell types were found randomly throughout the 3 dimensional lentoid structures due to their mixed expression of lens specific CRYA, CRYB and CRYG.

More recently, human ES cells were cultured in a chemically defined, serum-free medium containing recombinant human insulin and transferrin, with selenium. The protocol generated the production of both neural and non-neural ectoderm and mesoderm\textsuperscript{129}. Fluorescence-activated cell sorting was then used in a trial and error fashion to select for and against a variety of cell types and allow the purification of ‘lens epithelium like cells’, that when cultured formed lentoids. The method described was complex, requiring targeted depletion and then further selection of cells based on cell surface antigens p75, HNK-1, CD15 c-Met/HGFR and CD44. A
shortcoming of this protocol is its complexity, such a technique requires extended cell handling to first stain with multiple antibodies and then further perform multiple-laser sorting. Additionally, the c-Met/CD44-based approach was shown to be inefficient, with only 0.2 to 1.5% of the total cells positive for these markers. This report does, however, highlight an area requiring further study and it would be of great interest if LEC surface antigens were defined. These receptors or surface proteins could be used as future candidates permitting simple one step purification protocols that require minimal time and handling and therefore best suited to scalability.

1.2.12 Human ES cell derived lens cells for identification of developmental mechanisms
Currently, some of the factors responsible for driving lens regeneration and differentiation have been discovered for example, FGFs, but little is known of the molecular mechanisms and networks that control LEC and LFC behaviour. Access to a plentiful supply of normal or diseased (i.e., cataractous) human ES cell derived lens cells would allow scientists to better investigate the molecular mechanisms controlling lens required processes like differentiation, proliferation, migration and apoptosis.

1.2.12.1 Human ES cell derived lens cells to identify anti-cataract drugs
As the incidence of age-related cataract increases so too does the requirement for surgery and its associated costs and complications. Treatment strategies that delay or inhibit the progression of cataract whilst avoiding primary cataract surgery or
secondary cataract formation, and are inexpensive and widely available would allow treatment regimes that keep up with increasing cataract numbers. A 10-year delay in cataract formation is postulated to a greatly reduce associated surgery costs on the public health sector by reducing the number of required cataract operations by half\textsuperscript{131}. A new LEC based model derived from human ES cells would benefit the development of such novel strategies and aid our understanding of the mechanisms involved in cataract formation and therefore help develop anti-cataract drugs.

The development of high-throughput screening methods has enabled rapid and quantitative methods for drug discovery in the pharmaceutical industry\textsuperscript{132-134}. The potential for lens-based drug screening systems has already been demonstrated for human in vitro capsular bag models used to test for anti-cataract drugs\textsuperscript{135, 136}. However, this method was not designed to cope with the high-throughput nature of experiments required to identify anti-secondary cataract drugs as the capsular bags require donor lenses and are too technical challenging to enable scale up. Therefore what is needed is a novel method to produce purified populations of LECs that can be scaled to enable high-throughput screening assays that assess large chemical libraries for anti-secondary cataract properties. For example, LECs produced from human ES cell differentiation schemes could be applied in high-throughput screening methods to assess for drugs that either prevent their proliferation or differentiation to LFCs.
1.2.13 Summary

The incidence of cataract is increasing globally and so too are the associated social burdens and financial costs. Due to global population ageing, new methods of prevention and treatment are needed to cope with the projected increase as well as to improve treatment outcomes (e.g., avoidance of PCO). The establishment of a simple, efficient and scalable method for generating purified LECs from human pluripotent stem cells (i.e., human ES cells and or induced pluripotent stem cells) would provide a new humanised model for understanding lens and cataract formation as well as providing a new tool for anti-cataract drug discovery.
CHAPTER TWO:

Growth factor optimisation for production of lens epithelial cells from human embryonic stem cells
2.1 ABSTRACT

Purpose: A critical step towards new cataract treatments is the development of scalable human lens cell culture systems that enable better understanding of lens and cataract formation. A recently published 3-stage growth factor treatment has been shown to differentiate human embryonic stem (ES) cells into impure populations of lens progenitor-like cells. Further optimisation of this protocol is required to minimise and/or eliminate the generation of non-lens cells to produce large numbers of pure human lens epithelial cells (LECs) that can be maintained in culture and directed to form lens fibre cells (LFCs) in a controlled manner.

Methods: A published, 3-stage lens differentiation protocol was used as the starting differentiation protocol. This consisted of: noggin (Stage 1; 5 days); BMP4, BMP7 and FGF2 (Stage 2; 13 days); then FGF2 and WNT3A (Stage 3; 17 days). Additional growth factors thought to be involved in embryonic lens development were tested in an attempt to improve the generation and maintenance of desired LECs and their precursors by successive modification of the 3 Stages. This included: 1) maximising neuroectoderm production via increased noggin signalling together with inhibition of SMAD signalling; 2) optimising production of lens placode cells via secreted Frizzled-related proteins (SFRP1, SFRP2, SFRP3, SFRP4), Dickkopfs (DKK1, DKK3, DKK4), or Activin A; and 3) avoiding spontaneous generation of LFCs via a reduced FGF2 concentration or use of vitreous fluid.

Results: The published growth factor method was reproduced, yielding similar expression profiles of lens specific transcripts and lentoid production. Stage 1 modifications increased lentoid production and expression of LEC development genes (e.g. PAX6, CRYAB). Stage 2 modifications delivered no improvement as seen by morphological analysis and expression of LEC development genes. Stage 3
modifications via low FGF2 decreased spontaneous lentoid production but sustained
CRYAB expression.

**Conclusions:** Stage 1 and 3 modifications improved production of LECs, however,
the presence of lentoids and mixed morphologies within cell culture and expression
of the LFC marker CRYBB3 indicated the cultures still contained mixed populations
of lens and non-lens cells. Additional modifications are needed to generate pure
populations of LECs for research and drug discovery.
2.2 INTRODUCTION

To facilitate the expansion of our present understanding of lens and cataract formation as well as develop new cataract treatments, a method to reliably produce a scalable human lens cell culture system is necessary. A recent report demonstrated a 3-stage growth factor treatment that differentiates human embryonic stem (ES) cells into lens progenitor-like cells. This study additionally reported the production of 3-dimensional lens-like structures termed “lentoids”. These lentoids were shown by ultrastructural analysis as having an array of both cuboidal and elongating cells at different stages of development. Furthermore immunofluorescence demonstrated that the mixed cells within lentoids co-expressed classic lens epithelial cell (LEC) and lens fibre cell (LFC) markers including CRYAB and CRYBB respectively. Limitations of this protocol include the generation of non-lens cells, as well as the uncontrolled generation of LFCs from LECs as indicated by the spontaneous appearance of lentoids. The disorganised structure of these lentoids also does not reflect the highly-organised lens architecture required for normal lens function, specifically, a monolayer of LECs in contact with a mass of parallel-aligned, elongated LFCs. Further optimisation of this protocol is required to minimise or eliminate the generation of non-LECs whilst producing large numbers of pure human LECs that can be maintained in culture and directed to form human LFCs in a controlled manner.

Growth factors are known to be master regulators of cell proliferation, survival during development and differentiation towards more mature specialised cell types. Major growth factor families, including FGFs, TGFβ/BMPs and Wnt, have all been shown to be involved in regulating lens development. The
published 3-stage lens differentiation protocol was developed using trial and error testing of different growth factor combinations, ultimately resulting in sequential human ES cell exposure to: noggin, BMP4/BMP7/FGF2, and finally FGF2 and WNT3A. These growth factor combinations were chosen to mimic in vitro the successive generation of neuroectoderm, lens placode cells, LECs and LFCs that is known to occur in vivo during embryonic lens development. Further examination of the literature, however, indicates the involvement of additional growth factors during embryonic lens development, suggesting that their inclusion at an appropriate stage of this 3-stage lens differentiation protocol may enhance it by improving LEC production while simultaneously decreasing production of LFCs and non-lens cells.

During embryonic development both the lens and neural tissues arise from embryonic ectoderm, therefore, some of the early steps used to encourage neural differentiation in a laboratory setting might be suitable for stimulating lens differentiation in vitro. For example, Chambers and colleagues reported a step-wise protocol for differentiating human ES cells to neural cells by first optimising high levels of neuroectoderm formation\textsuperscript{149}. Neuroectoderm was induced by treating human ES cells with both high concentrations of noggin and a chemical inhibitor of TGF\beta/SMAD signalling (SB431542). Since noggin-based neuroectoderm formation is the first step of the reported 3-stage lens differentiation protocol, SB431542 was included in the first Stage 1 modification to increase the efficiency of neuroectoderm formation as a way of increasing human LEC production.

Additional signalling pathways postulated to be involved in lens placode
formation in vivo (and previously not tested in the published 3-stage lens differentiation method) include inhibition of Wnt signalling\textsuperscript{150} and activation of Activin signalling\textsuperscript{151}. Thus optimisation of Stage 2 (lens placode production) of the 3-stage lens differentiation method involved testing modulation of these growth factor pathways including the inclusion of Dickkopfs (DKKs) (antagonists of Wnt signalling pathways)\textsuperscript{152}, and secreted Frizzled-related proteins (SFRPs) (Wnt signalling activators)\textsuperscript{153}, as well as addition of Activin A\textsuperscript{142}.

Varying concentrations of FGF have been documented to either maintain LECs in culture i.e. low concentrations of FGF, or stimulate LFC formation i.e. high concentrations of FGF\textsuperscript{117}. The inclusion of high FGF concentrations within the final stage of the 3-stage lens differentiation method is thought to drive LFC production from LECs. Wnt/catenin signalling has been indicated as a requirement for the formation, proliferation and maintenance of anterior LECs. For instance, mouse embryos homozygous for a mutation in the \textit{Lrp6} gene have lenses that fail to form a normal anterior monolayer of epithelial cells\textsuperscript{145}. Conversely constitutive activation of the Wnt/β-catenin pathway results in lenses with enhanced epithelial cell cycle progression and incorrect regulation of cell cycle exit. These LECs incorrectly migrate and populate the posterior compartment of the lens\textsuperscript{154, 155}. Therefore Wnt-3a levels were not modified from the published Stage 3 levels in this study in order to provide optimal conditions for maintenance of the LECs produced in the growth factor modified test lens differentiation strategies used here. However, to avoid stimulation of LFC formation and allow maintenance of LECs, low concentrations of FGF were used.
In the present study, a variety of growth factors involved in lens development were tested in the reported 3-stage lens differentiation protocol to; 1) Maximise neuroectoderm production; 2) Optimise lens placode cell production; and 3) Avoid the generation of LFCs; effects were observed using light microscopy and real-time-PCR. These trial and error manipulations of the published lens differentiation protocol resulted in a greater production of lentoids with a corresponding increase in LEC specific markers. Additional modification of Stage 3 resulted in statistically lowered expression of early LFC transcripts with stabilised expression of LEC markers. The results indicate that modifications to the published 3-stage lens differentiation protocol improved the production and maintenance of LECs in vitro, however, further improvements are required to eliminate contaminating LFCs and non-lens cells.
2.3 METHOD

2.3.1 Cells

CA1 human ES cells were provided by A. Nagy\textsuperscript{156}. Approval for use of these cells as described was obtained from the University of Western Sydney Human Research Ethics Committee.

2.3.2 Reagents and consumables

All cell culture was performed in a Class II biosafety cabinet. Reagents used for cell culture including mTeSR1\textsuperscript{TM}, Dulbecco’s Modified Eagles Medium (DMEM), Dulbecco’s Modified Eagles Medium: Nutrient Mixture F-12 (DMEM: F12), dispase and trypan blue were acquired from Stem Cell Technologies (Tullamarine, Australia). Reagents used in cell harvesting including TrypLE\textsuperscript{TM} Express and Dulbecco’s Phosphate Buffered Saline –Ca/Mg (-PBS), were acquired from Life Technologies (Mulgrave, Australia) unless stated otherwise. Matrigel\textsuperscript{TM} required to coat tissue culture plates for cell adherence was acquired from BD Biosciences (North Ryde, Australia). All tissue culture plates (6-well, 96-well, 35 mm and 60 mm) and pipette tips used for general cell culture were acquired from Greiner-Bio-one (Frickenhausen, Germany).

2.3.3 General cell culture

CA1 human ES cells were maintained and cultured using the defined, feeder cell-free medium mTeSR1\textsuperscript{TM} \textsuperscript{157}. Human EC cells were passaged every 7 days using 1 mg/mL dispase and plated as aggregates on tissue culture dishes pre-coated with Matrigel (0.1 mg/mL) for a minimum of 30 min at room temperature after which time the Matrigel was removed\textsuperscript{158}.
2.3.4 Preparing human ES cells to be plated as single cells

CA1 cells cultured as aggregates in 60 mm tissue culture plates had their medium changed to 3 mL of mTeSR1™ containing 10 µM Rho-kinase inhibitor (ROCK inhibitor) (Merck, Kilsyth, Australia) to increase cell viability as single cells and thus plating efficiency. The cells were then incubated for a minimum of 1 h at 37°C, 5% CO₂. Single cell suspensions were generated using the enzyme TrypLE™ (7 min, 37 °C, 5% CO₂) as it allows the generation of single cells without requiring inhibition (i.e. as opposed to trypsin). The cells were collected and placed into a 15 mL tube; 3 mL of -PBS was used to wash each plate; this was then collected into the same 15 mL tube. The cells were centrifuged (300 × g, 5 min), supernatant discarded, and cell pellet re-suspended in 1 mL mTeSR1™ containing 10 μM ROCK inhibitor. Cell counts were performed by diluting 10 μL of each cell sample into with 40 μL of the dead cell stain trypan blue 0.4% (w/v). Diluted cell sample were placed in a hemocytometer (Bright-Line; Hausser Scientific, Pennsylvania, USA) and cell counts performed using a light microscope. For experiments, 2 × 10⁵ cells were re-plated in a Matrigel-coated 6-well tissue culture plate or 35 mm tissue culture dish containing 2 mL of mTeSR1™ with 10 μM ROCK inhibitor. The mTeSR1™ medium was replaced the following day without ROCK inhibitor and every day after for 4 days. Once a confluent monolayer of cells was established the lens differentiation growth factor treatment began (see Table 2.1).

2.3.5 Stage 1: Differentiation towards neuroectoderm (Day 0 to day 5)

The differentiation of human ES cells toward LEC fate was performed using the published 3-Stage 1 neuroectoderm induction medium or the modified medium
shown in Table 2.1. Each 35 mm well received 2 mL of medium which was refreshed every other day up until day 5. All plates regardless of treatment were analysed under the light microscope to observe morphological changes induced by the Stage 1, 2 and 3 growth factor modifications in comparison to the published growth factor treatment.

2.3.6 Stage 2: Generation of lens placode cells (Day 5 to Day 18)

At day 5, the published or modified Stage 2 lens placode induction medium (Table 2.1) was added. Each well was washed with 2 mL of -PBS (room temperature) then 2 mL stage 2 medium was added per 35 mm well. The medium was changed every other day up till day 18.

2.3.7 Stage 3: Generation of LEC and fibre cell types (Day 18 to Day 35)

At day 18, the published or modified Stage 3 LEC and LFC induction medium was added (Table 2.1). The Stage 2 medium was removed and each well was washed with 2 mL of -PBS (room temperature) before 2 mL of Stage 3 medium was added to each 35 mm well. The fresh medium was changed every other day up to 35 days. The plates were analysed every other day by light microscope to check for morphology’s suggestive of LEC and fibre cell types.

2.3.8 Lentoid counts

At days 14, 18, 26 and 35 experimental plates were analysed under the light microscope to check for the appearance of lentoids. These were defined as circular or irregular multilayered and transparent structures (Figure 2.1F-K). Each well of
<table>
<thead>
<tr>
<th>Published 3-stage lens differentiation media(^{127})</th>
<th>Stepwise modifications to published protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Differentiation stage</strong></td>
<td><strong>Standard media components</strong></td>
</tr>
<tr>
<td>Stage 1: Neuroectoderm induction</td>
<td>Noggin (100 ng/mL)</td>
</tr>
<tr>
<td>(Day 0 to 5)</td>
<td>N2</td>
</tr>
<tr>
<td></td>
<td>B27</td>
</tr>
<tr>
<td></td>
<td>DMEM:F12</td>
</tr>
<tr>
<td>Stage 2: Lens placode induction</td>
<td>BMP4 (20 ng/mL)</td>
</tr>
<tr>
<td>(Day 5 to 18)</td>
<td>BMP7 (20 ng/mL)</td>
</tr>
<tr>
<td></td>
<td>FGF2 (100 ng/mL)</td>
</tr>
<tr>
<td></td>
<td>DMEM:F12</td>
</tr>
<tr>
<td>Stage 3: LEC and LFC induction</td>
<td>FGF2 (100 ng/mL)</td>
</tr>
<tr>
<td>(Day 18 up to 35)</td>
<td>Wnt-3a (20 ng/mL)</td>
</tr>
<tr>
<td></td>
<td>DMEM:F12</td>
</tr>
</tbody>
</table>

*final growth factor concentrations shown, all growth factor from R&D Systems (Minneapolis, USA)
interest was divided up into quadrants by drawing lines on the base of the culture dish using a permanent marker, and the 10x objective was used to systematically scan for lentoids in one quadrant of each well. A cell counter was used to aid lentoid quantification in the quadrant in order to estimate the number of lentoids per dish.

2.3.9 RNA collection

RNA was collected at regular intervals between days 0, 5, 14, 18, 26 and or 35. RNA was extracted using RNA Lysis Buffer R (Bioline, Sydney, Australia) and stored at –80°C until required. RNA was purified using the RNA mini-kit according to manufacturer’s instructions (Bioline, Sydney, Australia). To ensure the removal of genomic DNA, RNA samples underwent DNase treatment kit according to the manufacturer’s instructions (Promega, Sydney, Australia).

2.3.10 cDNA synthesis via reverse transcription

Each RNA sample was used for 2 cDNA reactions: i) RNA and reverse transcriptase (+RT), ii) RNA without reverse transcriptase (-RT) to test for genomic contamination (in the later real-time PCR reactions), A third reverse transcription reaction was performed for each cDNA set that had iii) no RNA, with reverse transcriptase, and with H₂O as a non-template control (NTC) testing for DNA contamination of PCR reagents. The first stage of cDNA production required 500 ng of purified RNA, 2 µL of random hexamer primers (Bioline) and RNase/DNase free water for a final volume of 15 µL in a PCR reaction tube and mixed briefly with a QikSpin Personal Centrifuge (2000 × g, 1 min; Edwards, Sydney, Australia). The sample was placed in the Mastercycler™ (70 °C, 5 min; Stratagene, La Jolla, CA). The second stage of cDNA production required 6 µL 5 X reverse transcriptase (RT)
buffer, 1.5 µL 10 mM dNTPs, 1.5 µL 40 U/µL RNase inhibitor and 6 µL RNase/DNase free water for a final volume of 15 µL. To the +RT and NTC samples, 0.4 µL 200 U/µL Bioscript (Bioline) was added. The samples were mixed briefly with a QikSpin Personal Centrifuge (2000 × g, 1 min) and the sample was placed in the Mastercycler™ at the following settings: cycle 1 (42 °C, 60 min), cycle 2 (70 °C, 10 min). The resulting cDNA were then used for real-time PCR amplification testing for specific mRNA transcripts.

2.3.11 Primer design

Forward and reverse gene primers were designed utilising the Primer3 web page (http://frodo.wi.mit.edu/primer3/). Exon sequences for specific genes were obtained using the UCSC genome browser (http://genome.ucsc.edu/) and placing these sequences into a Microsoft Word Document™. For each gene the exon sequence from the 3’ end of one exon to the 5’ end of the next exon was copied into Primer3 and primer design was initiated using the parameters 60°C melting temperature and 80 - 200 base amplicon length. The resulting predicted primers were searched against the entire known human genome and transcriptome using the BLAST nucleotide web page to identify primers sets that did no bind anywhere else in the genome (the process was repeated until unique primer pairs were obtained for each gene of interest) (Table 2.2).

2.3.12 PCR amplification of mRNA transcripts

Each PCR primer (Geneworks, Hindmarsh, Australia) was tested with a trial PCR reaction at temperatures between 55 – 60 °C. Each PCR reaction consisted of 5 µL of 5 X Go-Taq Flexi PCR Buffer, 1.5 µL 25 mM MgCl₂, 1 µL 10 mM dNTPs, 2 µL
Table 2.2 List of primers used to assess lens differentiation strategies

| Primer  | Forward                     | Reverse                               |
|---------|                            |                                      |
| GAPDH   | CCCATCCACCATCTTCCAGGAG      | CTTCTCCATGGTGGTGTAAGACG              |
| PAX6    | CCCCACATATGCAGACAC          | TCACTTCCGGAACTTGAAC                  |
| CRYAB   | TGATTGAGGTGCATGGAAAA        | ATCAGCTGGGATCCGGTATT                |
| CRY BB3 | CCGCAAGATGGAGATAGTG         | ACTCATAGCCAACCACGTC                 |
12.5 µM Forward and Reverse Primers, 14 µL RNase/DNase free water, 0.5 µL Go-Taq Flexi and 1 µL of cDNA (+RT, -RT and NTC) to appropriate wells. The samples were then placed into the Mastercycler® using the appropriate temperature profile for primers. The PCR reaction was run for 40 cycles. Once the PCR run was complete, the PCR products were loaded into a 2% agarose gel with 5 µL of 10,000 X Gel Red nucleic acid stain (Jomar Diagnostics, Stepney, South Australia) against a 100 bp DNA ladder (4 µL of 50 µg/µL) (Axygen Scientific, Union City, California). The agarose gel was run at 100 V, 300 mA, 50 W for 40 min. The gel was then imaged on a Gel Dock Transiluminator (Vilber Lourmat) using the E-Box software. Analysis of the gel was based on intensity of the bands visible within the gel image at the 100 bp level in the + RT lanes. PCR samples were stored at 4 °C and cDNA samples stored at -20 °C.

2.3.13 Real-time PCR

Primers (Geneworks) were optimised with trial real-time PCR temperatures between 55-60 °C. The efficiency of each primer was determined and recorded for the genes of interest as well as the housekeeping gene GAPDH. A master mix for the real-time-PCR reaction (2 µL 5 X Go-Taq Flexi PCR buffer, 1 µL 1 mM MgCl₂, 1 µL forward and reverse primers, 1.5 µL 10 mM dNTPs, 0.3 µL DMSO, 0.1 µL Go-Taq flexi 5 U/µL and 0.1 µL 100 X SYBR Green) (Bioline, Sydney, Australia) was prepared for each sample to be tested; all samples were tested in triplicates. Firstly, 6 µL of master mix for each sample was aliquoted into a 96-well plate. Samples were then added, testing 4 µL of cDNA in triplicates (+RT, -RT and NTC) and centrifuged (1000 × g, 1 min) to remove air bubbles. The 96-well plate was then placed in the Mx3005P real-time-PCR System (Agilent Technologies, Sydney, Australia) and
programmed using the MxPro real-time-PCR software. The optimal temperature for primer binding was input into the MxPro software and samples were grouped and labelled as +RT, -RT and NTC and samples were amplified. GAPDH was used as the internal control.

2.3.14 Analysis of real-time PCR data

Cycle threshold (Ct) values of primer expression represent the number of cycles required for the fluorescent signal to surpass the threshold level of gene expression. The Ct values of triplicate technical replicates for each primer set obtained during real-time PCR for the gene being assessed, also known as the gene of interest (GOI), were averaged and calculated against the gene of interest efficiency to determine the gene of interest Quantification (Q) value:

Q value = Efficiency of Gene of Interest (GOI) \(^{(\text{Sample 1 - Sample 2 Ct values})}\).

The Q value of the gene of interest was normalised against the Q value of the housekeeping gene GAPDH:

\[
\frac{Q_{\text{GOI}}}{Q_{\text{Housekeeping Gene}}}
\]

A Student’s t-test was performed to determine whether any changes in gene expression levels were statistically significant (i.e. \(p < 0.05\)). If the results were statistically significant the P.value was reported, if not the null hypothesis is accepted and P.value only listed \(p<0.15\).
2.4 RESULTS

2.4.1 Stage 1 modification: optimisation of neuroectoderm production

Prior to starting Stage 1 modifications reproducibility of unmodified 3-stage protocol was assessed. Reproduction of the unmodified published 3-stage protocol yielded similar results to those seen in the published study as shown by the expression of LEC development genes (e.g., CRYAB and PAX6) (Figure 2.1B,C) and number and presence of lentoids (Figure 2.1E,F,G,H). This data demonstrated the method reproducible with a different human ES cell line while generating a similar response magnitude.

Optimisation of Stage 1 of the reported 3-stage growth factor protocol was performed by replacing the reported growth factor schedule over days 0 to day 5 with one hypothesised to increase neuroectoderm production (Figure 2.1A). The modified Stage 1 cocktail included 500 µg/mL noggin and 10µM SB431542 inhibitor to further increase inhibition of the TGFβ/SMAD signalling pathway. Knockout serum replacement was added in place of N2B27 medium. The benefit of using KOSR in place of N2B27 media is that it supports the growth of human ES cells and is chemically defined.

For the modified Stage 1 treatment the growth factor treatments for Stages 2 and 3 (Days 5 to 35) followed that of the published protocol. Each Stage 1 modified differentiation experiment was set up in parallel with an unmodified 3-stage growth factor differentiation program performed as published to serve as a control (similarly for each Stage 2 and Stage 3 modification). This strategy allowed every protocol modification to be compared against the ability to improve upon the reported
protocol. Analysis of the modified Stage 1 experiments showed an increased expression level of neuroectoderm marker PAX6 (Figure 2.1B) as well as a statistically significant increase of LEC development genes CRYAB (Figure 2.1C). Additionally, from days 14 onwards modifications saw an increased expression of CRYBB3 (Figure 2.1D) as well as a statistically significant increased production of lens-like structures (i.e., lentoids) (Figure 2.1E). These lentoids had similar morphologies to the published protocol (Figure 2.1F-K), i.e. tightly packed swollen irregular-shaped cells. These improved Stage 1 conditions were therefore used for all further experiments.

2.4.2 Stage 2 modification: optimising lens placode cell production

Optimisation of lens placode production began by testing the inclusion of a variety of growth factors thought to be involved in lens placode development including SFRP1, SFRP2, SFRP3, SFRP4, DKK1, DKK3, DKK4, and Activin A. Experiments began using the improved Stage 1 modification from days 0 to 5 followed by individual assessment of each of the 8 modifications to the Stage 2 medium between days 5 to 18 (Table 2.1).

A variety of concentrations of Activin A were tested (i.e., 1, 10 and 100 ng/mL) (Figure 2.2A), as Stage 2 modifications were followed by high FGF in Stage 3, improvement of LEC production would additionally result in an improvement or maintenance of LFC gene expression and lentoid production. However, the incorporation of this treatment saw a decreased expression of neuroectoderm marker PAX6 (Figure 2.2B) as well as no statistically significant increase in the production of CRYAB or CRYBB3 (Figure 2.2C,D) levels by comparison to the modified Stage
Figure 2.1 Stage 1 modification increased LEC production.

A) Schematic diagram of the published 3-stage differentiation protocol with Stage 1 modification. B) The modified Stage 1 protocol (red) increased expression of the neuroectoderm marker PAX6 by comparison to the replicated 3-stage protocol (blue). C) Additionally modification saw an increase of LEC marker CRYAB (D26: $p=0.034$; D35: $p=0.041$). D) Early LFC marker CRYBB3 increased (D18 $p=0.15$). E) The modified protocol produced lens-like structures (i.e., lentoids) with similar morphology to the published protocol but in greater numbers, results were statistically significant (D14: $p=0.011$; D18: $p=0.032$; D26: $p=0.010$). F-K) Microscopic analysis of lens differentiation program comparing published 3-stage differentiation program (F,G,H) and modified Stage 1 protocol (I, J, K) (at both 10x and 20x magnification; phase contrast H,K).
A) SMAD inhibitor
   ↑ Noggin
   hPSCs ➔ Neuroectoderm ➔ Lens placode ➔ Lens cells
   Day 0-5

B) PAX6 Relative Expression

C) CRYAB Relative Expression

D) CRYBB3 Relative Expression

E) Lentoid Counts

1 result. Additionally, the treatment of Activin A resulted in the absence of lentoids in culture (Figure 2.2E).

Inclusion of the SFRPs 1 to 4 at varying concentrations (Figure 2.3A, 2.4A, 2.5A, 2.6A) resulted in a decreased level of PAX6 expression up to day 26 (Figure 2.3B, 2.4B, 2.5B, 2.6B). Additionally, no increase in expression levels of CRYAB was observed (Figure 2.3C, 2.4C, 2.5C, 2.6C). SFRP3 resulted in a 38 fold reduction of CRYAB levels by comparison to the modified Stage 1 result (Figure 3.5B). Cell death was observed from day 24 and therefore cultures were not progressed beyond day 26. CRYBB3 expression was reduced in SFRP1-3 treatments (Figure 2.3D, 2.4D, 2.5D) and similar to unmodified results with SFRP4 exposure (Figure 2.6D). Lentoid counts were all reduced (Figure 2.3E, 2.4E, 2.5E, 2.6E). Stage 2 modifications did not result in an improved production of LECs as seen by no increase in CRYAB in addition to increase and or maintenance of CRYAB expression and lentoid frequency.

Testing of all 3 DKKs in Stage 2 (Figure 2.7A, 2.8A, 2.9A) resulted in decreased PAX6 expression at all concentrations used (Figure 2.7B, 2.8B, 2.9B). DKK1 and DKK3 had similar levels of CRYAB expression (Figure 2.7C, 2.8C) compared to the modified Stage 1 data; for DKK4 CRYAB levels were slightly lower by comparison (Figure 2.9C). CRYBB3 expression was reduced in DKK1 and DKK4 treatments (Figure 2.7D, 2.9D and similar to unmodified results with DKK3 exposure (Figure 2.8D). DKK3 treatment resulted in relatively similar production of lentoids in culture at 3 ng/mL and reduced numbers after 30 mg/mL concentrations (Figure 2.8E). DKK1 and DKK4 lentoid counts were lower at all concentrations.
Figure 2.2 Stage 2 modifications with Activin A did not increase LEC production.

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the inclusion of Stage 2 modification, Activin A, at concentrations of 1, 10 and 100 ng/mL. B) Addition of Activin A, at concentrations of 1, 10 and 100 ng/mL (blue, purple, green respectively) did not increase expression of neuroectoderm marker PAX6. C) Activin A did not increase expression of LEC marker CRYAB. D) CRYBB3 expression was similar to Stage 1 modified protocol profile. E) Activin A did not increase lentoid production compared to the Stage 1 only modification.
Figure 2.3 Stage 2 modifications with SFRP1 did not increase LEC production.

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 2 modification SFRP1 at concentrations of 100 ng/mL and 10 μg/mL (blue, orange respectively). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed a decrease of LEC marker CRYAB. D) Real-time PCR assessment showed a decrease of the LFC marker CRYBB3. E) Fewer lentoids were produced.
Figure 2.4 Stage 2 modifications with SFRP2 did not increase LEC production.

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 2 modification SFRP2 at concentrations of 100 ng/mL and 10 μg/mL (blue, pink respectively). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed a decrease of the LEC marker CRYAB. D) Real-time PCR assessment showed a decrease of the LFC marker CRYBB3. E) Fewer lentoids were produced.
Figure 2.5 Stage 2 modifications with SFRP3 did not increase LEC production

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 2 modification, 100 ng/mL SFRP3 (green). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed a decrease of LEC marker CRYAB. D) Real-time PCR assessment showed a decrease of the LFC marker CRYBB3. E) Fewer lentoids were produced.
Figure 2.6 Stage 2 modifications with SFRP4 did not increase LEC production.

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 2 modification SFRP4 at concentrations of 100 ng/mL and 10 ug/mL (green, purple respectively). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed no increase of LEC marker CRYAB. D) Real-time PCR assessment showed similar expression of the LFC marker CRYBB3. E) Fewer lentoids were produced.
tested (Figure 2.7E, 2.9E). None of the trialled Stage 2 modifications showed an increase in lentoid production or expression of LEC development genes. As none of the assessed Stage 2 modifications increased LEC gene expression or lentoid production, none of the above mentioned growth factors were included for the Stage 3 modification testing.

2.4.3 Stage 3 modification: optimisation of LEC maintenance

Since high FGF2 concentrations are known to differentiate LECs into LFCs\textsuperscript{117,162,163}, Stage 3 concentrations of FGF2 were decreased from 100 ng/mL to 20 ng/mL commencing at day 18 (Figure 2.10A). Stage 3 modification saw a decreased expression of PAX6 by day 26 (Figure 2.10B). Additionally, expression of CRYAB was maintained by comparison to Stage 1 modifications (Figure 2.10C) suggesting maintenance of LECs. Furthermore, early LFC marker CRYBB3 expression was decreased by day 26 (Figure 2.10D), and lentoid production was also significantly decreased (Figure 2.10E,F), however, mixed cell morphologies were observed within culture (Figure 2.10F-H).
Figure 2.7 Stage 2 modifications with DKK1 did not increase LEC production.

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 2 modification DKK1 at concentrations of 3 ng/mL and 30 ng/mL (purple, violet respectively). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed no increased expression of LEC marker CRYAB. D) Real-time PCR assessment showed a decrease of the LFC marker CRYBB3. E) Fewer lentoids were produced.
Figure 2.8 Stage 2 modifications with DKK3 did not increase LEC production:

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 2 modification DKK3 at concentrations of 3 ng/mL and 30 ng/mL (blue, orange respectively). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed no increased expression of LEC marker CRYAB. D) Real-time PCR assessment similar expression of the LFC marker CRYBB3. E) Fewer lentoids were produced.
Figure 2.9 Stage 2 modifications with DKK4 did not increase LEC production. A) Schematic diagram of the Stage 1 modified differentiation protocol with the additional Stage 2 modification DKK3 at concentrations of 3 ng/mL and 30 ng/mL (green, blue respectively). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Real-time PCR assessment showed a decrease expression of LEC marker CRYAB. D) Real-time PCR showed a decreased expression of LFC marker CRYBB3. E) Fewer lentoids were produced.
Figure 2.10 Stage 3 modification of low FGF2 increased LEC production.

A) Schematic diagram of the Stage 1 modified differentiation protocol (red) with the additional Stage 3 modification of 20nm/ml FGF2 (green). B) Real-time PCR assessment showed a decrease of the neuroectoderm marker PAX6. C) Expression of the LEC marker CRYAB remained unchanged compared to the Stage 1-only modification, where p>0.4 at D26 and p>0.2 at D35. D) Expression of early fibre cell marker CRYBB3 was reduced at day 26 by comparison to Stage 1-only modification (p=0.08). E) The Stage 3 modification decreased lentoid production compared to the Stage 1-only modification as desired, this was statistically significant at day 26 (p=0.036). F-H) Light micrographs showing mixed morphologies of cells within low
A) SMAD inhibitor
↑ Noggin
hPSCs → Neuroectoderm → Lens placode → Lens cells
Day 0-5  Day 5-18  Day 18+

B) PAX6 Relative Expression

C) CRYAB Relative Expression

D) CRYBB3 Relative Expression

E) Lentoid Counts

F) 100 μm

G) 100 μm

H) 100 μm
2.5 DISCUSSION

The strategy employed by Yang et al\textsuperscript{127} was to choose testable growth factors known to be involved in embryonic lens development. For this reason noggin was used as a way of inhibiting BMP signalling and encouraging ectoderm development. Furthermore to encourage the development of lens progenitor cells and avoid neural fate BMP4 and BMP7 were used. FGF2 was used at multiple stages, as FGF signalling has been shown to be involved throughout lens development\textsuperscript{117}. WNT3A was used due to its involvement in the canonical Wnt signalling pathway, demonstrated to be essential to the formation of lens epithelium\textsuperscript{145}. Utilising this same method of trial and error growth factor testing based on proposed regulatory pathways involved during stages of embryonic development, testable hypotheses were generated and assessed within the appropriate stages of the Yang protocol. Furthermore, a select set of markers utilised in the Yang protocol (PAX6, CRYAB and CRYBB) were additionally employed here to allow comparisons to be drawn between the reported method and any modifications made in this study.

Chambers et al.\textsuperscript{149} previously documented that over 80\% of human ES cells in culture could be converted to neuroectoderm in the presence of high noggin and SB431542 compared to <10\% when used on their own. This finding motivated Stage 1 modifications. Use of high noggin and SB43154 at Stage 1 saw an increase in neuroectodermal marker PAX6 and a statistically significant increase in LEC marker CRYAB. CRYBB3 expression also increased in parallel with the statistically significant increase in lentoid production. When included in Stage 1, these modifications increased the production of lens development markers as well as
lentoids and suggested a more efficient production of LECs most likely as a result of a greater starting population of ectodermal cells entering into Stage 2.

In the published 3-stage growth factor method, BMP and FGF signalling was used to stimulate differentiation of neuroectoderm into lens placode cells. An additional signalling event suspected to be involved in lens placode formation in vivo includes inhibition of Wnt signalling\textsuperscript{150, 164, 165}. In an attempt to replicate this in vitro, two divisions of Wnt antagonists SFRPs and DKKs were implemented. SFRPs have been detected during mouse lens morphogenesis, for example Sfrp2 has been shown to be restricted to the lens placode, pit and the presumptive epithelium\textsuperscript{166}. Dkks have been found to be expressed primarily in the lens epithelium from the early lens vesicle stages through to the postnatal stages\textsuperscript{167, 168}. DKK1 is one such protein that has been shown to be critical for lens separation from the surface ectoderm via β-catenin mediated Pdgfra and E-cadherin expression\textsuperscript{169}. While literature supported the use of these growth factors to optimise lens placode production, in this stem cell condition, testing of all DKKs and SFRPs did not improve lens placode production. This was demonstrated by a reduced PAX6 expression and/or decreased expression of CRYAB expression and reduced production of lentoids.

While BMPs play multiple roles in development they are essential for lens formation although the mechanisms surrounding this are incompletely understood\textsuperscript{141, 142, 170-173}. BMP4 and BMP7 members of this family have demonstrated their importance to lens formation in both knockout studies\textsuperscript{141, 174} as well as human ES lens differentiation protocols\textsuperscript{127}. Previous studies have focused on identifying the downstream effectors of BMP signalling and how their functions in ectoderm effect lens development.
Activin A type 1 receptor, (Acvr1) a type 1 BMP receptor was deleted in mice, this study revealed that cell proliferation was positively regulated by this receptor as mice developed abnormal small lens phenotypes. When both Acvr1 and Bmpr1a, an additional type 1 BMP receptor, were deleted lens formation was prevented, suggesting the potential Activin signalling involvement in lens induction via BMP signalling. This literature supported the idea that inclusion of Activin A would encourage lens placode development, however, regardless of their apparent regulatory significance in animal models of lens development, inclusion of various Activin A concentrations in Stage 2 did not appear to promote lens placode production, as shown by a reduction of PAX6 and CRYAB expression as well as the absence of lentoids.

A limitation of the published lens differentiation protocols is that the generation of LFCs cells from the in vitro derived LECs was not controlled. In the published differentiation method, the addition of WNT3A and high concentrations of FGF2 were used in the Stage 3. Wnt signalling is believed to be involved in LEC proliferation, where inactivation results in absence of LECs, conversely, constitutive activation results in their inappropriate proliferation and migration. In the present study WNT3A levels were therefore maintained in the modified Stage 3 medium. FGFs have been shown to be important at multiple stages of lens development such as the maintenance of LECs and differentiation of LFCs. Different concentrations of FGF2 have been documented to both maintain LECs in culture (low concentrations) and stimulate LFC formation (high concentrations).
Therefore, to aid maintenance of LECs and discourage their differentiation towards early LFCs, FGF2 was reduced 5-fold (20 ng/mL). Reduced FGF2 saw maintenance of the LEC marker CRYAB in addition to a fall in CRYBB3 expression by comparison to modified Stage 1 alone, demonstrating a more optimal condition to sustain LEC growth without favouring LFC production. However, lentoid production (indicative of uncontrolled LFC generation), as well as expression of the LFC marker CRYBB3 still occurred suggesting that uncontrolled LFC production was still occurring. During embryological development LECs give rise to LFCs. Since the goal of this project was to generate a monolayer of LECs to use for future drug and disease modelling such as secondary cataract, it was logical to begin by generating a pure population of LECs. In this way the derived LECs could be used to directly study the effects on these cell types and to therefore avoid diluting the effect seen due to other cell types present. Downstream applications including drug development, toxicology screens and cell therapy require pure populations of LECs that can be maintained in culture, scaled for high-throughput purpose and induced in a controlled manner to differentiate into LFCs. The ability to generate both of these cell types for full lens regeneration would be interesting for future work and is beyond the scope for this thesis. The expression of LFC markers and appearance of lentoids, indicates that neither the published Yang Stage 3 conditions nor the low FGF2 Stage 3 conditions used here are optimal for maintenance of the produced LECs without LFC differentiation.

Additional growth factors involved in maintaining LECs without inducing production of LFCs could be tested, such as epidermal growth factor (EGF)\textsuperscript{177} and platelet derived growth factor (PDGF)\textsuperscript{178}. EGF receptors have been highly localized
to the lens epithelium and their ligand EGF has been found in the human lens\textsuperscript{179, 180} and in the aqueous humor of patients undergoing intraocular surgery\textsuperscript{181}. PDGF has been shown to influence LEC proliferation in rat explant lens models\textsuperscript{178} as well as migration of human epithelial cell line HLE-B3. Additionally, as Wnt has been shown to influence differentiation toward LFC fate\textsuperscript{145, 146} levels of WNT3A could be varied to favor LEC maintenance over LFC production. However, results of the present analysis suggests that testing the ability of these growth factors to maintain pluripotent stem cell-derived LECs, would most effectively be done once conditions for establishing purified LECs are established.

An alternative initiative for future differentiation systems may also be to inhibit pathways proposed to support LFC survival. One such pathway is phosphatidylinositol 3-kinase (PI3K) signalling. PI3K activity inhibitor LY294002 resulted in the marked inhibition of lentoid formation but the continued proliferation of a monolayer of quail LECs\textsuperscript{182}. This study suggested that the PI3K survival mechanism in LFCs involves PI3K phosphorylation of GSK3 and that PI3K is therefore an important survival signal in differentiating LFCs. Therefore, one future strategy of controlling LFC fate in a culture setting may be via the inhibition of PI3k pathway, thereby allowing LEC maintenance and discouraging differentiation to LFCs.

In conclusion, the overall aim of the studies conducted within this thesis was to identify more efficient and selective methods for promoting the controlled differentiation of embryonic stem cells towards generating homogenous populations of human LECs. Stage 1 and 3 modifications have improved the production of LECs
in vitro, however, the presence of lentoids, mixed morphologies (Figure 2.11) within the cell culture as well as expression of CRYBB3 showed that the cultures still contained mixed populations of LECs, LFCs and non-lens cell types. The overall outcome of this chapter was the realisation that the trial and error approach to solving the problem of identifying effective modifications to the protocol would be very time consuming. Moreover, given the fragmentary and incomplete understanding of the factors and conditions required for the differentiation of lens cells from the embryonic ectoderm, that the trial and error approach applied would not achieve the desired outcome within a reasonable timeframe. Thus while the Stage 1 and 3 modifications represent an improvement over the currently published method, additional (as yet unknown) modifications are needed to generate pure populations of LECs for research and drug discovery. Additionally, more experiments are required to determine whether growth factors that seem to be necessary for non-human lens development are needed for human lens development. Further investigation of these avenues requires a method for producing purified human LECs, for example, via bioinformatic investigation of LEC plasma membrane proteins to identify candidate proteins for antibody-based LEC purification.
Figure 2.11 Mixed morphologies found in human ES cell lens differentiation methods. A-B) Light micrographs of Day 26 cultures following the 3-stage protocol shows heterogeneous cell populations. C-D) Light micrographs of Day 26 cultures following the modified Stage 1 protocol shows heterogeneous cell populations.
CHAPTER 3:

Identification of ELK1 as a novel lens transcription factor.
3.1 ABSTRACT

**Purpose:** A method of transcriptome analysis was developed to identify novel candidate transcription factors (TFs) involved in regulating gene expression within the ocular lens.

**Methods:** An Excel®-based macro was developed to analyse publically available human lens gene expression data obtained from the Gene Expression Omnibus (GEO). The macro output was then analysed with the bioinformatic tool PASTAA as well as David Gene Ontology (GO) to identify novel lens transcription factors. PCR and Western blotting was used to confirm lens expression of one of the predicted novel lens TFs.

**Results:** The human ocular lens GEO dataset GSE2256 was analysed using a custom built Excel® macro. In total 5387 transcripts were identified to be expressed by the lens. Of these 1301 transcripts were designated specific to lens epithelial cells (LECs), 604 were specific to lens fibre cells (LFCs) and 3482 were considered common between cell types. PASTAA-based promoter analysis generated a list of candidate transcription factors proposed to be involved in generating LEC- and LFC-specific gene expression patterns. Assessment of this list identified numerous transcription factors supported by literature to be associated with the lens, in particular BACH2. A novel lens transcription factor, ELK1, was also identified as a candidate regulator of LECs and LFCs. PCR and Western blot analysis showed ELK1 expression by FHL124 human lens cells. Gene ontology (GO) analysis of the PASTAA-predicted ELK1 targets revealed genes with previously reported associations to ELK1 in other tissues.

**Conclusions:** Biological confirmation of ELK1 expression in lens cells demonstrates the utility of this customised Excel® macro for discovery of novel gene expression
regulators. The known involvement of ELK1 within FGF and MAPK mediated signalling in other tissues suggests assessment of the role of ELK1 and its targets within the lens will further define the molecular mechanisms of lens development.
3.2 INTRODUCTION

Maintenance of proliferation of lens epithelial cells (LECs) as well as lens fibre cell (LFC) differentiation are highly regulated and complex processes, with Wnt/β-catenin, FGF, notch and TGFβ signalling pathways all having been implicated in these regulatory processes\textsuperscript{125}. The activation of each of these pathways is controlled globally via presence of specific growth factors (GFs), cofactors or inhibitors that interact via ligand mediated interactions with their specific receptor. At the nuclear level signal-regulated transcription factors (TFs) initiate transcription through their binding to specific DNA sequences or motifs within the promoters or enhancers of target genes\textsuperscript{183}. The activation of growth factor (GF) signalling pathways is further controlled via posttranslational modifications including phosphorylation and de-phosphorylation, as well as through the presence and or concentration gradient of individual growth factors, their cofactors, inhibitors and receptors\textsuperscript{184, 185}.

The crystalline lens is a unique model for differentiation studies because the lens is composed of a continuum of cell types that reaches different stages of differentiation. This includes anterior LECs exposed to aqueous fluid, equatorial LECs that have begun to differentiate into LFCs in response to exposure to vitreous fluid\textsuperscript{63}, and finally terminally differentiated LFCs found in the center of the lens. Early investigations into the GFs responsible for driving the growth and differentiation of lens cells determined that the concentrations of FGF within the ocular fluids were key\textsuperscript{106, 108-115, 186}. Exposure of LECs within cultured rat lens explants demonstrated lower concentrations of FGF2 support proliferation of LECs, and that higher concentrations of FGF2 induce migration and terminal differentiation of LFCs\textsuperscript{117, 187}.
While the need for FGF signalling in lens development and growth has been well established, little is known of the TFs that regulate FGF signalling within the lens. Identification of TFs that co-ordinate the response of LECs and LFCs to FGF and other GFs within the aqueous and vitreous fluids is an important step towards determining how the molecular mechanisms of different GF concentrations and their target pathways are integrated to produce normal lens development and growth. This information will also provide a model of GF regulation for investigation of abnormal lens development as well as normal/abnormal development of other tissues.

Bioinformatics is the application of computer science and information technology to the field of biology and medicine. In this study a bioinformatic based approach was used to provide testable molecular hypotheses, to extend upon our knowledge of signalling interactions within the lens. Publically available gene expression data can easily be accessed from repositories, such as the Gene Expression Omnibus (GEO). Analysis of these datasets by the wider science community can provide novel molecular biology insights across a range of fields. However, effective analysis of these gene expression datasets is often not possible for researchers with limited bioinformatics knowledge or experience. To overcome this limitation, an Excel®-based macro was developed to provide users with a simple alternative that offers a range of sorting and grouping capabilities familiar to most researchers (Figure 3.1A). Users download desired data from the GEO repository and simply condense it into a single spread-sheet for input into the macro; the minimum information required being a unique gene identifier e.g., Affymetrix identification numbers (ID’s) and an associated expression value (e.g., present and absent calls) from each microarray sample of interest. The macro, written in visual basic, then sorts each row to rank
genes from highest to lowest expression (or present/absent calls) across the replicate arrays of interest.

To determine whether the macro would facilitate the development of novel molecular hypotheses, published GEO datasets derived from human lens tissue were assessed using the macro and an associated pipeline of similarly simple, publically-available tools. The PASTAA Web server\textsuperscript{188} was used to identify TF binding sites within the groups of genes obtained via the Excel\textsuperscript{®} macro. PASTAA ranks genes by estimating the overall affinity of a position weight matrix (PWM) for sequence regions that are defined relative to the transcriptional start site of each gene in the submitted list. PASTAA interrogates groups of co-expressed genes (i.e., gene groups determined via the macro) and ranks their likelihood of being regulated by a previously established TF PWM. DAVID Gene Ontology (GO) analysis was used to identify common biological processes within the grouped list of genes and the predicted TF target genes. Together, this combined bioinformatics approach predicted a range of TFs to be involved in regulating gene expression in LECs and LFCs. In particular this included identification of ELK1, a known FGF-responsive transcription factor not previously associated with the lens. Analysis of RNA and protein expression profiles from the human lens cell line FHL124 showed ELK1 to be expressed.
3.3 METHODS

3.3.1 Cells and general cell culture

The FHL124 human fetal lens cell line was obtained from the American Type Culture Collection (ATCC). Approval for their use as described was obtained from the University of Western Sydney Human Research Ethics Committee. FHL124 cells were seeded on 60 mm dishes at 200,000 cells and maintained in 3 mL of 10 % FBS (fetal bovine serum) in DMEM with medium replaced every other day. Cells were passaged by exposure to the enzyme TrypLE™ (Invitrogen, Mulgrave, Australia), (7 min at 37 °C, 5 % CO₂) as it allows the generation of single cells without requiring inhibition (i.e. as opposed to trypsin).

3.3.2 Development of an Excel®-based macro

The gene expression dataset used were obtained from the GEO database as part of record GSE2256. The Excel®-based macro written in visual basic was designed to accommodate simple use and offers a range of sorting and grouping capabilities of pre-processed data, see Supplementary File 3.1 for macro code. If the macro-sorting identified a gene as "Present" (P) on all 3 microarray experiments for the LEC or cortical LFC samples (i.e., ‘3P’) then that gene was denoted as a high-confidence expressed gene and included as an expressed gene for the relevant tissue for the analyses here. If a gene was determined to be “Absent” (A) in one or more of the 3 LEC or LFC microarrays then that gene was denoted as an unexpressed gene for the relevant tissue and not included for that tissue in the analyses described here.
**3.3.3 GO analysis**

3P lists were selected and input into the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/). Level 5 GO analysis was performed using the program DAVID Bioinformatics Suite\(^1\). The selected gene list was uploaded to the ‘Functional Annotation Tool’ as Affymetrix IDs. The GO terms of these genes were output to an Excel\(^\circledast\) file for further analysis and categorisation. Due to a number of genes having more than one probe on a microarray chip, duplicate gene names were removed from the gene list of interest as well as between lists when expressed commonly to avoid biasing the GO categorisation, see Figure 3.1 for equation utilised in Microsoft Excel\(^\circledast\).

\[
=IF(ISNA(VLOOKUP(LIST1,LIST2!A:A,1,FALSE)),”NO”,”YES”)
\]

*Figure 3.1 Excel\(^\circledast\) equation used to determine duplicate genes between 3P lens lists.*

**3.3.4 PASTAA analysis and GenePaint**

PASTAA analysis was performed by converting the Official Gene ID’s for gene lists into ENSMBL ID’s using the DAVID Bioinformatics Database conversion tool. A small proportion of genes (< 5 %) did not have matching ENSMBL IDs and therefore were not converted. The ENSEMBL IDs were input for PASTAA analysis.
both 10,000 kb and +/- 400 kb upstream. Promoter regions predicted to be statistically significant were then candidates for further investigation.

### 3.3.5 RNA collection and cDNA synthesis

RNA was extracted using RNA Lysis Buffer R (Bioline, Sydney, Australia) and stored at 80°C until required. RNA was purified using the RNA mini-kit according to manufacturer’s instructions (Bioline, Sydney, Australia). RNA samples were further purified upon use utilising a DNase treatment kit according to manufacturer’s instructions (Promega, Sydney, Australia).

Each RNA sample requires 3 cDNA reactions to occur: i) RNA and Reverse Transcriptase (+RT), ii) RNA without Reverse Transcriptase (-RT) to test for genomic contamination and iii) a control without RNA with Reverse Transcriptase; a non-template control (NTC) testing for DNA contamination of PCR reagents. The first stage of cDNA production required 500 ng of purified RNA, 2 µL of Random Hexamer (Bioline) and RNase./DNase free water (variable volume) for a final volume of 15 µL in a PCR reaction tube and mixed briefly with a QikSpin Personal Centrifuge (2000 × g, 1 min). The sample was placed in the Mastercycler™ (70 °C, 5 min). The second stage of cDNA production required 6 µL 5 X Reverse Transcriptase (RT) buffer, 1.5 µL 10 mM dNTPs, 1.5 µL 40 U/ µL RNase Inhibitor and 6 µL RNase/DNase free water for a final volume of 15 µL. To +RT and NTC samples, 0.4 µL 200 U/ µL Bioscript (Bioline) was added. The samples were mixed briefly with a QikSpin Personal Centrifuge (2000 × g, 1 min) and the sample was placed in the Mastercycler™ at the following settings: cycle 1 (42 °C, 60 min), cycle 2 (70 °C, 10 min). The resulting cDNA were then used for PCR amplification testing for specific mRNA transcripts.
3.3.6 Primer design and PCR amplification of mRNA transcripts

Forward and reverse primers of specific genes were designed utilizing the Primer3 web page (http://frodo.wi.mit.edu/primer3/). Exon sequences for specific genes were obtained using the UCSC genome browser (http://genome.ucsc.edu/). A sequence from one end of the 5’ of the exon to the 3’ of the next exon was copied into Primer3 and primer design was initiated using the parameters such as 60 °C melting temperature and 80-200 base amplicon length. The resulting predicted primers were searched against the entire known human genome and transcriptome using the BLAST nucleotide web page to verify that the predicted primers were unique and did not bind anywhere close to the genome. Left and right primer sequences, respectively are as follows; ELK1: CCTGTCTGGAGGCTGAAGAG and TCTTCCGATTTCAGGTTTGG, BACH1: AGGCTGATGGAGAGCTGAAC and TTAGCAGTGTAGGCAAACTGAA, BACH2: GATCGTTCTCGGAAGCAGAC and ATCTGGAAATCGTTCTTTGG.

Each PCR reaction consisted of 5 µL of 5 X Go-Taq Flexi PCR Buffer, 1.5 µL 25 mM MgCl₂, 1 µL 10 mM dNTPs, 2 µL 12.5 µM Forward and Reverse Primers (Geneworks, Hindmarsh, Australia), 14 µL RNase/DNase free water, 0.5 µL Go-Taq Flexi and 1 µL of cDNA (+RT, -RT and NTC) to appropriate wells. The samples were then placed into the Mastercycler® using the optimal temperature profile for primers determined for all to be 60 °C.

The PCR reaction continued for 40 cycles, where cDNA was melted at high temperatures to allow for primers of interest to bind at optimal temperatures and for
the amplicon to extend. Once the PCR run was completed, the PCR products were loaded into a 2 % Agarose Gel with 5 µL of 10,000 X Gel Red Nucleic Acid (Bioline) against a 100 bp DNA ladder (4 µL of 50 µg/µL). The agarose gel was run at 100 V, 300 mA, 50 W for 40 min. The gel was then imaged on the Gel Dock Transiluminator (Vilber Lourmat) using the E-Box software. Analysis of the gel was based on the intensity of the bands visible within the gel image at the 100 bp level in the + RT lanes. PCR samples were stored at 4 °C and cDNA samples at -20 °C.

### 3.3.7 Protein collection

FHL124 cells were harvested as per the general passaging method and then centrifuged at 4 °C at 300 × g for 5 min. The supernatant was removed and the cell pellet was resuspended in 500 μL of total cell lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton-X 100, 1 mM Na Vanadate, 1 mM PMSF, 5 μg Aprotinin, 1 × Protease Inhibitor (PI), pH 7.4 and incubated on ice for 5 min with occasional vortexing. The mixture was then centrifuged at 4 °C at 300 × g for 5 min.

### 3.3.8 Protein concentration an quantification

Protein harvested was treated with a urea exchange resulting in the removal of detergents present in Total Protein Lysis Buffer and a more concentrated sample of protein. The collected protein was placed in a Millipore 3,000 molecular weight cut-off Falcon Tube (Millipore) and centrifuged (4,000 × g, 4 °C, 20 min). After the first spin, 1 mL of Urea Exchange Buffer (4 M Urea, 1 X PI) and centrifuged (4,000 × g, 4 °C, 35 min), this was repeated three times.

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Total membrane and soluble samples were measured against a bovine serum albumin standard using the EZ-Q protein quantitation assay (Molecular Probes, Eugene, Oregon). Briefly, 2 μL aliquots were blotted on filter paper in triplicate for each sample and air dried. The blot was fixed with 100 % MeOH for five min with agitation; fixing solution was discarded and the blot air dried. While protected from light, the blot was stained with EZ-Q protein stain (Molecular Probes, Eugene, Oregon) for 30 min with continuous agitation. The blot was de-stained, 3×25 s, with 10 % MeOH/7 % acetic acid, before imaging using the LAS-4000 imager and quantitation using Multi Gauge software v3.0 (FUJIFILM Corporation, Tokyo Japan).

3.3.9 Protein electrophoresis and staining

Isolated protein was diluted 1:1 with 1D sample buffer (2 % SDS, 25 mM tris (pH = 8.8), 12.5 mM Dithiothretretiol (DTT) and 5 % glycerol) having a final load of 20 μg. Prior to electrophoresis, samples were heated at 100 °C for 5 min and cooled to room temperature (approximately 5 min) before loading the required volume of protein into the Mini Protean® TGX, Long Shelf life Pre-Cast Gels (Bio-rad Laboratories). Electrophoresis was carried out in a cold room (4 °C) at 150V during migration into the stacking gel and then resolved at 90V to completion.

Following electrophoresis, gels were fixed with 10 % MeOH/ 7 % acetic acid for 1 h before staining in Neuhoff Coomassie Stain (0.1 % CBB-G250, 2 % phosphoric acid, 10 % ammonium sulfate and 20 % MeOH) for 20 h. Gels were then de-stained with 0.5 M NaCl (5 × 15 min washes) before being imaged using FLA-9000 (Fuji Film).
### 3.3.10 Western blotting

For Western transfer, proteins were transferred from Mini Protean® TGX, Long Shelf life Pre-Cast Gels (Bio-rad Laboratories) onto 0.2 μM pore-size polyvinylidene difluoride (PVDF) membrane (Merck) that had been pre-soaked in transfer buffer (25 mM Tris, 192 mM Glycine, 20 % MeOH, 0.025 % SDS). Electro-blotting of the PVDF membrane was carried out at 120V for 1 h (cool room - 4 °C). The membrane was placed in blocking buffer (1 X Phosphate buffered saline (PBS), 0.1 % Tween-20, 1 % PVP40, 5 % skim milk) for 1 h at room temperature. The blocked membrane then underwent 2 × 15 min washes with 0.1 % PBST (1 X PBS, 0.1 % Tween-20). The membrane was incubated with a 1/500 dilution of primary ELK1 mouse antibody (Sapphire Bioscience, Waterloo, Australia) in 0.1 % PBST overnight at 4 °C with constant agitation. The following day, the membrane was washed with 0.1 % PBST (2 × 15 min) before being treated with a 1/1000 dilution of Anti-Rabbit IgG Peroxidase Goat HRP (Sigma) in 0.1 % PBST for 1 h at room temperature with constant agitation. The membrane underwent 2 × 15 min washes with 0.1 % PBST followed by 1 × 15 min was with 1 X PBS. The membrane was then exposed to 1 mL of Illuminata Reagent (Millipore) for 1 min in the dark. The membrane was then imaged using the LAS-4000 by densitrometry then exported as a .tif file using Multi Gauge v3.0 (Fuji Film).

### 3.3.11 Statistical analysis

Tests for statistical significance were performed using either the single tailed two-sample T-test or single-tailed paired two sample T-test as appropriate. Statistical significance was assigned to p values < 0.05. If the results were statistically
significant the P.value was reported, if not the null hypothesis is accepted and
P.value only listed if necessary.
3.4 RESULTS

3.4.1 Comparative analyses of gene expression profiles

A macro was designed to partition gene transcripts based on their presence or absence of expression within the 22,215 genes represented by 22,283 separate probe sets (each probe set containing 10 perfect match and 10 one base pair mismatch probe sequences) of the HG_U133A microarray, with 6 of these microarrays used in the GSE2256 data. In the instance of this data set it further allowed all 6 arrays (i.e., 3 LEC microarrays, and 3 LFC microarrays) to be sorted within the one Excel® file. The macro-partitioned data identified 5387 transcripts as present (i.e., expressed) in LECs, LFCs or both. This included identification of all 2473 transcripts previously described as expressed at 2-fold or greater between the LEC and LFC samples within this dataset, thus indicating the macro was capable of accurately analysing the GEO formatted data. However, as all expressed transcripts within the LECs and LFCs were of interest for the present study to understand the signalling and transcriptional regulatory networks pertaining to all the expressed (i.e., 3P) transcripts within the LEC and LFC compartments. As a result, 2-fold cut offs in relative expression were not used here. Thus the macro sorting allowed the derivation of 3 working lists lens-expressed genes: 1) 3P in LEC samples, 2) 3P in LFC samples and 3) 3P in both tissue types (Figure 3.2B and Supplementary File 3.2).

3.4.2 PASTAA analysis to identify transcriptional regulators

The PASTAA webserver\textsuperscript{188} was used to analyse the promoters of the genes contained in the three separate 3P gene lists, to look for TFs that might participate in establishing cell type-specific expression patterns within the lens. Two separate PASTAA analyses were performed on each of the 3 macro-partitioned gene lists.
Figure 3.2 Macro facilitates the assessment of publically available gene datasets by bioinformatic tools.

A) Pipeline outlining bioinformatics approach taken. B) Lens dataset partitioned via Excel based macro, lists were allocated based on their presence (P) on all three of the array experiments in either LECs, LFCs or both to give 3P lists.
Figure 3.3. Promoter analysis of Exclusive gene lists via PASTAA reveals known transcriptional regulators of the lens. SP1, SP3, AP2, SMAD4 and BACH2 were all found in either proximal or distal analysis of 3P lens datasets. Their connection to the lens was validated via published data.
One involved interrogating a region extending 10-kb upstream from the transcription start site (distal analysis); the other interrogated a region ±400 bp on each side of the transcription start site (proximal analysis).

Predictions of known lens TFs were obtained from both the distal and proximal PASTAA analyses. This included BACH2, PAX family members, SP1, SP3, AP2 and SMAD4 (Figure 3.3). For example, PASTAA analysis of the distal 3P LFC gene list and Common gene lists both predicted a BACH2 PWM (p=0.0395, p=0.039; and see Supplementary File 3.2). Multiple PAX family members were predicted to be common between the two lens cell types (Figure 3.4), for example PAX5 PWMs were identified within PASTAA analysed 3P common (p= 0.0124), 3P LFC (p=0.00648, 0.0124) and 3P LEC (p=0.00322, 0.0105) gene lists (see Supplementary File 3.2).

Interestingly, PASTAA analysis of the 3P LEC gene list showed ELK1 PWMs to be highly ranked in the proximal analysis (p = 0.000005). PASTAA analysis of the 3P LFC gene list also predicted an ELK1 motif to be present in the proximal promoter region of the LFC genes (p = 0.00195). Analysis of the 3P common gene list predicted ELK1 motifs within the distal promoter regions of these genes (p = 0.0; 0.0304) (Figure 3.5; and Supplementary File 3.2).

3.4.3 Assessment of FHL124 lens cell line for expression of ELK1

To determine whether the in silico ELK1 predictions of ELK1 expression in lens cells are supported by ELK1 RNA and protein expression, analysis of the FHL124 cell line was performed together with an expression analysis of the known lens TF
Figure 3.4 Promoter analysis of 3P lens transcripts showing members of the Pax family. PAX5 (Red) was identified multiple times within proximal analysis of LEC, LFC and Common lists.
Figure 3.5 Promoter analysis of 3P lens gene lists via PASTAA predicted transcription factor ELK1 in all proximal analysis. ELK1 (Red) was identified multiple times within proximal analysis of LEC, LFC and Common lists.
BACH2 previously shown to be expressed mRNA in both LECs (Figure 3.6) and LFCs\textsuperscript{193}. Assessment of total RNA revealed that BACH2 was expressed (Figure 3.7A). Furthermore, a ELK1 amplicon was also detected within the FHL124 RNA (Figure 3.7A). Total protein from FHL124 cells was then assessed via Western blot and a commercial anti-ELK1 antibody. A distinct protein band was observed within the Western blot at the predicted molecular weight of 62 kDa (Figure 3.7B).

3.4.4 GO analysis of TF targets

To broadly determine the types of lens-expressed genes predicted to be regulated by BACH2 and ELK1, the predicted distal PASTAA targets for BACH2 (derived from 3P LFC and Common lists) as well as the predicted proximal PASTAA targets for ELK1 (derived from all 3P lists) TFs were submitted to DAVID bioinformatics suit enabling the statistically significant ($p<0.05$) clustering of all gene targets based on known biological processes (see Supplementary File 3.3). GO categories with relevance to lens biology were identified for the BACH2 target genes from the 3P Common gene list including: protein folding (GO:0006457), regulation of apoptosis (GO:0042981), cellular protein metabolic process (GO:0044267). GO categories identified for the BACH2 targets from the 3P LFC list included: regulation of cell morphogenesis involved in differentiation (GO:0000904), and positive regulation of cell migration (GO:0030335). Individual genes identified within these GO categories known to have relationship to the lens included; CRYAB, NQO1, FGFR1, CDH4, WNT7A and TGF-B1 (Table 3.1 and Supplementary File 3.3).
Figure 3.6 BACH2 is expressed within the LECs of the embryonic mouse lens. GenePaint in situ hybridisation of mouse embryo E14.5 indicates BACH2 localisation within the LECs, indicated by black arrow.
Figure 3.7 Biological validation of ELK1 predictions in human foetal lens cell line FHL124.

A) PCR reveals expression of ELK1, BACH2 in FHL124 lens cells, -Rt refers to no reverse transcriptase control. B) Analysis of FHL124 total protein shows presence of ELK1 protein at a molecular weight of 62 kDa. Key: M (Marker), SDS (SDS polyacrylamide gel electrophoresis), W (Western blot).
Table 3.1 Gene Ontology analysis of BACH2 targets

<table>
<thead>
<tr>
<th>Targets</th>
<th>Biologically relevant GO Categories:</th>
<th>Gene of interest:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common</td>
<td>Protein folding</td>
<td>Crystallin, alpha B (CRYAB)</td>
</tr>
<tr>
<td></td>
<td>Regulation of apoptosis</td>
<td>NAD(P)H dehydrogenase, quinone 1 (NQO1)</td>
</tr>
<tr>
<td></td>
<td>Regulation of programmed cell death</td>
<td>Fibroblast growth factor receptor 1 (FGFR1)</td>
</tr>
<tr>
<td></td>
<td>Cellular protein metabolic process</td>
<td>cadherin 4, type 1, R-cadherin (CDH4)</td>
</tr>
<tr>
<td>LFC</td>
<td>Regulation of cell morphogenesis</td>
<td>Wingless-type MMTV integration site family, member 7A (WNT7A)</td>
</tr>
<tr>
<td></td>
<td>involved in differentiation</td>
<td>Positive regulation of cell migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transforming growth factor, beta 1 (TGFβ1)</td>
</tr>
</tbody>
</table>
GO categories for the predicted ELK1 targets obtained from the 3P LEC proximal analysis included: positive regulation of programmed cell death (GO:0043068), regulation of mitosis (GO:0007088), regulation of signal transduction (GO:0009966), negative regulation of cell cycle process (GO:0045786), and regulation of apoptosis (GO:0042981). GO categories for the predicted ELK1 targets obtained from the 3P LFC proximal analysis included: regulation of cell morphogenesis involved in differentiation (GO:0000904), positive regulation of cell migration (GO:0030335), and positive regulation of cell motion (GO:0006928). GO categories for the predicted ELK1 targets obtained from the 3P Common proximal analysis included: regulation of cell morphogenesis involved in differentiation (GO:0000904), positive regulation of cell migration (GO:0030335), and positive regulation of cell motion (GO:0006928) (Supplementary File 3.2). Analysis of individual genes within these GO categories identified genes known to be involved in lens biology such as: GP1, EGF, BMP7, SMAD3, GAS1, sFRP1, ACVR1, WNT7A and TGFβ1 (Table 3.2 and Supplementary File 3.3).
### Table 3.2 Gene Ontology analysis of ELK1 targets

<table>
<thead>
<tr>
<th>Targets</th>
<th>Biologically relevant GO Categories:</th>
<th>Gene of interest (Gene Symbol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LEC</strong></td>
<td>Positive regulation of programmed cell death</td>
<td>Glutathione peroxidase 1 (GP1)</td>
</tr>
<tr>
<td></td>
<td>Regulation of mitosis</td>
<td>Epidermal growth factor (EGF)</td>
</tr>
<tr>
<td></td>
<td>Regulation of signal transduction</td>
<td>Bone morphogenetic protein 7 (BMP7)</td>
</tr>
<tr>
<td></td>
<td>Negative regulation of cell cycle process</td>
<td>SMAD family member 3 (SMAD3)</td>
</tr>
<tr>
<td></td>
<td>Regulation of apoptosis</td>
<td>Growth arrest-specific 1 (GAS1) Secreted frizzled-related protein 1 (SFRP1)</td>
</tr>
<tr>
<td><strong>Common</strong></td>
<td>Cellular protein metabolic process</td>
<td>Activin A receptor, type I (ACVR1)</td>
</tr>
<tr>
<td><strong>LFC</strong></td>
<td>Regulation of cell morphogenesis involved in differentiation</td>
<td>Cadherin 4, type 1, R-cadherin (CDH4) Wingless-type MMTV integration site family, member 7A (WNT7A)</td>
</tr>
<tr>
<td></td>
<td>Positive regulation of cell migration</td>
<td>Transforming growth factor, beta 1 (TGFB1)</td>
</tr>
<tr>
<td></td>
<td>Positive regulation of cell motion</td>
<td></td>
</tr>
</tbody>
</table>
3.5 DISCUSSION

The primary interest of this research was to generate new leads about potential regulatory mechanisms involved in the lens from existing GEO lens microarray data. To facilitate this discovery an Excel® based macro was designed to allow the generation of three relevant gene lists (3P LEC, 3P LFC and 3P Common) that were then analysed via an array of publically available bioinformatic tools. Excel® has been reported to introduce gene name errors that have the potential to contaminate bioinformatic results\textsuperscript{194}. These limitations were known prior to analysis, official gene symbols such as DEC1 or SEPT1 were affected and converted to date format. To overcome this the analysis in this study included the use of multiple gene identifiers such as full gene names, Affymetrix ID’s and ENSEMBL ID,s that are not affected by this formatting function in Excel®. As a result, the pipeline of methods used, both individually and in combination, consistently resulted in the identification of genes known from other studies to be involved in the regulation of cell signalling within the lens. For example, the identification of known lens TFs whose identification here provided positive controls for the identification of lens-relevant TFs. These positive control TFs included; SMAD4, SP1, SP3, AP2, and BACH2, thus validating the approach taken. For example, Smad4 is required for the development and maintenance of the lens in mice\textsuperscript{195}, and studies using DNase I footprinting analysis have found that purified Sp1, Sp3 and AP2 transcription factors interact with several domains of the human LFC major intrinsic protein (MIP) promoter sequence and therefore may be involved in regulation of its transcription in the lens\textsuperscript{196-198}. Furthermore, previous studies have shown expression of BACH2 mRNA within LECs of E14.5 mouse embryos, additionally, immunohistochemical studies using
affinity-purified anti-Bach2 antibodies in E12.5 mouse embryos have localised Bach2 protein expression within the cytoplasm of LFCs193.

The present analysis predicted BACH2 to be involved in regulating the expression of the distal 3P LFC and 3P Common lists further validating that the approach used in this study provides biologically relevant insights. FHL124 are a foetal immortalised LEC cell line that have been characterised to retain 99.5% homology when compared to primary human LECs after multiple passages. Furthermore, this cell line maintains phenotypic LEC characteristics such as the expression of CRYAA, PAX6 and FOXE3199. As previous studies confirm BACH2 expression within foetal LECs this sample was assessed. Further validation of this approach is supported by the identification of PAX-family TF binding sites within the macro-sorted gene lists. For instance, it is known that the PWM-based motif identification method used by PASTAA allows for the identification of PWMs related to particular TF families but may not always distinguish between specific members of TF factor families that bind to the same sequence200. For example, in this analysis multiple PAX family PWMs were predicted including PAX5 within the distal analysis of all 3P lens gene lists. While PAX6 was not predicted here, it is known to share highly similar DNA-binding PWM and therefore both are highly likely to bind to this PWM201.

Here in this study promoter analyses predicted ELK1 to be involved in regulation of genes within LECs and LFCs. RNA and Western blot analysis of the human LEC line FHL124 confirmed the presence of ELK1 RNA transcripts and protein with molecular mass consistent with the reported molecular mass of ELK1. Further analysis of
published Gene expression profiles of postnatal day 13 mice lenses shows that Elk1 is expressed in both the epithelial and fibre cell types of the lens\textsuperscript{202}. Therefore ELK1 is likely expressed in both subpopulations of the lens as supported by PCR and western blot analysis and published mouse lens data\textsuperscript{202}.

The predicted ELK1 LEC and LFC targets were then grouped by GO analysis in order to identify biological processes required by the lens, such as apoptosis, mitosis, cell cycle and signal transduction. LFC-specific biological processes investigated related to those involved in differentiation and cell migration. Common biological processes included any GO terms identified as any biological processes involved in the lens i.e. those already mentioned for LECs and LFCs. Interestingly, within these groupings individual target genes contained were identified that are known to be regulated by ELK1 in other tissues, as well as genes known to be involved in lens development but not previously reported as ELK1 targets. Previously reported ELK1 target genes identified here within the LFCs included TGFβ3, and AGT. Within LEC the previously reported ELK1 target IQGAP1 was identified. TGFβ3 has been shown to regulate anchoring junction dynamics in the seminiferous epithelium of the rat testis via the Ras/ERK signalling pathway in which ELK1 was implicated\textsuperscript{203}. AGT, an angiotensin precursor, once cleaved by the enzyme angiotensin-converting enzyme it is converted to angiotensin II which has been shown to activate the transcription factors EGR1, ELK1, JUN and FOS in human adrenocortical cells\textsuperscript{204}. Lastly, ELK1 has been implicated in the control of a network of actin/migration-related genes of which IQGAP1 was named\textsuperscript{205}. All the other targets with no previous links to ELK1 thus may play a novel role in ELK1 mediated
regulation within the lens, and their investigation could provide useful information for comparison of ELK1-mediated gene expression regulation in other tissues.

ELK1 targets identified here that have documented links with LECs include: EGF/EGFR, SFRP1, GAS1 and BMP7. EGF and EGFR\textsuperscript{206} are expressed in the native human lens epithelium\textsuperscript{177, 207} throughout life, and signalling via EGF/EGFR is associated with capsule opacification\textsuperscript{180, 208}. Furthermore, SFRP1\textsuperscript{209} has been shown to be required for maintenance of Wnt/β-catenin signalling in LECs. GAS1\textsuperscript{210} has been proposed to play a role in proliferation, where mouse Gas1 mutant eyes are small and contain minimal or no structures resembling the lens. Finally, BMP7 has been shown to be involved in the development of lens placode in both mouse and human embryonic stem cell-derived lens cells\textsuperscript{127}. All these genes were identified by GO analysis of the 3P LEC ELK1 target genes. An ELK1 target identified as being common to both LECs and LFCs included ACVR1, a bone morphogenetic protein receptor which when knocked out in mouse results in lenses with abnormal proliferation and cell death in epithelial and cortical fibre cells\textsuperscript{175, 176}. LFC associated ELK1 targets included CDH4, TGFβ1 and WNT7A. CDH4 has been demonstrated to be expressed within the central LFCs and in the newly differentiated LFCs of the adult zebrafish. TGFβ1 has been proposed to induce epithelial-mesenchymal transition in the lens, a process suspected to be involved in cataract development\textsuperscript{211}. While none of these ELK1 predicted targets have been reported to be regulated by ELK1 in other tissues, their known role in normal or pathological lens biology makes the role of ELK1 in regulating their expression within the lens worthy of further investigation. Interestingly WNT7A has both a reported link to the lens and ELK1. In chicken lenses Wnt7ahas been shown to be expressed by differentiating
LFCs and comparative genomics of Wnt7a previously identified conserved ELK1 binding sites within exon 1 among mammalian Wnt7a promoters. These studies further validate the strategy used in this study to identify novel lens biology.

BACH2 a previously identified lens related TF was additionally identified by this study though no established lens regulatory mechanism has been reported BACH2 targets. Interestingly TGFβ1, WNT7A, CRYAB, FGFR1 and CDH4 were identified by this method of analysis. The role of BACH2 in regulating the expression of these genes is also worthy of further investigation.

Maintenance of the lens architecture and its distinctive polarity are essential for normal function. Development and preservation of these features is achieved by the surrounding ocular fluids where the aqueous humor bathes the anterior LEC monolayer and the vitreous humor the posterior LFCs. Characterisation of the responsible factors within ocular fluids has identified members of several growth factor families including, IGF, FGF, PDGF, EGF, HGF, and VEGF. Of these FGF has been shown to independently induce LFC differentiation in rat lens epithelial explants as seen by the accumulation of known LFC features including cell elongation, the loss of cytoplasmic organelles, de-nucleation, and the accumulation of fibre specific CRYB and CRYG. Furthermore, the effect of FGF has been demonstrated to be dose dependent, where LEC proliferation was induced by low doses and high doses resulted in differentiation to LFCs.
While FGF involvement in the lens has been widely established, TFs that regulate the response of lens cell types to FGF within the aqueous and vitreous fluids are insufficiently defined. However, FGF has previously been established to be involved in the regulation of the MAPK pathway in the lens, a cascade that ends with extracellular regulated kinases 1/2 (ERK1/2). From other systems, ERKs are known to phosphorylate nuclear TFs comprising the AP-1, ETS and MAF families. The TF ELK1 belongs to the ETS family that mediates serum-induced expression of a class of immediate early genes including c-fos and egr-1. Although ETS families have been suggested as plausible downstream targets of the MAPK signalling pathways in the lens, ELK1 has not been specifically mentioned or investigated.

Like insulin and IGF-1, FGF has been shown to induce activation of ERK in both rat and chick whole lens cultures. ERK1/2 signalling is essential for both lens cell proliferation and differentiation where both low and high doses of FGF stimulate ERK activation in rat LECs, with higher doses of FGF stimulating greater levels of ERK activation. Furthermore, the duration of ERK1/2 activation has been shown to determine whether LECs proliferate or undergo differentiation, however, the exact mechanisms regulating and responding to these processes is not known. In non-lens cell types it has been proposed that the duration of ERK1/2 activation may regulate stimulation of downstream targets of ERK1/2, such as ELK1. For example, sustained ERK1/2 activation induced by nerve growth factor results in PC12 cell differentiation, and this is postulated to be due to its greater stimulatory effect on ELK1. By contrast, EGF induced PC12 cell proliferation is believed to result from a weaker effect of ELK1 due to the transient phosphorylation of ERK1/2. A direct link between ELK1 and FGF
signaling has additionally been identified using rat hippocampal neuronal H19-7 cells, here FGF was shown to stimulate phosphorylation of Elk1\textsuperscript{231}. Therefore, while no association between the lens and ELK1 has been reported, links between signalling in addition to ELK1 and ERK1/2. Therefore, ELK1 may play likely role with FGF signalling in the lens.

In conclusion, this study used bioinformatics (consisting of gene expression partitioning and promoter analyses), together with Western blotting, to identify ELK1 as a novel lens TF. The data that supports its potential involvement in lens gene expression has provided novel clues into the molecular mechanism of this TF in the lens. This research demonstrates the utility of the Excel\textsuperscript{®} macro to enable non-bioinformaticians to discover new candidate regulators of gene expression within the lens or other tissues. Future research is required to understand if FGF mediated signalling in the lens effects ELK1 expression and/or activity, if downstream ERK1/2 kinases are involved in ELK1 regulation, and if ELK1 is involved in cataract or PCO. Since mouse Elk1 knockouts exist future immunohistochemical and in situ hybridization experiments comparing normal mouse models would be beneficial to further discovery, but were not plausible for the time frame of this thesis. Further investigation of the role of BACH2 in lens development is also worthwhile.
CHAPTER 4:

Large-scale production of purified human lens epithelial cells for anti-cataract drug screening
4.1 ABSTRACT

Purpose: The development of new cataract preventions or treatments requires a scalable source of human lens epithelial cells (LECs). Human pluripotent (PS) stem cells cell-derived lens cultures are one such source, however, current differentiation protocols produce heterogeneous cell populations of lens and non-lens cells together with uncontrolled production of LECs and lens fibre cells (LFCs).

Methods: An Excel®-based macro was developed to analyse publically available gene expression data. The macro was then applied to a published human lens gene expression dataset to generate a list of expressed LEC surface proteins for subsequent testing of LEC purification via magnetic activated cell sorting (MACS). This list was shortened to include only those genes with relatively restricted expression to LECs, by first analyzing the genes within the list for embryonic tissue specificity within E14 mouse embryos via the GenePaint in situ hybridization webserver.

Results: The Excel®-macro facilitated the identification of 1052 candidate surface proteins expressed by lens cells; of these 119 were receptors. Manual assessment of these receptors via GenePaint identified only 2 as being expressed within the lens epithelium of E14 mouse embryos (ROR1 and GPR161). These two genes were shown to be expressed, via PCR, within FHL124 cells as well as human embryonic stem (ES) cell cultures differentiated towards lens cells. Use of commercially-available anti-human ROR1 antibodies together with magnetic activated cell sorting (MACS) was shown to enable the purification of LECs from cultures of differentiating human ES cells. ROR1 positive cells showed expression of LEC specific transcripts at both the RNA and
protein level. Treatment of ROR1 cultured cells with hydrogen peroxide (H₂O₂) was shown to be highly toxic to these purified human LECs.

**Conclusions:** This study resulted in the identification of a simple, robust and scalable LEC purification method. The ability of ROR1 to selectively target LECs has future application to large-scale production of purified human LECs for developmental biology investigations. These purified human LECs also have the potential to enable development of lens toxicology screening assays and anti-cataract drug screening assays.
4.2 INTRODUCTION

The ability of human embryonic stem (ES) cells to self-renew and to be differentiated into cells from all three embryonic germ layers highlights them as a promising source of cells for research and drug discovery applications. For the lens, a critical step towards the development of anti-cataract drug screening assays is the development of a scalable human lens epithelial cell (LEC) culture system. A recent publication reported a 3-stage growth factor treatment that differentiates human ES cells into heterogeneous populations of human lens epithelial progenitor-like cells, 3-dimensional lens-like structures termed “lentoids”, and non-lens cells. One of the major hurdles related to the in vitro generation of purified human ES cell-derived LEC is the generation of undesired non-lens cells, as well as spontaneous production of lentoids\textsuperscript{127-129}. These lentoids comprise a mixture of lens fibre cells (LFCs) and LECs that do not correctly mirror the complex architecture of the lens, namely, a monolayer of anterior epithelial cells overlying a compacted mass of parallel-aligned, elongated LFCs\textsuperscript{2}. Therefore a new method that generates purified LECs free of LFCs and non-lens cells is required. Optimally, this method should enable simple and robust purification of large numbers of purified human LECs that can be maintained in culture and directed to form human LFCs in a controlled manner.

One isolation technique used in many laboratories to enable cell purification is flow cytometry via a fluorescent activated cell sorting (FACS). A recent publication reported the application of FACS to human derived LEC cultures\textsuperscript{129}. This method was complex in nature requiring cells to be cultured for up to 30 days before being processed by multiple-laser sorting. This technique requires time-consuming staining, expensive reagents/equipment and the need for a trained operator.
Furthermore a low yield of purified LECs was reported, with only 0.2 to 1.5% of the total cells positive for the used LEC associated markers (c-Met/CD44) being captured, and non-lens cell morphologies in the purified cell populations were seen. These limitations suggest this technique is not scalable for downstream high-throughput applications.

In comparison to multi-laser flow cytometry, a simple and cost effective approach to cell purification involves the use of magnetic activated cell sorting (MACS). For the present study, in order to use MACS a LEC specific surface marker was required. Central to this approach was the use of bioinformatics and publically-available gene expression resources to identify two cell surface receptors with lens-specific protein expression, as well as the identification of commercially-available antibodies that enabled application of MACS for LEC purification. This work therefore represents the first report of a simple, robust and efficient LEC purification method. This discovery paves the way for establishment of human ES cell-derived LECs as an important and highly relevant tool for lens and cataract research, lens toxicity screening and anti-cataract drug screening.
4.3 METHODS

4.3.1 Cells

CA1 human ES cells were obtained from Prof. Andras Nagy of The University of Toronto, Canada\textsuperscript{156}. The FHL124 human fetal lens cell line was obtained from the American Type Culture Collection (ATCC). Approval for their use as described was obtained from the University of Western Sydney Human Research Ethics Committee.

4.3.2 Reagents and consumables

Reagents used for cell culture including mTeSR1\textsuperscript{™}, Dulbecco’s Modified Eagles Medium (DMEM), Dulbecco’s Modified Eagles Medium: Nutrient Mixture F-12 (DMEM:F12); as well as dispase and trypan blue were acquired from StemCell Technologies (Melbourne, Australia). Reagents used in cell harvesting and antibody staining, including TryPLE\textsuperscript{™} Express and Dulbecco’s Phosphate Buffered Saline - Ca/Mg (-PBS), were obtained from Invitrogen Corporation (Mulgrave, Australia) unless stated otherwise. Matrigel\textsuperscript{TM} required to coat tissue culture plates for cell adherence was acquired from BD Biosciences (North Ryde, Australia). All tissue culture plates and pipette tips used for general cell culture were acquired from Greiner-Bio-one (Frickenhausen, Germany).

4.3.3 General FHL124 cell culture

FHL124 cells were passaged every 7 days using TryPLE\textsuperscript{™}, whereby 3 mL was added to each 60 mm dish and incubated (7 min at 37 °C, 5 % CO\textsubscript{2}). The cells were collected and placed into a 15 mL tube; 3 mL of -PBS was used to wash each plate; this was then collected into the same 15 mL tube. The cells were centrifuged at (300
× g, 5 min), the supernatant discarded, and the cell pellet re-suspended in 1 mL of 10 % fetal bovine serum in DMEM. Cell counts were performed by placing 10 μL of single cells into a well of a 96-well tissue culture plate containing 40 μL of 0.4 % (w/v) trypan blue. The mixture of cell sample and trypan blue was then placed into a hemocytometer (Bright-Line; Hausser Scientific, Pennsylvania, USA) and a cell count was performed using a light microscope. Cells were then seeded on 60 mm dishes at 2 × 10^5 cells in 3 mL of 10 % fetal bovine serum in DMEM; medium was replaced every other day^{189}.

4.3.4 Human ES cell maintenance, cell harvest and differentiation

CA1 human ES cells were maintained and cultured using the defined, feeder cell-free medium mTeSR1™^{157}. Human ES cells were passaged every 7 days using 1 mg/mL dispase and plated as aggregates on tissue culture dishes pre-coated with Matrigel™ (0.1 mg/mL in DMEM) for a minimum of 30 min at room temperature after which time the Matrigel™ was removed^{158}.

To prepare CA1 cells as single cells, maintenance plates were incubated with 10 μM Rho-kinase inhibitor (ROCK inhibitor) (Merck, Kilsyth, Australia) in mTeSR1™ for a minimum of 1 h at 37 °C, 5 % CO₂^{159}. Single cell suspensions were generated using the enzyme TrypLE™ (7 min at 37 °C, 5 % CO₂). The cells were collected and placed into a 15 mL tube; 3 mL of -PBS was used to wash each plate; this was then collected into the same 15 mL tube. The cells were centrifuged at (300 × g, 5 min), the supernatant discarded, and the cell pellet re-suspended in 1 mL mTeSR1™ containing 10 μM ROCK inhibitor. Cell counts were performed as mentioned above for FHL124 cells.
For differentiation, 2 × 10^5 cells were re-plated in a Matrigel™ coated 6-well tissue culture plate containing 2 mL of mTeSR1™ with 10 μM ROCK inhibitor. The mTeSR1™ medium was replaced the following day without ROCK inhibitor and every day after for 4 days. Once cells were confluent and a clear monolayer of cells was visible growth factor treatment began (Table 4.1). Briefly, 2 mL of Stage 1 medium containing noggin (R&D Systems, Minneapolis, USA) was added to each 35 mm well and refreshed every other day up until day 5. At day 5, each well was washed with 2 mL of room temperature -PBS, followed by addition of 2 mL Stage 2 medium containing FGF2, BMP4 and BMP7 (all, R&D Systems) added per 35 mm well. The medium was changed every other day up till day 18.

### 4.3.5 RNA collection and cDNA synthesis via reverse transcription

RNA was extracted using RNA Lysis Buffer R (Bioline, Sydney, Australia) and stored at -80 °C until required. RNA was purified using the RNA mini-kit according to the manufacturer’s instructions (Bioline, Sydney, Australia). Genomic DNA was removed through the use of a DNase treatment kit according to the manufacturer’s instructions (Promega, Sydney, Australia).

For each RNA sample 3 cDNA reactions were performed: i) RNA and Reverse Transcriptase (+RT), ii) RNA without Reverse Transcriptase (-RT) to test for genomic contamination, and iii) no RNA, with reverse transcriptase as a non-template control (NTC) to test for DNA contamination. The first stage of cDNA production required 500 ng of purified RNA, 2 µL of Random Hexamer (Bioline) and RNase/DNase free water for a final volume of 15 µL in a PCR reaction tube and
Table 4.1: Stage 1-2 of the Published 3-stage differentiation medium and their components

<table>
<thead>
<tr>
<th>Differentiation medium</th>
<th>Medium Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1: Neuroectoderm induction medium</td>
<td>Noggin (100ng/mL)</td>
</tr>
<tr>
<td></td>
<td>N2/B27 supplement</td>
</tr>
<tr>
<td></td>
<td>DMEM:F12</td>
</tr>
<tr>
<td>Stage 2: Lens placode induction</td>
<td>BMP4 (20ng/mL)</td>
</tr>
<tr>
<td></td>
<td>BMP7 (20ng/mL)</td>
</tr>
<tr>
<td></td>
<td>FGF2 (100ng/mL)</td>
</tr>
<tr>
<td></td>
<td>DMEM:F12</td>
</tr>
</tbody>
</table>

*final growth factor concentrations shown, all growth factor from R&D Systems (Minneapolis, USA)
mixed briefly with a QikSpin Personal Centrifuge (2000 × g, 1 min; Edwards, Sydney, Australia). The sample was placed in the Mastercycler™ (70 °C, 5 min; Stratagene, La Jolla, CA). The second stage of cDNA production required 6 µL 5 X Reverse Transcriptase (RT) buffer, 1.5 µL 10 mM dNTPs, 1.5 µL 40 U/µL RNase inhibitor and 6 µL RNase/DNase free water for a final volume of 15 µL. To RT and NTC samples, 0.4 µL 200 U/µL Bioscript (Bioline) was added. The samples were mixed briefly with a QikSpin Personal Centrifuge (2000 × g, 1 min) and placed in the Mastercycler™ at the following settings: cycle 1 (42 °C, 60 min), cycle 2 (70 °C, 10 min). The resulting cDNA were then used for PCR amplification testing for specific mRNA transcripts.

4.3.6 Primer design

Forward and reverse primers for specific genes were designed utilising the Primer3 web page (http://frodo.wi.mit.edu/primer3/). Exon sequences for specific genes were obtained using the UCSC genome browser (http://genome.ucsc.edu/). For each gene the exon sequence from the 3’ end of one exon to the 5’ end of the next exon was copied into Primer3 and primer design was initiated using the parameters 60°C melting temperature and 80 - 200 base amplicon length. The resulting predicted primers were searched against the entire known human genome and transcriptome using the BLAST nucleotide web page to determine whether predicted primers bound anywhere else in the genome; only primers uniquely complementary to the gene of interest were used (Table 4.2).
Table 4.2 List of primers used to assess lens differentiation strategies

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROR1</td>
<td>GCACATGCAAGGGAAATAG</td>
<td>AAGGAATGGCGAACTGAGAA</td>
</tr>
<tr>
<td>GPR161</td>
<td>GCCCTCCTCTACCTGCTGAT</td>
<td>CGGTTCCCTGTGATCTTCAT</td>
</tr>
</tbody>
</table>
4.3.7 PCR amplification of mRNA transcripts

Each PCR primer (Geneworks, Hindmarsh, Australia) was tested with a trial PCR reaction at temperatures between 55 – 60 °C to determine their optimal PCR temperature. Each PCR reaction consisted of 5 µL of 5 X Go-Taq Flexi PCR Buffer, 1.5 µL 25 mM MgCl₂, 1 µL 10 mM dNTPs, 2 µL 12.5 µM Forward and Reverse Primers, 14 µL RNase/DNase free water, 0.5 µL Go-Taq Flexi and 1 µL of cDNA (+RT, -RT and NTC) to appropriate wells. The samples were placed into the Mastercycler® using the appropriate primer temperature. The PCR reaction was run for 40 cycles. Once the PCR run was complete, the PCR products were loaded into a 2 % agarose gel with 5 µL of 10,000 X Gel Red nucleic acid stain (Jomar Diagnostics, Stepney, South Australia) together with a 100 bp DNA ladder (4 µL of 50 µg/µL) (Axygen Scientific, Union City, California). The agarose gel was run at 100 V, 300 mA, 50 W for 40 min. The gel was then imaged on a Gel Dock Transiluminator (Vilber Lourmat) using the E-Box software. Analysis of the gel was based on band intensity of the bands visible within the gel image at the 100 bp level in the + RT lanes. PCR samples were stored at 4 °C and cDNA samples stored at - 20 °C.

4.3.8 Development of an Excel® based macro

GSE2256 lens expression dataset was obtained from the GEO database. The Excel®-based macro written in visual basic was designed to facilitate identification of expressed genes (see Supplementary File 3.1 for macro code). To allow sorting the macro identifies genes that are “Present” (P) and “Absent” (A). If sorting resulted in a gene being "Present" (P) on all 3 microarray experiments for the LEC or cortical LFC samples (i.e., ‘3P’) then that gene was denoted as a high-confidence expressed
gene and included as an expressed gene for the relevant tissue for the analyses here. Conversely, if a gene was determined to be “Absent” (A) in one or more of the 3 LEC or LFC microarrays then that gene was denoted as an unexpressed gene for the relevant tissue and not included for that tissue in the analyses described here.

4.3.9 GO analysis

Genes having 3 present calls (3P) were selected and input into the DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/) Gene ontology (GO) analysis was performed using the program DAVID Bioinformatics Suite 191. The selected gene list was uploaded to the ‘Functional Annotation Tool’ as Affymetrix identification numbers (ID’s). The selected genes were analysed using the GO category Cellular Compartments and specific subsets of genes within the terms; integral to membrane, integral to plasma membrane and cell surface receptor linked signal transduction, were used to identify surface proteins. An additional text search for receptor was used to identify cell surface receptors within the Excel®-partitioned Gene Expression Omnibus (GEO) file as well as those identified via DAVID GO analysis. The cell surface protein and receptor gene lists were copied into an Excel® file for further analysis and categorisation.

4.3.10 Magnetic activated cell sorting

A range of commercially available antibodies were tested for LEC purification including: rabbit polyclonal GPR161 antibody (ab58679, Sapphire Bioscience, Waterloo, Australia); rabbit polyclonal ROR1 antibody (ab135669, Sapphire Bioscience); rabbit polyclonal ROR1 antibody’s (PAB3383 and PAB3384, Abnova™, Taipei City, Taiwan); goat polyclonal ROR1 antibody (AF2000, R&D
Systems). The goat polyclonal ROR1 antibody (AF2000, R&D Systems) was used in MACS and was biotinylated using a One-Step Biotinylation kit as per the manufacturer’s instructions (Miltenyi Biotech North Ryde, Australia). Briefly 100 µL of 0.1 mg/mL of ROR1 antibody was added to one vial of the biotin kit and left to incubate for 24 h at room temperature. Biotinylated antibody was stored at 4 °C for future use. Cells were collected as single cells utilising ROCK inhibitor as previously described and re-suspend in 40 µL of 0.1 mg/mL biotinylated ROR1 antibody per $3 \times 10^6$ cells. MACS protocol was followed as per manufactures instructions using anti-biotin micro beads (Miltenyi Biotech) and Miltenyi Biotech MS column protocol.

4.3.11 Flow cytometry

FACS was used to asses day 18 human ES cell differentiation cultures pre- and post-MACS. To achieve this cells were harvested as single cells as describe above. The cells were then centrifuged (300 × g, 5 min), the supernatant removed, and the residual supernatant used to re-suspend the cell pellet. To assess for expression of CRYAB protein $2 \times 10^5$ cells per sample was used; for assessment of ROR1 protein expression $1 \times 10^5$ cells was used. Cells were additionally processed specifically for each antibody, if cells were being assessed for ROR1 pre and post MACS the cells were left alive, however, intracellular staining for CRYAB required fixation by adding 2 % para-formaldehyde on ice for 15 min. After incubation, 1 mL of -PBS was added to the suspension before centrifugation (300 × g, 5 min). The supernatant was removed and the pellet re-suspended in 10 % FBS in PBS.
4.3.12 Antibody staining with ROR1 and CRYAB

The cell number required for either intracellular or extracellular staining is shown in Table 1 and was added to 1.5 mL tubes. Samples for extracellular staining were placed in the QikSpin Personal Microfuge (Edwards Instrument Co., Narellan, Australia) (2000 × g, 1 min), the supernatant was removed, and the cell pellet re-suspended in 10 % FBS in -PBS then placed on ice. Samples designated for intracellular staining were centrifuged using the QikSpin Microfuge (2000 × g, 1 min), supernatant was removed, and the cells re-suspending in 500 µL of 0.1 % saponin permeabilisation buffer; containing 0.1% saponin, 100 mL -PBS and 10 % bovine serum albumin for 15 min on ice. The intracellular samples were then placed in the QikSpin Personal Centrifuge (2000 × g, 1 min), and the supernatant removed from each sample.

For each test sample, 100 µL of diluted primary antibody was added to the cells. For extracellular antigens, ROR1 a 1:10 dilution was used (R&D Systems); for intracellular staining, 100 µL of a 1:50 dilution of CRYAB antibody was used (Sapphire Bioscience). All samples stained with primary antibody were placed in the 4 ºC fridge for 20 min to allow for antibody binding to occur. After incubation, 1 mL of 10 % FBS in -PBS was added to the extracellular stained samples and 1 mL of 0.1 % saponin was added to the intracellular stained samples. These samples were then centrifuged using the QikSpin Personal Centrifuge (2000 × g, 1 min) followed by removal of 1 mL of supernatant. A dilution of 1 in 10 secondary antibody was then added to all test and FACS-control samples, followed by incubation at 4 ºC for 20 min. The secondary antibody used for extracellular ROR1 detection was Alexa Fluor 488 anti-goat IgG (isotype Rabbit) secondary antibody (Invitrogen), while Alexa
Fluor-488 anti-rabbit IgG antibody (isotype Goat) secondary antibody (Invitrogen) was used for intracellular samples both used at 1:1000 dilutions. After incubation, 1 mL of 10 % FBS in -PBS was added to the extracellular staining samples and 1 mL of 0.1 % of saponin was added to the intracellular samples. All samples were centrifuged in the QikSpin Personal Centrifuge (2000 × g, 1 min), followed by the removal of 1 mL supernatant. For live cells an additional Propidium iodide stain was used at 1:1000 for 5 min followed by three additional rinses and spins. The volume for each sample was then made up to 300 µL by the addition of 200 µL of 10 % FBS in -PBS for extracellular samples and 200 µL of 0.1 % saponin for intracellular samples. The samples were then kept on ice until analysis.

4.3.13 Flow cytometry data acquisition and analysis

The antibody-stained samples were analysed using a MACSQuant® Analyser Flow Cytometer (Miltenyi Biotec). To enable assessment of the correct cell populations, cells were gated in accordance to specific parameters. Firstly propidium iodide vs side scatter (SSC-A) was used to ensure live cells were captured by excluding cell fragments and dead cells. To further ensure that only single cells were analysed for antigen expression two forward-scatter parameters (area and height: FSC-A vs. FSC-H) were used. Finally, FITC-H detector (488 nm) was used to detect secondary antibody fluorescence. All FACS data was analysed using FlowJo software (Tree Star, Ashland, OR, USA) as described previously.200,232
4.3.14 Culture of MACS-purified cells

ROR1+ cells were seeded at $2.1 \times 10^4$, $2.1 \times 10^5$ and $4.7 \times 10^5$ cells/cm$^2$ on tissue culture dishes pre-coated with Matrigel$^\text{TM}$ in Medium 199 (M199; Life Technologies, Mulgrave, Australia) containing 5 ng/mL FGF2 and 10 µM ROCK inhibitor. The cells were maintained at 37 °C with 5% CO$_2$ and the medium was refreshed 24 h later to remove ROCK inhibitor then changed twice weekly as necessary.

4.3.15 Toxicology assays and fluorescence imaging

ROR1+ cells were seeded at $2.1 \times 10^5$ cells/cm$^2$ and allowed to proliferate to confluence. At confluence, the cultured ROR1+ cells were harvested from 3 wells of a 24 well plate and re-plated between 6 wells, 3 days later cells were exposed to H$_2$O$_2$ at 0.2 mM in 5 ng/mL FGF2 M199 media, or control conditions of 5 ng/mL FGF2 M199 media at 37 °C 5% CO$_2$ incubator. After 3 h of exposure to these conditions cell death was visualised via light microscopy. To determine total cell numbers and % dead cells hoechst 33342 and propidium iodide were used. Hoechst 33342 was diluted to 5 µg/mL in M199 medium containing 5 ng/mL FGF2 and added to all wells to stain for 15 min at 37 °C 5% CO$_2$ incubator. Wells were rinsed with -PBS. Propidium iodide was diluted to 500 nM in M199 medium containing 5 ng/mL FGF2 and used to stain the cells for 5 min at 37 °C 5% CO$_2$. A final wash with -PBS was performed then -PBS was added to wells to allow clear imaging via fluorescent microscopy (Olympus).

4.3.16 Statistical analysis

Tests for statistical significance were performed using either the single tailed two-sample T-test or single-tailed paired two sample T-test as appropriate. Statistical
significance was assigned to p values < 0.05. If the results were statistically significant the P.value was reported, if not the null hypothesis is accepted and P.value only listed if necessary.
4.4 RESULTS

4.4.1 Defining receptors in the lens

Current human ES cell lens differentiation protocols produce heterogeneous populations of lens and non-lens cells (Supplementary Figure 2.1). To enable MACS-based purification of LECs an unbiased, transcriptomics-based approach was used to identify lens-specific cell surface proteins. Central to this method was the development of an Excel®-based macro to sort and group human lens gene expression data set (GSE2256) from publically available pre-processed gene expression datasets stored in the GEO. When applied to this dataset the macro analysed 22,275 genes and grouped these into genes detected with high confidence expression (i.e., expressed in all 3 replicate microarrays per tissue, or ‘3P’). This resulted in the identification of 1301 LEC and 604 LFC expressed genes, with 3454 genes commonly expressed by both tissues (Figure 4.1 B). GO analysis of the partitioned list further identified 1026 candidate cell surface proteins (Supplementary File 4.1). To further refine this list cell surface receptors were assessed; in total 44 receptors were found in the LEC list, 15 in LFC list and 60 were common (Figure 4.1 B).

4.4.2 Assessment of receptor locations

In situ hybridization was used to determine whether any of these receptors have lens-specific expression during embryonic development. In situ hybridization in mouse embryos was accessed via GenePaint (Max Plank laboratory, http://www.genepaint.org) to determine the embryonic tissue-specific expression profile for each receptor in the lens and elsewhere in the mouse embryo. In situ hybridisation for a variety of lens development stages could be examined, i.e. lens pit
(E10.5), lens vesicle (E11.5), primary LFCs (E12.5), and differentiating secondary LFCs (E14.5). As the published 3-stage lens differentiation protocol is thought to mimic embryonic lens development via induction of neuroectoderm, lens placode and then LECs and LFCs\textsuperscript{127}, stage E14.5 mouse embryos were assessed via GenePaint (i.e., near when LFCs are first produced). From this analysis two genes were identified, ROR1 and GPR161, whose expression was reasonably restricted to the lens and no other tissues within the embryo. ROR1 showed a strong anterior LEC gene expression with little expression in LFCs or other embryonic tissues (Figure 4.1 C). GPR161 showed strong LEC and lesser staining in differentiating LFCs and other tissues of the embryo (Figure 4.1 D).

### 4.4.3 Assessment of expression in culture

The foetal human LEC line FHL124 was cultured and found to express both ROR1 and GPR161 via PCR (Figure 4.2 A, B). Additional assessment of RNA from human ES cell-derived lens differentiation samples at time points day 14, 18, 26 showed expression of ROR1 (Figure 4.2 C) and GPR161 (Figure 4.2 D).

### 4.4.4 Cell purification

The FHL124 cell line was then used for simple assessment of a panel of 3 commercial anti-ROR1 antibodies and 1 anti-GPR161 antibody to identify optimal concentrations for flow cytometry and MACS. FHL124 cells were also used to optimise a CRYAB antibody to detect LECs. No working antibody was found for GPR161. However, an ROR1 antibody (R&D Systems) was identified that resulted in optimal FHL124 cell staining and was therefore used for MACS.
Figure 4.1 Macro facilitates the identification of LEC specific surface proteins.

A) Macro enabled further assessment of partitioned gene lists and those isolated from text sort via a pipeline of bioinformatic tools. First the Gene Ontology tool within DAVID bioinformatic suite was used; this allowed genes isolated on the cell membrane to be found. GenePaint (*in situ hybridisation*) further assessed expression of lists in the embryonic mouse lens epithelium and fibres (www.genepaint.org). B) Lens dataset partitioned via Excel® based macro, lists were allocated based on their presence (P) on all three of the array experiments in either LECs, LFCs or both. A further text sort was used to highlight any potential receptors (R) within the lens. C-D) GenePaint analysis of gene lists (generated using the Excel®-based macro) identified ROR1 (C) and GPR161 (D) as having highly-restricted lens expression patterns in E14 mouse embryos. NR, neural retina; RPE, retinal pigment epithelium.
Figure 4.2 ROR1 and GPR161 are expressed in lens culture conditions.

A) A light micrograph showing FHL124 cells in culture (10 x magnification). B) PCR analysis reveals FHL124’s express ROR1 and GPR161. Lane 1 ROR1 expression, lane 2 No RT (reverse transcriptase) control, lane 3 GPR161 expression and lane 4 No RT control. C-D) PCR analysis at 14, 18 and 26 days shows that the 3-stage differentiated human pluripotent stem cell culture expresses both ROR1 (C) and GPR161 (D).
Day 18 of the lens differentiation time-course was chosen due to the reported maximal expression of CRYAB and maximal production of lentoids compared to earlier and later time-points, with this data suggesting that LEC production was at its height by day 18\textsuperscript{127}. Similar day 18 data were reproduced here using an alternate human ES cell line. However, generation of a complete and uniform single cell suspension was difficult at this time point, with large cell aggregates unable to be separated. To enable MACS, a 40 μM cell strainer needed to be used to produce a single cell suspension; this resulted in a large number of cells captured by the cell strainer and therefore a large proportion of cells not being analysed by MACS or FACS. Of the cells that could be analysed by flow cytometry at day 18, both ROR1\textsuperscript{+} and ROR1\textsuperscript{-} cells were detected in the presorted cell samples (Figure 4.3A). Magnetic cell separation using the anti-ROR1 antibody resulted in isolation of the ROR1\textsuperscript{+} population (Figure 4.3B) with a purity of 97\% ± 0.94\% as determined by post-MACS ROR1 FACS (Figure 4.3D). Flow cytometric analysis of the ROR1\textsuperscript{+} cell fraction collected via MACS showed 97\% ± 1.75\% of the ROR1\textsuperscript{+} cells also expressed the LEC marker CRYAB (Figure 4.3 C,D). The negative portion of cells was not assessed by FACS but PCR, this is because the MACS technology may not fully collect all ROR1\textsuperscript{+} cells and therefore would not confidently be specific to ROR1\textsuperscript{-} cells alone. This trend is supported by the low expression of CRYAB and PAX6 in the ROR1\textsuperscript{-} cells. PCR analysis of the MACS-sorted cells however, showed a higher expression of CRYAB and PAX6 in the ROR1\textsuperscript{+} cells compared to the ROR1\textsuperscript{-} and unsorted fractions. Additionally CRYBB3, an early marker of LFCs, was more highly expressed in unsorted and ROR1 negative cell populations (Figure 4.3E).
Figure 4.3 ROR1 enabled purification of human ES cell derived LEC cells.

Flow cytometry analysis shows that performing MACS using commercially-available antibodies against ROR1 enables lens cell purification. A) Pre MACS population of ROR1⁻ and ROR1⁺ populations. B) Post MACS flow cytometry assessment of ROR1⁺ population. C) Post MACS flow cytometry assessment of ROR1⁺ population for CRYAB expression where grey is secondary antibody and black is CRYAB population. D) Post-MACS ROR1 FACS of Day 18 cells from n=4 experiments resulted in isolation of the ROR1⁺ population with a purity of 97% ± 0.94%. Additionally ROR1⁺ cell fraction showed 97% ± 1.75% of the ROR1⁺ cells also expressed the LEC marker CRYAB. E) Real-time PCR analysis shows that ROR1 purified lens cells (+ MACS) possess increased transcript levels for lens epithelial cell biomarkers (e.g., CRYAB, PAX6) and lower levels of lens fibre cell biomarkers (e.g., CRYBB3) compared to non-expressing (- MACS) cells and an impure starting cell population (‘Day 18 pre-MACS’). F) A light micrograph showing MACS-purified lens cells in culture (derived from differentiating human pluripotent stem cell cultures; 20x magnification).
4.4.5 Cell culture post MACS

The ROR1\textsuperscript{+} MACS-sorted LECs were assessed for their ability to be maintained in culture. These LECs were seeded after MACS at differing cell densities and cultured in M199 containing 5ng/ml FGF2 (i.e., LEC maintenance conditions rather than LFC differentiation conditions), then assessed for the appearance of lentoids as the cultures grew to confluence. Seeding of the ROR1\textsuperscript{+} cells at 2.1 x 10\textsuperscript{5} cells/cm\textsuperscript{2} enabled confluence within 3 days, with few “lentoid-like” structures generated of which the first of these structures only appearing at 8 days of culture (Figure 4.3F). A higher density of 4.7 x 10\textsuperscript{5} ROR1\textsuperscript{+} cells/cm\textsuperscript{2} resulted in confluence one day post plating, however, “lentoid-like” structures appeared after 3 days (Figure 4.4A, B). Seeding the ROR1\textsuperscript{+} cells at 2.1 x 10\textsuperscript{4} cells/cm\textsuperscript{2} did not allow cells to reach confluence, instead “lentoid-like” structures appeared within 3 days and large numbers of non-adherent/dead cells were visible (Figure 4.4C).

4.4.6 Toxicology/anti-cataract screening using ROR1\textsuperscript{+} LECs

To investigate whether the MACS-purified ROR1\textsuperscript{+} LECs are suitable for applications such as high throughput drug or toxicology screening, a toxicology assay was performed utilising hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). After 3 hours of H\textsubscript{2}O\textsubscript{2} exposure, cell death was visualised under the microscope in H\textsubscript{2}O\textsubscript{2} treated wells only. All wells were stained with both propidium iodide and hoechst allowing cell death and cell nuclei respectively to be visualised (Figure 4.5A-D). Total cell numbers and percentage cell death between control and H\textsubscript{2}O\textsubscript{2} treated wells established that H\textsubscript{2}O\textsubscript{2} killed all cells well inside the 3 hour treatment time (Figure 4.5E).
Figure 4.4 Seeding densities of ROR1\(^+\) cells.

ROR1\(^+\) cells were seeded at different densities on Matrigel\(^\text{TM}\) coated plates in 5 ng/mL FGF2 in DMEM:F12. A) Light micrograph 3 days post seeding cells at 4.7 x 10\(^5\) ROR1\(^+\) cells/cm\(^2\), lentoids indicated by arrows. B) Phase contrast image of micrograph 3 days post seeding 4.7 x 10\(^5\) ROR1\(^+\) cells/cm\(^2\), lentoids indicated by arrows. C) Light micrograph 3 days post seeding ROR1\(^+\) cells at 2.1 x 10\(^4\) cells/cm\(^2\) resulted in lentoids, cell death and minimal proliferation.
Figure 4.5 Proof of principal, the culture of (MACS) purified LECs enables lens drug and toxicology screening.

A-D) ROR1\(^+\) cells purified via MACS were cultured till confluence and then exposed to hydrogen peroxide (H\(_2\)O\(_2\)), (blue = Hoechst nuclear stain for total cells; red = propidium iodide staining for dead cells): control-treated cells retain high cell numbers (A) with little cell death (B), whereas cell treated with 2 mM H\(_2\)O\(_2\) (C) results in massive cell death within 3 hours (D) leaving only residual dead cells. E) Quantification of the effects of H\(_2\)O\(_2\)-treatment on purified lens cells shows the effects of H\(_2\)O\(_2\) are highly significant.
4.5 DISCUSSION

A source of purified LECs would better enable the study of human lens development and enable both toxicology and anti-cataract drug screening. Current human ES cell differentiation protocols are able to produce LECs in culture, however, these methods additionally produce contaminating LFCs and non-lens cells. An attempt has been reported for LEC purification via flow cytometry, relying on literature-based identification of surface proteins known to be expressed by LECs. While this approach did allow a degree of LEC purification, the approach was suboptimal due to the complexity of the multi-laser technique used, the poor yield of LECs, the high material and equipment costs, and the relatively large amount of time required for staining. Therefore the study presented here was transcriptomics-based to allow the identification of a variety of surface proteins that would enable a single step purification of LECs by way of MACS. Over 1000 cell surface proteins were identified as expressed by human lens cells, and in situ hybridization assessment allowed identification of cell surface receptors with expression isolated to the lens and specifically the LECs. This approach identified ROR1 and GPR161 to be expressed primarily within LECs of E14.5 mouse embryos. This lens-specific expression pattern was supported by PCR identification of ROR1 and GPR161 expression within both FHL124 human LECs and human ES cells differentiated towards lens cells. These findings are supported by data within the literature that reported ROR1 expression via whole mount in situ hybridisation, with ROR1 localisation within the LECs of E12-E13 mouse embryos\textsuperscript{233,234}. Furthermore, published in situ hybridisations for GPR161 show expression within the lens pit at E10.5, lens vesicle at E11.5 and within primary lens fibre cells at E12.5\textsuperscript{235} of mouse embryos.
Screening of commercially available ROR1 and GPR161 antibodies using FHL124 human lens cells identified an ROR1 antibody that successfully stained FHL124 cells. Testing showed that this antibody also detected ROR1+ cells within human ES cell lens differentiation cultures. Application of this ROR1 antibody with MACS allowed the purification of populations of human ES cell-derived LECs, as demonstrated by the expression of traditional LEC markers (i.e., PAX6, CRYAB) via both FACS and PCR. Importantly technical limitations of this study, such as the inability to create single cell suspensions without cell filtering, likely resulted in an overestimate of the size of the ROR1+ population in the unsorted bulk differentiation cultures. This was due to the need to filter out cell aggregates during the cell harvest procedure that would have affected the subsequent MACS and FACS experiments (e.g., ROR1− LFC within the Day 18 lentoids, as well as ROR1− non-lens cells), but resulting in these ROR1- cells not being assessed in pre-MACS FACS. Regardless of this technical limitation, a statistically significant increase in the number of ROR1+ cells was seen with MACS purification that resulted in almost 100% of the retained cells being ROR1+. The small percentage of apparently ROR1- cells within this population is likely a technical artefact due to the reduced stoichiometry of FACS secondary antibody binding caused by the anti-biotin micro beads; this idea is supported by the almost 100% CRYAB staining of the ROR1+ cells and suggests alternate MACS-based purification should be investigated. For example, PE or APC conjugation of the ROR1 antibody, followed by MACS with anti-PE or anti-APC micro beads, would enable the resulting MACS-purified cells to be immediately assessed for purity via flow cytometry without compromised ROR1 detection.
Irrespective of these technical limitations, the ROR1 method for human LEC purification shown here represents a drastic improvement on all previous attempts to generate purified populations of human LECs from pluripotent stem cells. By comparison, the previously reported 3-stage lens differentiation protocol that generates mixed populations of LECs, LFCs and other cell types has reported only a 40% CRYAB positive population by flow cytometry compared to the nearly 100% CRYAB positive ROR1+ population shown here.

The MACS purified LECs were then shown to be maintained in culture in low FGF2 conditions\textsuperscript{137}. Furthermore, plating densities were observed to be critical to the maintenance of the ROR1 purified cells, where too low or too high plating densities encouraged lentoid production. Future assessment of the molecular characteristics of these structures, via FACS or by immunocytochemistry analysis of lens structural proteins (CRYAB, CRYBB, CRYG) and even ROR1 is necessary.

Cataract has been reported as a side effect of use of some prescription medications\textsuperscript{236}, for example valproic acid, an antiepileptic drug\textsuperscript{237}, use of MACS purified LECs therefore may additionally be used to screen for any toxic effect of currently used medications. Cataract-induced blindness affects 100 million people globally and is currently only treated surgically\textsuperscript{13}. Cataract surgery involves removal of the non-transparent biological lens and implantation of an intraocular lens. A frequent complication of this is posterior capsule opacification (PCO)\textsuperscript{44}. PCO results from abnormal growth and migration of residual LECs post primary cataract surgery, which results in wrinkling and clouding of the posterior capsule leading to eventual vision loss and requirement of additional laser surgery\textsuperscript{53}.
A review of the literature identified hydrogen peroxide (H2O2) as one substance readily found in the lens that may affect the proliferation of LECs. H2O2 levels within the aqueous humour of the normal human lens have been recorded between 0.03 to 0.05mM and for cataract patient’s levels as high as 0.6mM are documented\textsuperscript{238-240}. Furthermore, a dose dependant effect of H2O2 on LEC proliferation has previously been demonstrated using a rabbit LEC line, TOTL-86. Ohguro and colleagues concluded that proliferation of LECs was concentration dependent with low doses (1μM) leading to proliferation and high doses (1mM) killing the cells, over a 48 hour time frame\textsuperscript{241}. Additionally, the age of LECs has been demonstrated to also influence cell survival, where cultured LECs from older rabbits were shown to be more susceptible to H2O2 insult than younger cells\textsuperscript{242}. It was shown that this was due in part to reduced activity of the enzyme glutathione reductase and to a diminished response of the hexose monophosphate shunt in the older cells\textsuperscript{242}. The strategy utilised by this thesis was to design a screen that demonstrates the utility and scalability of the sorted hESC derived LECs. These cells provide an alternative to nonhuman cell lines for future secondary cataract screens. In the developed screen, H2O2 was chosen 1) because of its potential relevance to secondary cataract and 2) past studies have already characterised the effect of H2O2 on LECs over a range of concentrations previously mentioned\textsuperscript{241, 243, 244}. A concentration of H2O2 aimed to quickly result in LEC death and therefore their elimination was chosen. 2mM was chosen because 0.6mM levels have been documented within human aqueous and 1mM has been shown to kill the previously mentioned cell line TOTL-86\textsuperscript{241}. Furthermore, based on the assumption that our derived LECs are potentially immature a slightly higher concentration of H2O2 i.e. 2mM was selected. Secondary cataract is the migration of residual LECs after
primary cataract removal. For this reason only ROR1\(^+\) cells were used as these best represent the purest form of LECs. ROR1\(^-\) or other cell types were not included in this assay, this was because only the effect on LECs was of interest, therefore demonstrating the potential of this tool to model secondary cataract. Hoechst and Propidium Iodide stains alongside fluorescence microscopy, allowed the collection of statistical counts of live/healthy, apoptotic, and necrotic cells. While most high-throughput assays would utilise automated high content plate readers i.e. In Cell 6000 with In Cell developer Toolbox (GE Healthcare) to give relative measurements, fluorescence microscopy was used in this instance to gain a closer look at the actual cells and staining patterns in relation to cell morphology.

Furthermore, the AlamarBlue assay was not chosen as it does not measure rate of cell death but only measures the cells that survive, in this instance a percentage of dead vs alive cells was wanted. No Z-factor was established, however, it is accepted that this is the widely accepted coefficient to monitor the quality of cell based assays. For future, larger screens this will be included. Future use of these ROR1\(^+\) LECs in more sophisticated high throughput drug development schemes is achievable. Of particular interest are selective and pharmacologically diverse “drug-like” small molecules that target key signalling systems thought to be involved in LEC proliferation, ranging from proteasome regulation, epithelial-mesenchymal transition, Wnt, TGF\(\beta\) and FGF signalling\(^{117, 145, 245-248}\).

This discovery made in this study has broad clinical and commercial applications, as limited access to human lens cells has severely inhibited the development of needed new cataract treatments. The discovery of a simple LEC purification method will enable the use of presently suboptimal differentiation methods to supply a large and
continual supply of LEC for lens research, toxicology screening, and anti-PCO drug screening.

Impending future work will be based around overcoming technical difficulties associated with successful generation of a single cell suspension. Alternative enzymes such as Collagenase or Accutase® will be trialed to avoid the need for filtering. These enzymes dissociate cells by breaking the key structural peptides within collagen, as collagen IV is the structural protein making up the lens capsule. Furthermore, alternative human ES cell lines will be assessed for their ability to be used in this method of differentiation and purification, to determine if there are any cell specific limitations of this method. Lastly, purified LECs will be assessed for their ability to be differentiated into LFCs in a controlled manner.

In conclusion, the use of bioinformatics and MACS as presented here enabled the purification of ROR+ LECs from heterogeneous cultures of differentiating human ES cells. This simple, scalable method has the potential to allow assessment of lens toxicity for drugs currently under development, as well as high-throughput assessment of drug libraries to discover anti-secondary drugs.
CHAPTER 5: GENERAL DISCUSSION
5.1 Limitations of current lens cell differentiation strategies

Presently the limiting factor for lens differentiation models derived from human pluripotent stem (PS) cells (i.e., embryonic and induced pluripotent) is that multiple lens and non-lens cell types are present in culture. Clearly, more efficient and selective methods are needed to direct the controlled differentiation of homogenous populations of stem cell derived lens epithelial cells (LECs). This will greatly facilitate studies of the molecular mechanism of lens and cataract development. In Chapter 1, the inability to produce pure populations of LECs by way of growth factor modifications demonstrates the complexity of signalling involvement in the lens, and flags the need for additional knowledge of signalling networks occurring during human lens formation and growth. Investigation of the signalling pathways involved in ES cell differentiation to LECs, and the integration of these signals to determine lens-restricted gene expression programs, is of much interest.

5.2 Advances in LEC production and biological insight from this thesis

New and exciting insights into lens development have been identified through the studies presented in this thesis. Chapter 1 showed that trial and error growth factor additions to the reported 3-stage lens differentiation protocol gave some improvement, but they did not yield purified LECs. Chapter 2 showed that use of transcriptomics and bioinformatics has the potential to reveal key players involved in lens development, and that this application has the potential to expand our current knowledge of regulation within the lens by providing novel testable hypotheses that may be implemented within the lens differentiation protocols. Discovery of the ELK1 transcription factor and validation of its expression by FHL124 cells
demonstrates the potential value of this approach, and further research will provide new information on the expression of ELK1 in ROR1-purified LECs and its importance to lens development (e.g., does it aid lens specific biological processes such as proliferation, apoptosis, cell migration). Finally Chapter 4 showed a further application of this bioinformatic-based strategy which lead to the identification of ROR1 and its suitability for use in developing a strategy for LEC purification. The simple, publically available bioinformatic method used in this research illustrates its potential for use by non-bioinformaticians interested in the lens or other tissues to generate other new biological hypotheses for testing.

5.3 Future technical developments

Due to unavoidable external time-constraints imposed by the Australian PhD system, there was not enough time to investigate in greater detail specific technical refinements that will allow unambiguous determination of the LEC purity after magnetic activated cell sorting (MACS). For example, as mentioned in Chapter 4, the use of anti-biotin micro beads to detect the ROR1 antibodies for MACS followed by the use of a fluorescent anti-IgG secondary antibody to detect the same ROR1 antibodies for FACS. This arrangement was necessary as a starting point for establishing the MACS technique, however, it causes a reduced stoichiometry of secondary antibody binding that decreases maximal detection of the ROR1+ cells by FACS. Future use of a phycoerythrin (PE) conjugated ROR1 antibody would not require additional staining of a secondary antibody post MACS, and thus could be tested to determine the most accurate measurement of ROR1 levels in the MACS-separated populations. Additionally, alternative means of creating a more efficient single cell suspension would also be of minor interest to possibly increase the yield.
of purified cells in addition to providing a better estimate of the percentage ROR1
population in the bulk differentiation culture. Finally, now that this technique of
purifying LECs from an initial heterogeneous cell population has been developed it
should also be trialed in alternative human embryonic and induced stem cell lines to
determine how robust the protocol is. Furthermore, while chapter 3 importantly
identified ELK1 many other potential lens candidates were identified. Future work
may involve 1) further assessing their expression profile in the lens using GenePaint.
2) Reviewing published literature and mouse knock out data to further develop
potential testable hypothesis. 3) Finally, the expression profile of these interesting
candidates could then be assessed in either rat lens samples, FHL124 cell culture or
the purified and un-sorted derived LEC culture.

5.4 Future applications for purified human LECs to understand lens
and cataract development

Previously, rat lens explants have been used to discover key molecular insights about
lens development and growth, such as the discovery of FGF signalling, as well as the
in vitro conditions for regeneration of lens-like structures capable of focusing and
magnifying light\textsuperscript{124}. An unlimited source of human ES cell-derived LECs are ideally
suited for studies of differentiation and early embryonic development. For example,
they have the potential to overcome the limited supply, technical difficulties and
animal research costs associated with isolation of primary animal lens tissues.
Moreover, any discoveries made through the use of human ES stem cell-derived lens
cells would likely be more directly translatable to human lens development than
animal based models. There is however, the possibility that the human ES cell-
derived purified LECs are embryonic-like or foetal-like (not adult like) in terms of
their gene expression profile and behaviour. This has been shown to occur for other cells derived from human ES cells, such as red blood cells which typically produce embryonic and foetal globins. Future characterisation is required to determine whether the ROR1+ LECs are an appropriate cataract model at this stage or whether further research is needed to define conditions that ensure their maturation to adult-like LECs in order to study adult posterior capsule opacification (PCO) or secondary cataract.

A scalable source of human derived LECs also has the potential to speed up drug discovery. For the first time this scalable source will enable high-throughput chemical screening for molecules that slow the migration of LECs, inhibit their aberrant differentiation and/or cause LEC apoptosis and therefore identify potential anti-PCO drugs as demonstrated in Chapter 4. For example application of the ROR1-purified LECs to small molecule cell-based phenotypic or pathway-specific screens of synthetic chemical or natural product libraries may allow the identification of pathways that control lens and cataract fate. With recent advances in automation and detection technologies, millions of discrete compounds can be screened rapidly at low cost further justifying the suitability of these stem cell-derived LECs for drug discovery. Ideally, the properties of a chemical library are optimised to interact with the specific biomolecules or collection of biomolecules of interest. In this study, a list of receptors were identified in the lens and by using this concept various naturally occurring and synthetic molecules known to interact with proteins involved in cell signalling pathways associated with these cell surface receptors could be screened to identify pathways regulating developmental processes required for development of the lens and secondary cataract.
While anti-PCO drug screening only requires a scalable source of LECs, future applications developing human age relate nuclear (ARN) cataract models from these purified LECs may also be possible. Previously, rat lens explant pairs have been shown to regenerate to form lens-like structures capable of focusing and magnifying light\textsuperscript{124, 163}. Additionally, continued culture of these in vitro lenses led to the development of lens opacities mirroring age-related nuclear (ARN) cataract. As a result, the explant-pair in vitro lens regeneration system was proposed as a new system to aid investigation of the molecular mechanisms driving the formation of human nuclear cataract\textsuperscript{124, 162}. It may be possible to humanise this explant model now that a scalable source of LECs are available, thereby creating an in vitro human lens displaying ARN cataract morphologies. However, before this is possible investigation into the minimal requirements to build a lens must be identified. Specifically, what scaffolds would correctly mirror functions of the natural lens capsule (e.g. left-over human capsule after cataract surgery; artificial scaffolds) and what defined growth factors within vitreous best drive formation of a full functional lens needs to be investigated.

### 5.6 Concluding remarks

This PhD thesis presents a logical progression of work based on the current knowledge of signalling molecules that operate during lens embryogenesis. In doing so it has provided new insights into lens signalling (e.g., ELK1; LEC and LFC receptor catalogues) and a simple new bioinformatics tool and pipeline that will enable other non-bioinformaticians to investigate freely available gene expression datasets. Furthermore, this thesis presents a simple, efficient and scalable method for
purification of LECs derived from differentiating human ES cell cultures. These purified LECs have many applications including for models of developmental biology and potentially anti-PCO drug discovery. This thesis also provides clear avenues for further investigation of lens and cataract development, including further characterisation of the purified LECs as embryonic-like or adult-like via transcriptomics and proteomics, as well as human in vitro lens formation and related disease modelling.
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