HISTAMINE REGULATION OF THE NORMAL AND PATHOLOGICAL LENS

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Abstract

Purpose: Histamine is an important inflammatory molecule. Following injury to the eye, such as cataract surgery, levels are likely to rise and could contribute to the development of posterior capsule opacification (PCO). Consequently, we investigated the effect of histamine on signalling pathways and growth rates of human lens cells.
Methods: Gene expression in the native human lens was determined by analysis of Illumina bead array data sets. Calcium signalling was investigated through imaging of FURA-2 loaded cells while phosphorylation of ERK, p38 and JNK was assessed using the BIOPLEX suspended bead array system. Levels of phosphorylated ERK were further assessed using immuno-cytochemistry. Growth rate was assessed in FHL 124 cells by changes in protein level detected by coomassie blue dye extraction and ^{3}H-thymidine incorporation. In addition, the human capsular bag model was employed; in this system, cell growth was measured as coverage of the central posterior capsule.
Results:
Non-Pathological Lens:
Analysis of array data from the human lens revealed that only the H1-receptor sub type is detectable; this is exclusively expressed in the epithelial cell population. It was also found that the lens had no detectable endogenous ability to produce histamine as it lacked histidine decarboxylase, however the lens does express large amounts of histamine releasing factor (HRF). Moreover, histamine breakdown genes encoding histamine N-methyltransferase and diamine oxidase are expressed.
Pathological Lens:
Application of histamine to FHL 124 cells produced a dose-dependent increase in intracellular calcium, with detectable responses observed from 1μM histamine. Responses to 100μM histamine were ablated with the use of 10μM triprolidine (an H1 antagonist) and not ranitidine (an H2 antagonist) or thioperamide (an H3 antagonist). Addition of 100μM histamine induced significant elevation of phosphorylated ERK and P38; pJNK was also elevated, but this change was not significant. In each case, peak response was observed 10 minutes following stimulation. The pattern for levels of pERK was also observed using Immuno-cytochemistry with a peak response between 5-10mins. Addition of histamine (≥30μM) significantly stimulated FHL 124 cell growth which was inhibited with the use of 100μM triprolidine. Moreover the rate of cell coverage of the posterior capsule in capsular bags was accelerated by 100μM histamine.
Conclusions: The dominant histamine receptor present in native lens epithelial cells is the H1 receptor sub-type. Activation of this receptor induces significant changes in calcium and MAPK signalling pathways. In addition, histamine stimulates an accelerated growth response that could contribute to PCO progression; therefore, histamine receptor antagonists could provide therapeutic benefit following cataract surgery.

Abbreviations

AAH	Artificial Aqueous Humour
ABL1	Abelson murine leukemia viral oncogene homolog 1
ABP1	Amiloride binding protein 1
ADRBK	Adrenergic, beta, receptor kinase
Akt kinases	serine/threonine-specific protein kinase
AM	acetoxymethylester
AOC2	Amine oxidase, copper containing 2
AU	Arbitrary Units
BCR	Breakpoint cluster Gene
BSA	Bovine serum albumin
Ca ATPase	Calcium Adenosine Trisphosphate
CaCl2	Calcium Chloride
cAMP	Cyclic adenosine Monophosphate
CDC25B	CDC25 family of phosphatases
CDC25C	CDC25 family of phosphatases
CNS	Central Nervous System
CSF2	Colony Stimulating Factor
CTNNB1	Catenin (cadherin-associated protein), beta 1
DAG	Diacylglycerol
DAO	Diamine Oxidase
DAPI	4'-6-Diamidino-2-phenylindole
DEFB4	Defensin, Beta 4
EGF	Epidermal Growth Factor
EMEM	Eagles Minimum Essential Medium
ERK	Extracellular Signal-Regulated Kinase
FCER1A	Fc fragment of Ig-E, high affinity I, receptor
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
GABRB3	GABA-A receptor beta-3 subunit
GM-CSF	Granulocyte macrophage colony-stimulating factor
HDC	Histidine Decarboxylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth Factor
HNMT	Histamine N-Methyltransferase
HRF	Histamine Releasing Factor (TPT1/TCTP)
HRH1-4/H1-4	Human Histamine Receptor
Ig-E	Immunoglobulin E
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL -13	Interleukin 13

IOL	Intraocular Lens
IP-10	Interferon-inducible protein 10
IP3	Inositol 1,4,5- trisphosphate
JAK	Janus Kinase
JNK	Jun N-terminal Kinase
KCl	Potassium Chloride
LASP1	LIM and SH3 protein 1
MAPK1/3	Mitogen Activated Protein Kinase 1/3
MCP-1	Monocyte chemotactic protein-1
MgCL2	Magnesium Chloride
MIP-1a	Macrophage Inflammatory Protein 1 alpha
MIP-1b	Macrophage Inflammatory Protein 1 beta
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaHCO3	Sodium Bicarbonate
NAOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
Nd:YAG	Neodymium-doped yttrium aluminium garnet
NN1003A	Rabbit Cell Line
NO	Nitrous Oxide
p38	Class of mitogen-activated protein kinases
PBS	Phosphate Buffered Saline
PCO	Posterior Capsule Opacification
PDGF-bb	Platelet Derived Growth Factor
pERK	Phosphorylated Extracellular Signal-Regulated Kinase
pJNK	Phosphorylated Jun N-terminal Kinase
PLC	Phospholipase C
p-P38	Phosphorylated P38
PPP1R14A	Protein phosphatase1, regulatory subunit 14A
PRKCD	Protein kinase C Delta
PTGS2	Prostaglandin-endoperoxide synthase 2
PYK2	Proline-rich tyrosine kinase 2
RANTES	Regulated upon activation, normal T cell expressed and secreted
RNA	Ribonucleic Acid
STAT	Signal Transducers and Activators of Transcription protein
SYK	Spleen tyrosine kinase
TCA	Trichloroacetic acid
TGFâ	Transforming Growth Factor Beta
TNF-a	Tumour Necrosis Factor alpha
TPT1	Tumour Protein Translationally Controlled 1(TCTP, HRF)
VDR	Vitamin D Receptor
VEGF	Vascular-Endothelial Growth Factor
VKC	Vernal keratoconjunctivitis
VWF	Von Willebrand Factor

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1. Introduction

1.1 The Human Eye

The eye is a complex sensory organ that detects and interprets photic stimuli (see figure 1). The human eye can recognise electromagnetic waves with a wave length between 400 and 700nm a region known as visible light. The light is detected by two types of photoreceptors found in an epithelial layer at the back of the eye called the retina. The two types are rods which operate best in low light conditions and cannot recognise colours nor do they produce high quality images, and Cones which differ as they work best in lighter conditions, recognise colours and produce high quality images {Davson, 1990 #92}.

The structure of the eye is made up of three concentric layers, the inner, middle and outer layers. These layers contain different structures and membranes which all serve important functions for sight {Davson, 1990 #92}. The outer layer known as the fibrous coat contains the sclera, conjunctiva and the cornea and is the attachment point for extraocular muscles which control eye movement. The sclera is the fibrous layer that makes up the whites of the eyes, the conjunctiva is a thin transparent membrane surrounding the sclera which protects the eye, and the cornea found at the front of the eye is a transparent membrane involved in focusing light onto the retina {Davson, 1990 #92}. The middle layer known as the vascular coat contains the iris, the ciliary body and the choroid. The iris is the coloured part of the eye which moves in response to light intensity controlling the amount of light that enters the eye. The

ciliary body produces the fluid which fills the spaces of the eye and also contains muscles which moves the lens so the eye can focus on objects at differing distances {Davson, 1990 #92}. Lastly, the choroid is a membrane which contains blood vessels providing nutrients to the retina. The inner layer known as the neural coat is also known as the retina. The retina covers the entire back of the eye and contains photoreceptors connected to nerve fibres. These nerve fibres all converge to form the optic nerve and where this occurs is the blind spot {Davson, 1990 #92}. The nerve fibres form electrical impulses and the optic nerve carries all the information to the brain where it is interpreted and an image is formed. The retina contains different regions such as the macula which is involved in high visual acuity and the fovea which is the most sensitive part of the macula {Davson, 1990 #92}. When the eyes are directed at an object the fovea is where all the light is focused. Apart from these external structures, the eye also contains fluids within it {Davson, 1990 #92}. Aqueous humor is actively secreted from the ciliary body and is found in the anterior and posterior chambers. This fluid helps to maintain the shape of the eye but also provides nutrients to parts of the eye which do not have access to blood vessels and removes waste products {Davson, 1990 #92}. The bulk of the internal space of the eye contains the vitreous humor. This is not produced or turned over as readily as the aqueous humor and is found in the rear of the eye and has a gel-like consistency. The vitreous humour is essential for maintaining the structure of the eye. Separating the two humours is the lens which is required to focus light successfully onto the retina through the process of accommodation {Davson, 1990 #92}.

1.2 The Lens

The lens is a transparent bi-convex disk found between the aqueous and vitreous humors. It is connected by suspensory ligaments (zonules), made from the protein fibrillin, to the ciliary muscles. The lens focuses light onto the retina and its thickness and curvature can be changed by the ciliary muscles to refract light more or less so objects at different distances can be focused {G. Duncan, 2001 #93}. The process of changing lens shape is called accommodation. Accommodation can be a reflex or consciously controlled and changes can occur within 350milliseconds. The ability of the lens to change its optical power diminishes with age and by the age of 60 nearly all people require aids for near vision. This is a condition known as presbyopia. The lens is derived from cells of surface ectodermal origin {G. Duncan, 1997 #53}. In the mature lens, the lens is enveloped by a collagenous capsule; on the anterior surface there is a monolayer of epithelial cells. Cells at the centre of this epithelium do not normally divide or undergo cell death. At the periphery of the epithelium, also known as the equator, cells do divide and differentiate into fibre cells {G. Duncan, 1997 #53} which form the bulk of the lens; these events are slow but persistent (Figure 1.1). New fibre cells are laid down upon the older ones which are internalised forming a fibre cell age gradient through the lens. This also forms a well ordered structure through the lens preventing light scattering. As the cells differentiate into mature fibre cells they lose there nucleus and organelles and accumulate the transparent proteins α, β and γ crystallins {G. Duncan, 2001 #93}. The crystallins provide refractive power of the lens and thus contribute to light focusing abilities of the lens. The lens contains no blood vessels and therefore all the nutrients required by the cells that make up the lens must come from the fluids which surround the lens; the aqueous and vitreous humors {I. M. Wormstone, 2006 #83}. The anterior epithelial layer of the lens is connected to

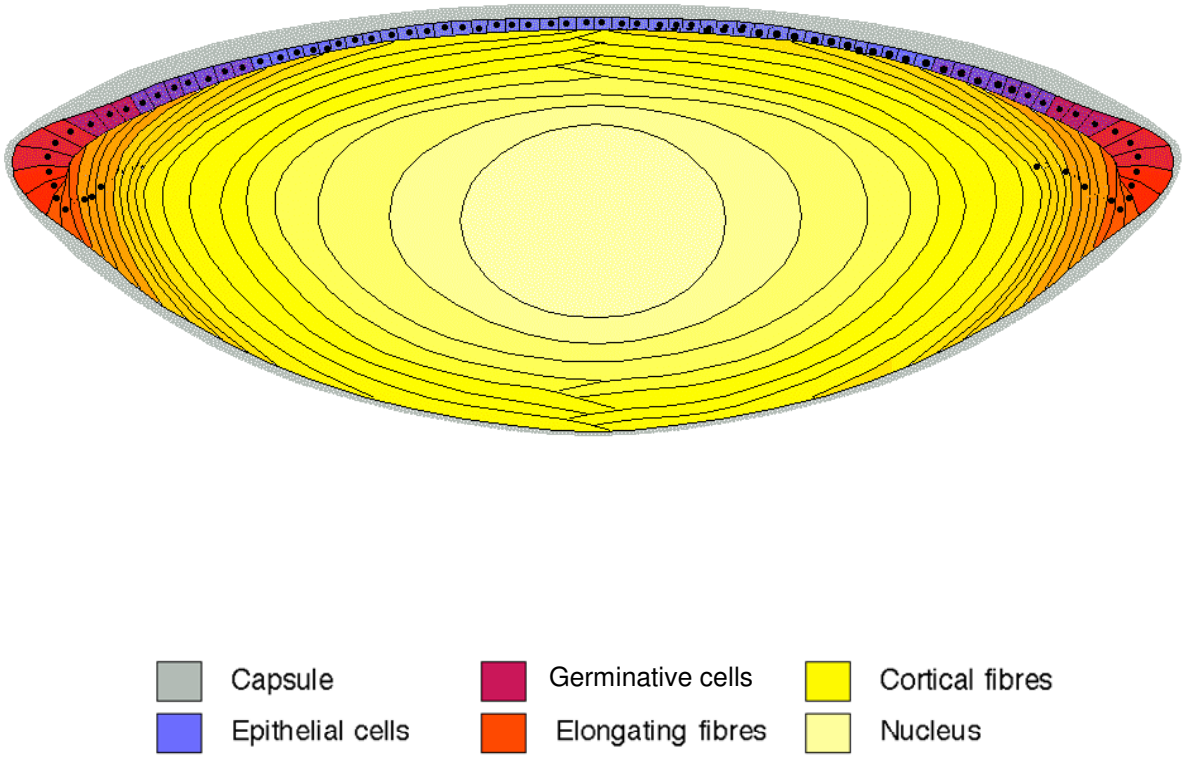


Fig. 1.1. A diagrammatic representation of a cross section of humans lens (Maidment et l 2004)

the fibre cells through gap junctions, mainly at the equator, and produce energy which allows for the transport of small molecules and metabolites to the fibre cells {G. Duncan, 2001 #93}. This maintains the correct ion and metabolite concentration and prevents the crystallins from aggregating. If the integrity of the lens is disrupted then cataract can result.

1.3 Cataract

Cataract is a condition defined by an impairment of vision due to opacity forming in the lens. There are many causes of cataract such as age, long term exposure to ultraviolet light, physical or surgical trauma and in some cases due to other diseases such as diabetes. There is also evidence of a genetic susceptibility and cataracts can run in families. There are many different types of cataract with three main types. Nuclear cataracts are classically associated with old age, form in the centre of the lens and are associated with a yellowing/browning (brunescene) of the lens. This is caused by proteins within the lens aggregating together which causes light scattering affects and blue light absorption {G. Duncan, 2001 #93}. Cortical cataracts are characterised by wedge shaped opacities which encroach into the centre of the lens from the periphery. They are thought to be cause by an electrolyte imbalance. Cortical cataracts have been associated with imbalances in osmotic pressure within the eye and from exposure to ultraviolet light {G. Duncan, 2001 #93}. The other relatively common form is posterior subcapsular cataract which is characterised by opacity in the posterior pole region of the lens. These are thought to be caused by damage to a fibre cell in the anterior pole region and the damage spreads from pole to pole. They have also been associated with irradiation, diabetes and retinal degradation {G. Duncan, 2001 #93}. There is no cure for cataract, and the only available treatment is surgery.

Modern cataract surgery adopts a procedure known as extracapsular cataract extraction {G. Duncan, 2001 #93}. This involves making an opening in the anterior of the lens and removing the cataract material. This leaves a capsular bag made up of the complete posterior capsule and a ring of anterior capsule. An artificial lens known as an intra-ocular lens (IOL) can be implanted, held in place with support loops, within the capsular bag, restoring vision. The success of cataract surgery is still affected by complications later in life such a retinal detachment and a secondary loss of vision resulting from post-surgical wounding, a condition known as posterior capsule opacification (PCO).

1.4 Posterior Capsule Opacification

PCO is the most common complication of cataract surgery affecting significant proportions of cataract patients {I. M. Wormstone, 2009 #81}. Immediately following surgery, light can pass along the visual axis through the IOL and the acellular posterior capsule without disruption. However, residual lens epithelial cells on the anterior capsule survive the rigours of surgery and begin to re-colonise the anterior capsule, the IOL and the posterior capsule and eventually make inroads across the visual axis. These cells also begin to undergo changes in organisation and the matrix is altered so that the light is scattered. If this continues to develop surgery is often required {I. M. Wormstone, 2009 #81} (Figure 1.2).

The incidence of PCO is influenced by a number of factors including age. With respect to age, rates are higher in younger patients (up to 70% after 4 years) compared with older patients, which was approximately 40% over the same time frame {J.

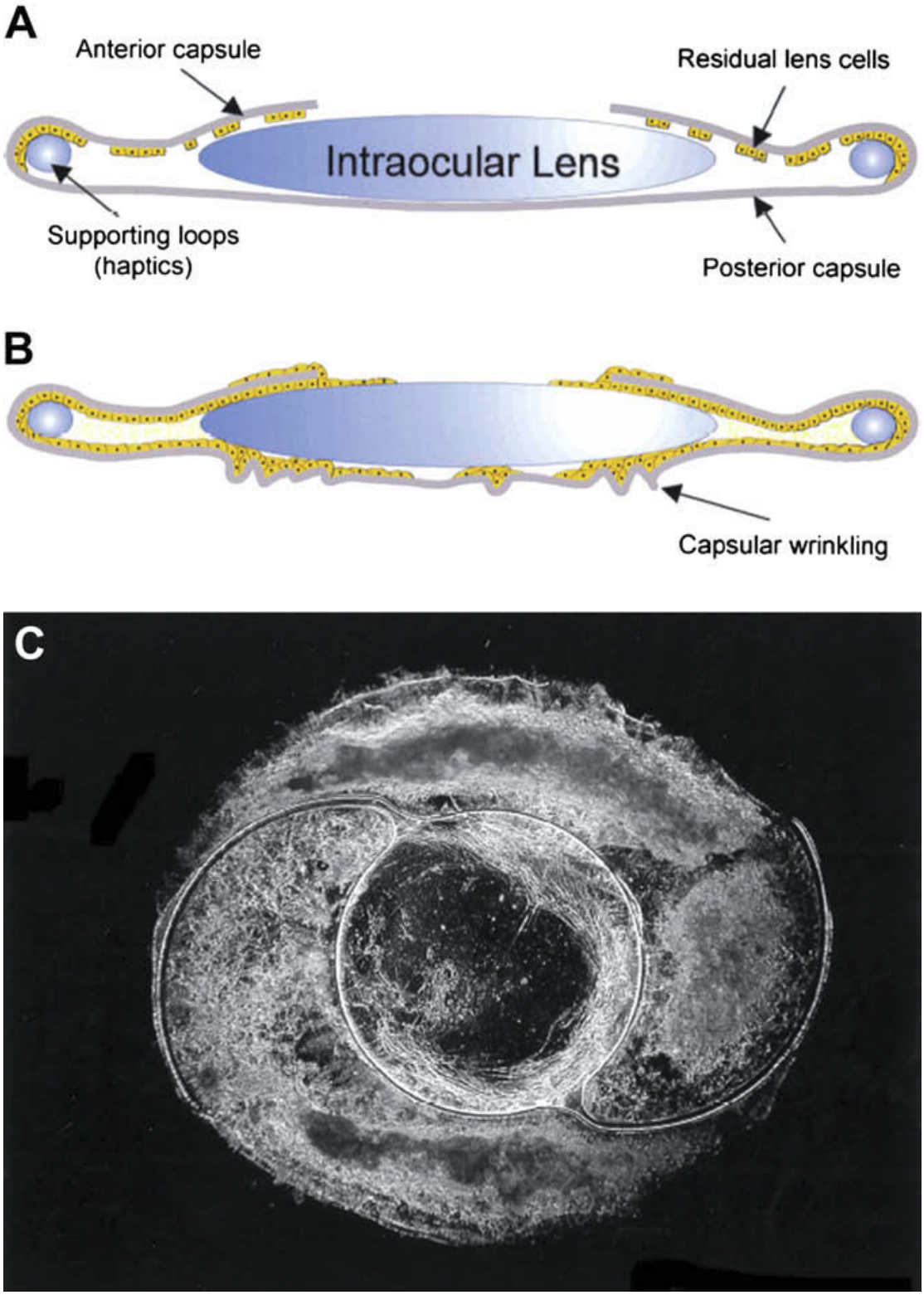


Fig. 1.2. Diagram of (A) the post surgical capsular bag and (B) proliferation and morphological changes that cause Posterior capsule opacification. (C) A dark-field micrograph of a capsular bag removed from a donor eye that had undergone cataract surgery prior to death that exhibits light scattering regions beneath an intraocular lens. Previously published in Wormstone, 2002 and Wormstone, 2009.

Moisseiev, 1989 #94}. In children the problem is even more severe {W. F. Astle, 2009 #95}. While there is clearly a greater incidence of PCO in the young, the frequency is still significant for all age groups. The majority of patients undergoing cataract surgery will be >60 years and an incidence of 10-30% in this group will mean millions worldwide are affected by PCO formation. Consequently, significant numbers require further surgery to correct the condition and restore vision through ablation of light scattering regions on the posterior capsule using a Nd:YAG laser; this is both expensive and as with all surgery has risks {I. M. Wormstone, 2009 #81}. Due to the importance of PCO, new ways of preventing this cellular growth and migration have been developed with mixed results.

Different strategies have been developed to improve management of PCO. The major areas of investigation are to improve surgical methods and IOL design. The second is to employ agents to control cell behaviour following surgery. i.e. to kill the cells, prevent growth, or inhibit , matrix modification. In all cases the aim is to reduce light scatter {I. M. Wormstone, 2009 #81}.

Improvements in IOL design led to the production of the “sharp edged” IOL. Through interaction with the capsule the edge of the IOL forms a physical barrier to prevent the epithelial cells growing on to the central posterior capsule {O. Nishi, 2007 #96}. This has helped to reduce the incidence of patients requiring further surgery. However this approach seems to delay the point at which PCO becomes clinically relevant, but does not prevent it with many patients still requiring laser treatment.

Other areas of research into preventing PCO include the use of molecular agonists to

kill or stop the growth of the epithelial cells {G. Duncan, 1997 #97} {G. Duncan, 2007 #98} {J. L. Walker, 2007 #99} {R. J. Stump, 2006 #100}. Such developments will benefit greatly from improved methods of drug delivery at the time of cataract surgery {G. Duncan, 2007 #98} {T. M. Rabsilber, 2007 #101}. The identification of novel biological targets allied to efficient delivery systems will allow effective targeted inhibition of lens cells, while reducing the risk of damage to other ocular tissues.

The focus of a large body of research is therefore on what mechanisms result in the cellular changes that lead to PCO. Cells respond to signals found in their environment and lens cells can respond to either signals they generate themselves (autocrine signals) or external signals from other ocular tissues (paracrine signalling) {Wormstone, 2002 #50}. Both are thought to contribute to PCO as a constant supply of signalling molecules are required to maintain growth and the health of the cells. The paracrine signals that underlie PCO are thought to be derived from an increase in proteins found in the aqueous humour following cataract surgery {I. M. Wormstone, 2009 #81}. These come from a disruption in neighbouring blood vessels and an inflammatory response. Many of these proteins are only found following surgery and are not usually present in the aqueous humour. Many of these proteins, especially cytokines, have been shown to increase both cell survival and cell proliferation {Y. Ishizaki, 1993 #102} {I. M. Wormstone, 2000 #103} {I. M. Wormstone, 2001 #104} {J. M. Maidment, 2004 #69}, two properties that play major parts in PCO formation (Figure 3). In addition, there is a great deal of focus on the possible role of Transforming Growth Factor beta (TGFβ) which is known to be able to induce morphological changes in many cell types and could induce the fibrotic changes

which cause light scattering in PCO {A. Leask, 2004 #105 {Massagué, 2000 #106} {R. Derynck, 2003 #107} (Figure 1.3).

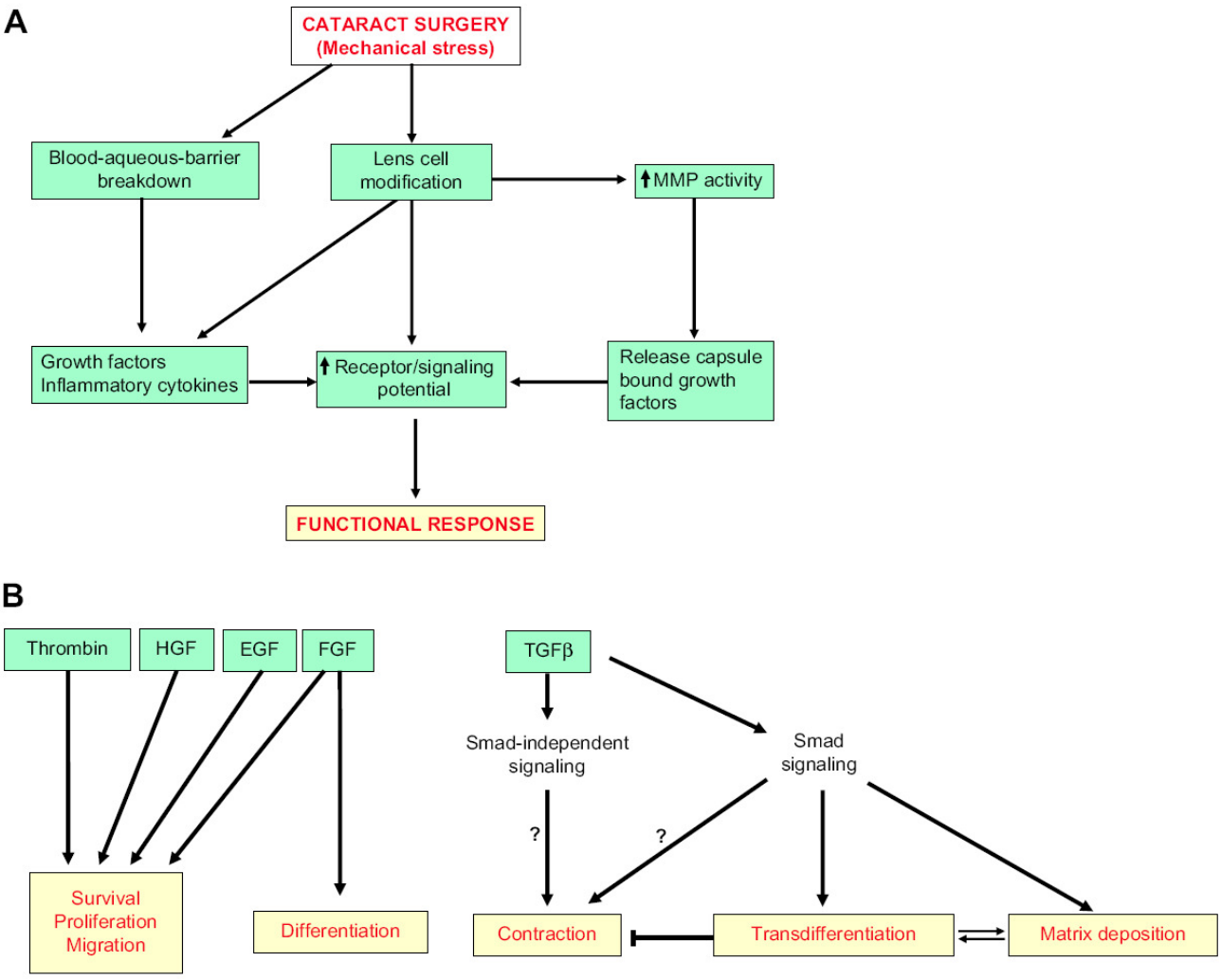


Fig. 1.3. Diagram of (A) the early stage events following cataract surgery that lead to PCO and (B) the functional outputs of different growth factors in relation to PCO (Wormstone, 2009).

The increased levels of proteins in the aqueous humour return to normal levels in the weeks and months after surgery. Interestingly however PCO can take years until surgery is necessary, therefore an autocrine system is likely to be driving PCO

development {Wormstone, 2002 #50}. This would take the form of the normal levels of protein in the aqueous humour driving PCO or that signals endogenously produced by the lens drive PCO. It is known that the inflammatory response following cataract surgery has the ability to change the composition of proteins in the aqueous humour and the pattern of protein expression in the lens {Wormstone, 2002 #50}. The lens is also known to have many autocrine systems. It has been shown that the lens is a large contributor to transferrin in the aqueous humour {M. C. McGahan, 1995 #109} and has many autocrine systems for the production of cytokines and receptors {I. M. Wormstone, 2001 #104} {E. H. Lee, 1999 #110}. These autocrine systems are demonstrated by the fact that cultured lens cells can survive and proliferate for greater than 100 days in serum free medium {I. M. Wormstone, 1997 #108}. This shows an autocrine signalling mechanism that can maintain lens cells without external regulation. This survival and proliferation has been shown to allow lens cells to respond to damage of the lens and grow across the posterior capsule of a capsular bag (a model for cataract surgery) (Figure 1.2). Capsular bags from young donors were shown to be colonised much quicker than those from older patients {I. M. Wormstone, 1997 #108}. Supplementing the capsular bags with 10%FCS sped up proliferation with the older donors but less so in the younger donors which means autocrine signalling is likely to diminish with age.{I. M. Wormstone, 1997 #108}.

In the formation of PCO, both paracrine and autocrine signalling is likely to contribute. At the early stages following cataract surgery exogenous proteins will cause an initial drive in wound-healing response (Figure 3). This will be maintained through the action of autocrine control systems. These systems are likely to drive PCO at a slower rate than the early stages as the level of protein will have dropped. Therefore each mode of regulation is a worthy area of investigation. The focus of the current study was to investigate the role of the inflammatory molecule histamine in relation to PCO.

1.5 Histamine

Histamine is a biogenic amine with a wide array of functions in all parts of the body. It plays major roles as a neurotransmitter, in gastric acid secretion and immune-regulation {S. J. Hill, 1997 #44}. Histamine was first identified to play a role in inflammation and smooth muscle contraction which led to the development of antihistamines to block the effect of histamine in these tissues {Hill, 1990 #11}. The fact that these antagonists did not affect the ability of histamine to affect other tissues showed that different histamine receptors played different roles in different tissues of the body. Histamine is now known to be produced in a large number of cells in the body including mast cells, basophils, platelets, enterochromaffin-like cells, endothelial cells, and neurons, {M. Jutel, 2002 #47} through histidine decarboxylation mediated by the enzyme histidine decarboxylase {Stark, 2007 #45}. Once produced histamine can be stored in mast cells and basophils so that it can be released in relatively large quantities when required. This means that large amounts of

histamine are available throughout the body. The levels of histamine are regulated through the actions of breakdown enzymes Diamine oxidase (DAO) and histamine N-methyltransferase (HNMT) {K. Yamauchi, 1994 #85} {Schayer, 1956 #86}.

1.6 Histamine receptors

Histamine regulates its functions through 3 main types of G-protein coupled receptors, HRH1-3. A fourth receptor has also been recently discovered called HRH4 {Stark, 2007 #45}. HRH1 is found in most smooth muscle, endothelial cells, adrenal medulla, heart, and the CNS. It is known to play roles in Smooth muscle contraction, stimulation of NO formation, endothelial cell contraction, increased vascular permeability, stimulation of hormone release, negative inotropism, depolarization (block of leak potassium current) and increased neuronal firing, inositol phospholipid hydrolysis and calcium mobilization, hyperpolarization by Ca^{2+} -dependent potassium current. HRH1 signals through a $G\alpha_{q/11}$ receptor. This causes the activation of phospholipase C (PLC}and an increase in inositol 1,4,5- trisphosphate (IP3) and 1.2-diacylglycerol (DAG) which leads to an increase in intracellular calcium concentration and/or cAMP {Stark, 2007 #45} {S. J. Hill, 1997 #44} {Hill, 1990 #11}.

HRH2 receptor is found in Gastric parietal cells, vascular smooth muscle, suppressor T cells, neutrophils, CNS, and the heart. It is known to play roles in the stimulation of gastric acid secretion, smooth muscle relaxation, stimulation of adenylyl cyclase, positive chronotropic and inotropic effects on cardiac muscle, decreased firing rate, hyperpolarization or facilitation of signal transduction in CNS, blocking of Ca^{2+} -dependent potassium conductance, increase of hyperpolarization-activated current,and

the inhibition of lymphocyte function. HRH2 signals through a G_s coupled receptor. This leads to the activation of adenylyl cyclase and an increase in cAMP. However it has also been observed that HRH2 can lead to activation of PLC and an increase in intracellular calcium concentration {Stark, 2007 #45} {S. J. Hill, 1997 #44} {Hill, 1990 #11}.

Histamine HRH3 receptor is found in the CNS, peripheral nerves (heart, lung, gastrointestinal tract), endothelium, and enterochromaffin cells. It is known to play roles in the inhibition of neurotransmitter release, inhibition of gastric acid secretion (dog), increase in smooth muscle voltage-dependent Ca^{2+} current, and inhibition of the firing of tuberomammilary (histaminergic) neurons. HRH3 signals through $G\alpha_i$ and $G\alpha_o$ coupled receptors. This leads to inhibition of adenylyl cyclase and the N^+/H^+ antiporter and activation of mitogenactivated protein kinase (MAPK), phospholipase A_2, and Akt kinases {Stark, 2007 #45} {S. J. Hill, 1997 #44} {Hill, 1990 #11}.

HRH4 receptor is found at low levels in most tissues and therefore is most likely involved in inflammatory processes. This is supported by the fact that anti-histamines that target HRH4 have anti inflammatory properties. Also levels of HRH4 receptor expression are regulated by various cytokines including IL-6, -10 and -13. HRH4 also signals through $G\alpha_i$ and $G\alpha_o$ receptors. This leads to inhibition of adenylyl cyclase and reduces levels of cAMP and activation of MAPK and phospholipase C causing an increase in intracellular calcium {Stark, 2007 #45} {S. J. Hill, 1997 #44} {Hill, 1990 #11}.

1.7 Histamine and the Eye

Histamine is stored in various compartments of the eye including the ciliary body and the iris {McMenamin, 1997 #55}{May, 1999 #34} {T. Nakagami, 1999 #35}. The availability of these stores is perhaps mostly widely drawn upon during acute and chronic inflammation. Histamine has been linked to a number of diseases in the eye including pterygia and uveitis {K. Yamashiro, 2001 #57} {N. Girolamoa, 2004 #15}. Levels of histamine released from mast cells have been shown to increase during the onset of uveitis {C. H. Lee, 1993 #56}. Histamine receptor HRH1 has also been found to be expressed in Pterygial derived fibroblasts {R. Maini, 2002 #60}. Pterygial fibroblasts and epithelial cells have a greater growth rate than normal conjunctival cells and a greater sensitivity to growth factors. There is also a greater density of mast cells in pterygial tissue compared to normal conjunctiva. Histamine signalling through HRH1 and HRH2 has been shown to cause both proliferation and migration and in sub-conjunctival fibroblasts {A. Leonardi, 1999 #74}; proliferation was inhibited with the use of an H1 antagonist though migration was not. Histamine has also been shown to play a possible role in vernal keratoconjunctivitis (VKC). Histamine stimulated VKC cells and normal conjunctival cells to grow at similar rates while VKC's usually grow faster. This growth was inhibited by both HRH1 and HRH2 antagonists {A. Leonardi, 1999 #74}. Histamine also promoted cell migration after wounding which was inhibited by HRH2 antagonists. Lastly histamine caused VKC fibroblasts to produce more PIP than normal cells and normal fibroblasts. This was not prevented by HRH1 or HRH2 antagonists and could therefore be caused by a different receptor {A. Leonardi, 1999 #74}.

Within the lens itself histamine has been shown to induce calcium mobilisation in

native human lens cells and this was inhibited with the use of a HRH1 antagonist {R. A. Riach, 1995 #6}. This demonstrates the lens ability to respond to histamine that is produced either endogenously or available in the surrounding environment.

1.8 Histamine Signalling

Histamine receptors can signal through the release of intracellular calcium (Ca^{2+}) (HRH1 and HRH2), by the increase in cyclic adenosine mono-phosphate (cAMP) (HRH2) or by a decrease in cAMP (HRH3 and HRH4) {S. J. Hill, 1997 #44} {Stark, 2007 #45}. This can lead to the activation of other signalling pathways such as MAPK cascades through signalling pathway crosstalk {A. J. Robinson, 2001 #61} {F. Hao, 2008 #14}.

Ca^{2+} is a versatile and powerful messenger in cells. It regulates multiple cellular functions involving many different signalling molecules {Clapham, 2007 #87}. Ca^{2+} can also react with other signalling pathways to create even more diversity in output. All the components of Ca^{2+} signalling form the calcium signalling 'toolkit'. Each component of the toolkit often comprises molecules with slight variations allowing cells to have a large number of Ca^{2+} signalling pathways. The components in the toolkit are divided into four main stages of calcium signalling:

- Stimulus that leads to signals for causing Ca^{2+} release.
- These ON signals lead to a release of Ca^{2+} into the cytoplasm
- Ca^{2+} acts as a messenger stimulating many Ca^{2+} sensitive processes.
- Feedback leads to OFF a signal which removes cytoplasmic Ca^{2+} through membrane pumps and exchangers.

Histamine, mainly through the HRH1 but also through the HRH2 receptor, is known to cause Ca^{2+} mobilisation through the activation of PLC which produces inositol-1,4,5-trisphosphate and diacylglycerol DAG {S. J. Hill, 1997 #44} {Stark, 2007 #45}. Inositol-1,4,5-trisphosphate then bind to receptors on the endoplasmic reticulum causing the release of Ca^{2+}. Ca^{2+} can then cause the activation of the MAPK pathway through the proline rich tyrosine kinase 2 (PYK2) and Ras. Ca^{2+} can also cause the expression of a metalloproteinase that would liberate EGF that could then signal through the MAPK pathway. MAPK signalling cascades are involved in a wide variety of cellular functions including proliferation and migration. Signalling occurs through the phosphorylation of a series of secondary messengers until the activation of several transcription factors lead to a cellular response. There are three MAPK pathways p38, JNK and ERK which all cause similar cellular responses but signal through different secondary messengers {M. J. Berridge, 2000 #3}.

To enable cell proliferation, a major process in PCO development, increased intracellular Ca^{2} must be maintained for a long period (>2 hours) and involves an influx of extracellular Ca^{2+} to maintain Ca^{2+} levels. If this can be maintained Ca^{2+} activates transcription factors which can then drive proliferation. If Ca^{2+} levels drop then transcription factors are quickly deactivated and translocated out of the nucleus {M. J. Berridge, 2000 #3}.

1.9 Interleukins and other cytokines

Cytokines are a group of polypeptides and glycoproteins that are similar to hormones

but are produced locally rather than part of the endocrine system. They act mainly as part of autocrine and paracrine systems and are potent in small concentrations {Stadnyk, 1994 #5}. The levels of cytokines can increase dramatically as one cytokine triggers the release of another and so on. Cytokines can also feedback to inhibit further release {K. Arai, 1990 #89}. Histamine is reported to cause the production of interleukins IL-6 and -8 and GM-CSF through the HRH1 receptor in conjunctival epithelial cells {J. M. Yanni, 1999 #46}. IL-6 and IL-8 are key mediators of the inflammatory response {A. Harada, 1994 #90} {Snick, 1990 #91}. IL-8 has been shown to promote cell survival and proliferation in endothelial cells {A. Li, 2003 #48}. IL-6 can signal through JAK/STAT, MAPK cascades and through PI3K signalling which all have the potential to promote cell proliferation {P. C. Heinrich, 2003 #65}. IL-8 signals through G-protein coupled receptors that activate PLC and the production of inositol 1,4,5-trisphosphate {D. Wu, 1993 #49}. As discussed earlier this is also linked with cell proliferation among many functions. Cytokines are a good avenue of investigation as they have a solid link with histamine signalling and are known to promote cell proliferation and survival in some cell lines.

1.10 Hypothesis

Histamine can induce functional changes in Lens cells that are linked with the development of PCO.

1.11 Aims and Objectives

The main aims of this project are to demonstrate that histamine can induce functional changes in the lens cell line FHL-124 cells that play a role in the development of PCO. The main early stage change in lens cells in PCO is an increase in proliferation

therefore the main focus of the project will be to investigate if histamine can induce proliferation in FHL-124 cells and which signalling pathways histamine signals through to cause this. This will be achieved through the use of a simple growth assay called a patch assay. This assay allows you to measure levels of cellular proliferation through measuring the size of the patch in relation to a control group and through measurement of total protein level. If this growth can be demonstrated then the signalling pathways triggered by histamine will be analysed to find which pathways are involved in histamine induced proliferation. First Ca^{2+} imaging will be used to see if histamine can mobilise intracellular Ca^{2+} in FHL-124 cells. Histamine receptor antagonists can be used to find which receptor is responsible for any response. It is expected that Histamine will produce a response as this has been demonstrated previously in native lens cells. BIOplex will also be employed to look at the expression of MAPK pathway proteins in response to histamine. If any of these pathways are found to be involved then these pathways can be blocked using antagonists or SiRNA during growth assays to see if they inhibit histamine induced proliferation. Away from this main focus gene array data from FHL-124 cells will be analysed to see if any genes that are associated with histamine are present in the lens. This will be achieved by using the NCBI website gene search function.

2. Materials and Methods

2.1 Cell culture of FHL-124's

The human lens cell line FHL-124 was used for all the experiments in this project. These were generated in the laboratory of Dr John Reddan (Oakland University, MI) from human capsule-epithelial explants {I. M. Wormstone, 2000 #103}. The FHL-124 cells were seeded on to 35mm culture dishes (BD Falcon, Oxford, UK) (patch growth assays, real time PCR and western blots) or on to cover slips (VWR International, Lutterworth, Leicestershire) (Immuno-cytochemistry). Cells were maintained in EMEM (Sigma, Poole, Dorset) supplemented with 5% foetal calf serum (FCS) (Gibco, Paisley, Scotland) until confluent and then typically maintained in serum free EMEM for 24 hours before exposure to experimental conditions.

2.2 Gene array analysis

A retrospective analysis of a gene array data set was performed as part of the investigations of histamine function in the lens. The original data set had been generated by Dr Lisa Hodgkinson, as part of her PhD studies in the Wormstone lab, and a collaborator James Hadfield at the Genomics Facility at The Cancer Research Institute, UK, Cambridge. A brief summary of the protocol that produced the original data set is as follows.

Four clear human lenses from donors aged 57, 76, 77 and 78 years were separated into three distinct regions, namely the central epithelium, the peripheral epithelium/equator and fibre cells. Samples were snap-frozen and stored in a -80C freezer prior to extracting RNA using the RNeasy® mini kit (Qiagen Ltd, Crawley,

UK). A microarray platform was selected to provide a comprehensive view of the gene activity in 3 regions of the human lens. It was also important that data could be generated from small samples to allow evaluation of gene expression in individual donors. To achieve this the commercially available Human-6 v2 Expression BeadChip (Illumina, San Diego, CA., BD-25-113) array platform was used in accordance with manufacturers instructions. Each BeadChip allows simultaneous analysis of 6 arrays, each with >46,000 probes derived from human genes in the National Centre for Biotechnology Information (NCBI) Reference Sequence. To quantitate mRNA levels 1.8 million beads are available on the Human- v2 BeadChip. Each bead is designed with an optimised gene specific 50mer oligo probe, each bead has several thousand copies of each probe on its surface. Concatenated to the probe is a short bead address sequence used during the manufacture process to identify the bead (Figure 2.1). The Bead chips were imaged using Illumina's Bead Array Reader. The raw data generated were analysed using Gene Spring software (Agilent Technologies UK Limited, South Queensferry, UK), which recognises the Illumina bead array platform and normalises the data using internal controls. Normalised signal data was then used to generate spreadsheets stored on Excel for future interrogation. In the current study this data set was analysed to determine gene expression patterns of genes involved in histamine regulation and function. Genes related to histamine were found using the NCBI website gene search function.

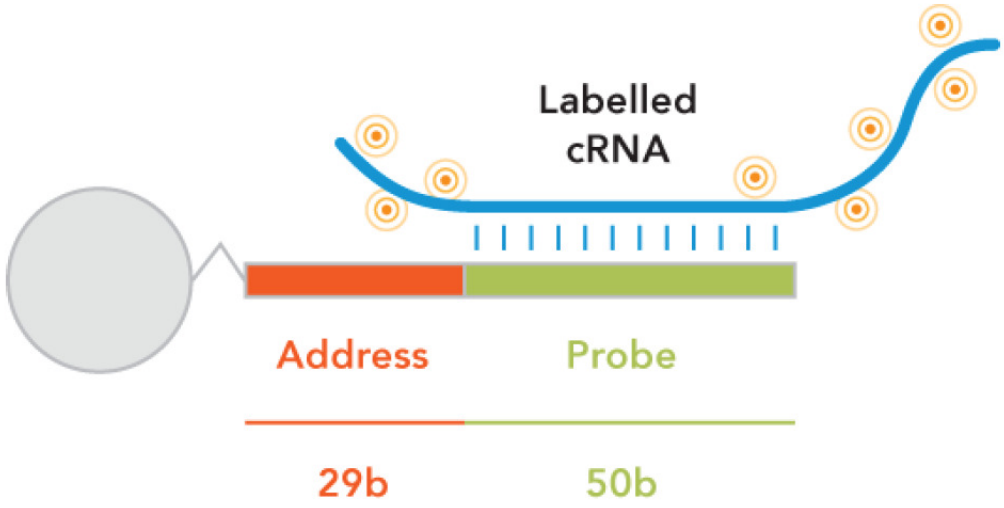


Fig. 2.1. Basic illumina bead design showing an identifier/address region (red) and a 50mer probe. http://www.switchtoi.com/pdf/GXHuman6-8v2Datasheet.pdf

2.3 Patch Growth Assay

5000 cells in 100µl EMEM (Sigma, Poole, Dorset) supplemented with 5% FCS were seeded at four distinct sites (Patches) on 35mm tissue culture dishes. The cells were left to adhere and until the patches were confluent. The medium was removed and cells placed in 1.5mls of serum free media (non-supplemented EMEM) for 24 hours. Four dishes were then fixed using 4% formaldehyde (Sigma, Poole, Dorset) for 30mins at room temperature to provide the t=0 reference controls. The remaining dishes were placed in experimental conditions the cells were maintained for a further 3 days. Following this period, the cells were fixed in 4% formaldehyde. The media was removed and the cells were stained using Coomassie Brilliant blue G 250 (Merck, Germany) protein dye and left at room temperature for 45 minutes. Excess Coomassie

blue dye was removed when the dishes were washed with PBS. Images of the patches were captured using gene snap software (Syngene, Cambridge, UK) and a CCD camera. The patches were then converted to binary images of black and white pixels using threshold techniques and measured using Scion Image to determine the number of black pixels, which corresponds to the area of cell coverage. To provide additional data, total protein was assessed to provide a measure of cell population. This was achieved by extraction of Coomassie Blue dye from the cells by addition of 1000µl of 70% (v/v) ethanol. The dishes were left on a shaker for 1 hour at room temperature. 100µl from each dish was then sampled and added to a 96 well plate (BD Falcon, Oxford, UK). The absorbance was measured at 550nm using a Wallac Victor 2 1420 plate multi-label counter with Elmer Workout (V15) software (Perkin Elmer, Cambridge, UK).

2.4 3H-Thymidine incorporation assay

One milliliter of a 1 x 10^4 cells/ml suspension of FHL124 cells in 10% FCS–EMEM was added to each well of a 24-well tissue culture plate (Beckton Dickinson labware, NJ, USA) and cultured for 48 hr. Following this period the medium was removed and replaced with serum free medium (EMEM (Sigma, Poole, Dorset)) and cultured for a further 48hrs. This medium was then removed and the cells placed in experimental conditions for a further 24hrs. During the final 4hrs of this culture period the cells were exposed to 1µci ml^{-1} ^{3}H-thymidine (Amersham, International) and 1µm cold thymidine. At the end of culture each well was washed twice with 1ml EMEM (Sigma, Poole, Dorset) to remove any radioactive ^{3}H-thymidine. One millilitre of 5% trichloroacetic acid (TCA) was added to each well. After 30min at room temperature, the TCA was removed and 1ml of 250mM NAOH was added to each well and left

overnight at 4ºC, 5ml of this NAOH was then sampled. 10 millilitres of scintillation fluid (Perkin Elmer, Cambridge, UK) was added to each sample and then measurements were taken using a scintillation counter (EG and G Wallac, UK).

2.5 Capsular Bags

After the removal of corneo-scleral discs for transplantation purposes, human donor eyes obtained from the East Anglian Eye Bank were used to perform sham cataract surgery, including continuous circular capsulorhexis (5 or 8 mm), hydroexpression of lens fibre mass, and aspiration of residual lens fibres. The capsular bag was then dissected free from the zonules and secured on a sterile polymethylmethacrylate Petri dish (BD Falcon, Oxford, UK). Six to eight entomological pins (Dl; Watkins and Doncaster, Kent, UK) were inserted through the edge of the capsular bag to retain its circular shape. Cultures were maintained in 1.5 ml of EMEM (Sigma, Poole, UK) supplemented with 10% foetal calf serum and 50 mg/1 gentamicin, and incubation was at 35 °C in a 5% CO_2 atmosphere. In the current invesitigation one eye from a donor was used as a control while one was exposed to 100µM histamine in order to limit the affect of biological or age variations between donors. The maintenance medium and histamine were replaced every other day. Ongoing observations were performed using phase-contrast and dark-field microscopy.

2.6 Calcium Imaging

FHL 124 cells originally seeded at a density of 1x 10^3 cells/100µl 5% FCS EMEM (Sigma, Poole, Dorset) on round glass cover slips (10 mm diameter) were imaged within 6 days. On the day of the experiment, cells were loaded with the

acetoxymethylester (AM) form of 2µM Fura-2/AM for 60 min at 35°C. The lens cells were then washed in artificial aqueous humor (AAH) for 20 min to allow complete de-esterification of the dye. The composition of AAH is (in mM): 130 NaCl, 5 KCl, 5 NaHCO3, 1 CaCl2, 0.5 MgCl2, 5 glucose, and 20 HEPES, adjusted to pH 7.25 with NaOH. The coverslip with the dye loaded cells was then placed in an imaging chamber which was mounted on the heated stage of an epifluorescence microscope (model TE-200; Nikon, Tokyo, Japan) fitted with a x20 objective. The imaging chamber with a depth of 3 mm was designed for calcium imaging, in which cells were continuously perfused with AAH at 35°C. Solutions were administered by a two-way tap using a flow rate of 1 ml/min. Different concentrations of histamine and molecular antagonist were added to the chamber through the irrigation tube. Cells were excited alternatively with light of 340- and 380-nm wavelengths. The emitted fluorescence was detected at 510 nm every 2s. After background subtraction the ratio of the light intensity monitored at 340/380 was used as an index for $Ca2^{+}$ release. A minimum of 5 cells were analyzed in each experimental condition, and all experiments were repeated on three further occasions to give N=4.

2.7 Suspended Bead Array (BIOPLEX) Analysis of cytokines and signalling molecules

FHL 124 cells were seeded onto 35-mm dishes at ~30,000 cells in 400µl of 5%FCS-EMEM (Gibco, Invitrogen Ltd, Paisley, UK) and were maintained in 1.5ml of 5%FCS-EMEM for 3 days. Following a 24 hour incubation period in non-supplemented EMEM cells were treated with Histamine at 100µM for 0, 2, 5, 10, 30, and 60 minutes respectively. At each time-point, the culture medium was removed

and stored before cells were lysed using a Bio-Plex Cell Lysis Kit, following manufacturers instructions (Bio-Rad, Hercules, CA). Lysates were pre-cleared by centrifuging at 4500 rpm at 4°C for 20 minutes. Equal amounts of protein per sample from cell lysates were analysed for phosphorylated and total levels of ERK1/2, P38 and JNK. The culture medium was analysed for cytokine levels. In each case commercially available Bio-Plex Suspended Multiplex Bead Array Assay kit (Bio-Rad) were employed. Data from the reaction was acquired using a Luminex X Map-100™ instrument and accompanying Bio-Plex Manager™ software (Bio-Rad, Hemmel Hempstead, UK). The median fluorescence intensity was used as a measure of detection.

2.8 Immuno-cytochemistry

FHL-124 cells were seeded onto glass cover slips (VWR International, Lutterworth, Leicestershire) in 35mm petri dishes (BD Falcon, Oxford, UK) in 100µl EMEM supplemented with 5% FCS. These were left to grow for 3 days until confluent at 35˚C/5%CO_2 and then transferred to serum free conditions supplemented with gentamycin (5µg/ml, Sigma) for 24 hours. The experimental conditions were added to each dish and they were incubated at 35˚C/5% CO_2 for 2 hours. The medium was aspirated off and the cells washed with PBS and then fixed with 1.5ml of 4% formaldehyde in PBS at room temperature for 30 minutes. The formaldehyde was then aspirated off and the cells given three washes with PBS/BSA0.02%/IGEPAL 0.05%. 1.5ml 0.5%-Triton-x-100 was then added to each dish for 30 minutes at room temperature in order to permeabalise the cells followed by another three washes with PBS/BSA0.02%/IGEPAL 0.05% for 10 minutes each with shaking. The edges of the coverslips were dried carefully with tissue to create surface tension when the solutions

were added. A blocking solution was made up and 40µl was added to each dish. A small plastic coverslip was placed on top in order to disperse the solution evenly and prevent evaporation of the solutions. The dishes were placed in square petri dishes with wetted tissue in the base and place in a 35˚C incubator for 1 hour. Forty microlitres of anti-pERK (mouse monoclonal antibody) (Cell Signalling Technology, Beverly, MA) (1:100 dilution in 1% BSA (Sigma, Poole, Dorset)) was added to the coverslips which were incubated at 35˚C for another hour. After an hour the cells were washed three times with PBS/BSA0.02%/IGEPAL 0.05% for 15 minutes each with shaking. Forty micro litres of ALEXA 488-conjugated anti-mouse secondary antibody (Molecular Probes, Leiden, Netherlands) was added and the dishes placed in square petri dishes covered in aluminium foil. The cells were left in secondary antibody for 1 hour at 35˚C. After the hour the cells were again washed three times with PBS/BSA0.02%/IGEPAL 0.05% for 15 minutes each with shaking. A mixture of Texas Red-X-phalloidin (Molecular Probes, Leiden, Netherlands) at 1:100 dilution with 1% BSA and DAPI (Sigma, Poole, Dorset) at a 1:50 dilution with 1% BSA was made up and 40µl was added to each coverslip. The cells were further incubated in the dark at 35˚C for 10 minutes. These two substances stain for F-actin and chromatin respectively allowing the cells and the nuclei to be visualised. A final series of three washes with PBS/BSA0.02%/IGEPAL 0.05% at 15 minutes each with shaking is given to the cells. To mount the coverslips a drop of hydromount mounting solution (National Diagnostics, Atlanta, GA, USA) was added to pre-cleaned slides and the coverslips placed cell side down on to the slide. The coverslips were left in the dark at room temperature for 20-30 minutes to allow the mounting solution to dry and then stored in the dark at 4˚C until imaged. The cells were imaged using a Zeiss Fluorescent microscope with an attached digital camera and Axiovision software

(Carl Zeiss Ltd, Welwyn Garden City, UK).

2.9 Statistical analysis

T-test analyses using Excel software (Microsoft, Redmand, WA) and a one way ANOVA with Tukey's post-hoc analysis using SPSS 12.0 for windows (SPSS Inc, Illinois) were performed to determine any statistical difference between experimental groups. Significance was assessed using a p value of ≤ 0.05.

3. Results

3.1 Gene Array

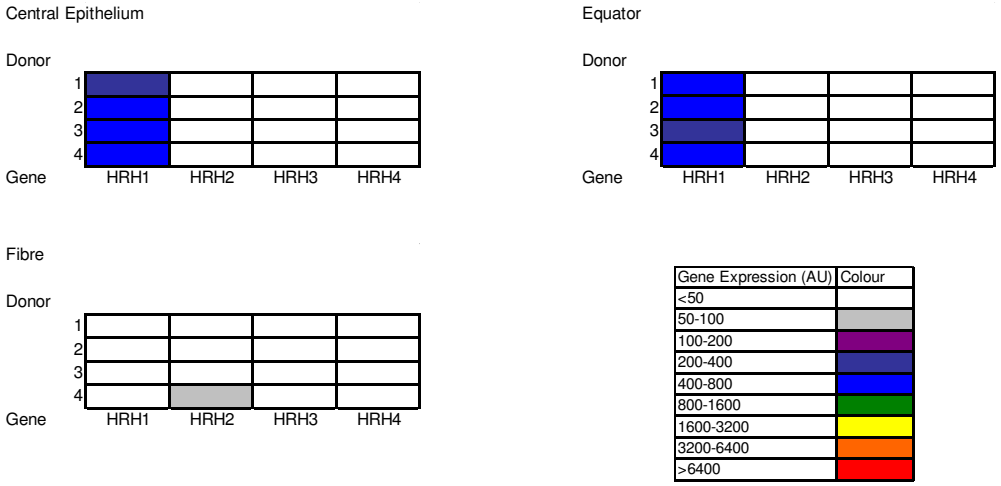
3.1.1 Analysis of Gene Array Data Derived from Quiescent, Dividing and Differentiated Regions of the Human Lens to Identify Genes Associated with Histamine

Gene array analysis was carried out for three distinct regions of the human lens: the equator; the central epithelium; the fibres. Using the NCBI website genes associated with Histamine were identified and expression levels determined from the array data. These genes were categorised by their functional role.

3.1.2 Histamine Receptor Expression

The levels of the genes encoding four histamine receptors are shown in figure 5. This clearly shows that *HRH1* is present in the epithelium with similar levels observed in the central and equatorial regions (Figure 3.1). However, *HRH1* was not detected at a significant level in the fibre cell population (Figure 3.1). None of the remaining genes encoding histamine receptors (*HRH2-4*) were detected in the epithelium. Expression of *HRH2* however was detectable in the fibre cells, albeit at a relatively low level this was still significant.

A.



B.

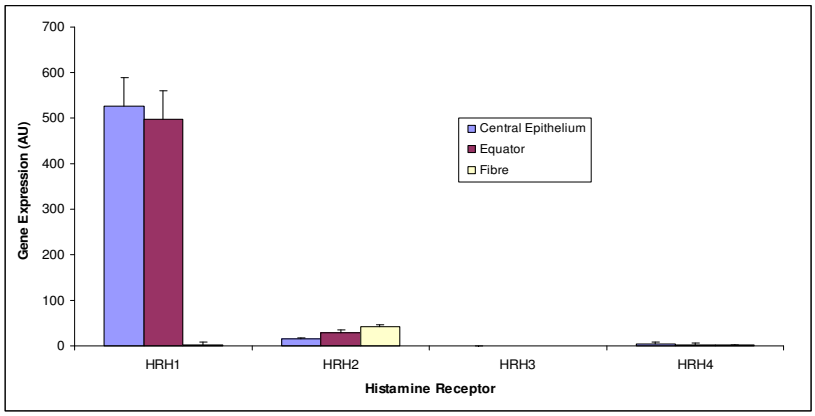


Fig. 3.1. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically histamine receptor gene expression in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled

3.1.3 Histamine Metabolism and Breakdown

Histidine decarboxylase (HDC), the gene encoding the primary enzyme responsible for histamine synthesis was not detected in any lens region (Figure 3.2). Conversely, gene expression of Histamine N-Methyltransferase (*HNMT*) which is involved in histamine breakdown was significantly detected in the central and equatorial epithelium; lower expression was observed in the fibre cells but this was still

significant. Other genes associated with histamine breakdown; Diamine D-amino-acid oxidase (*DAO*) and amiloride binding protein 1 (*ABP1*) were not detected in any region. Amine oxidase, copper containing 2 (*AOC2)* was not detected in the central epithelium or within fibre cells, but was significantly detected in the equatorial cells (Figure 3.2).

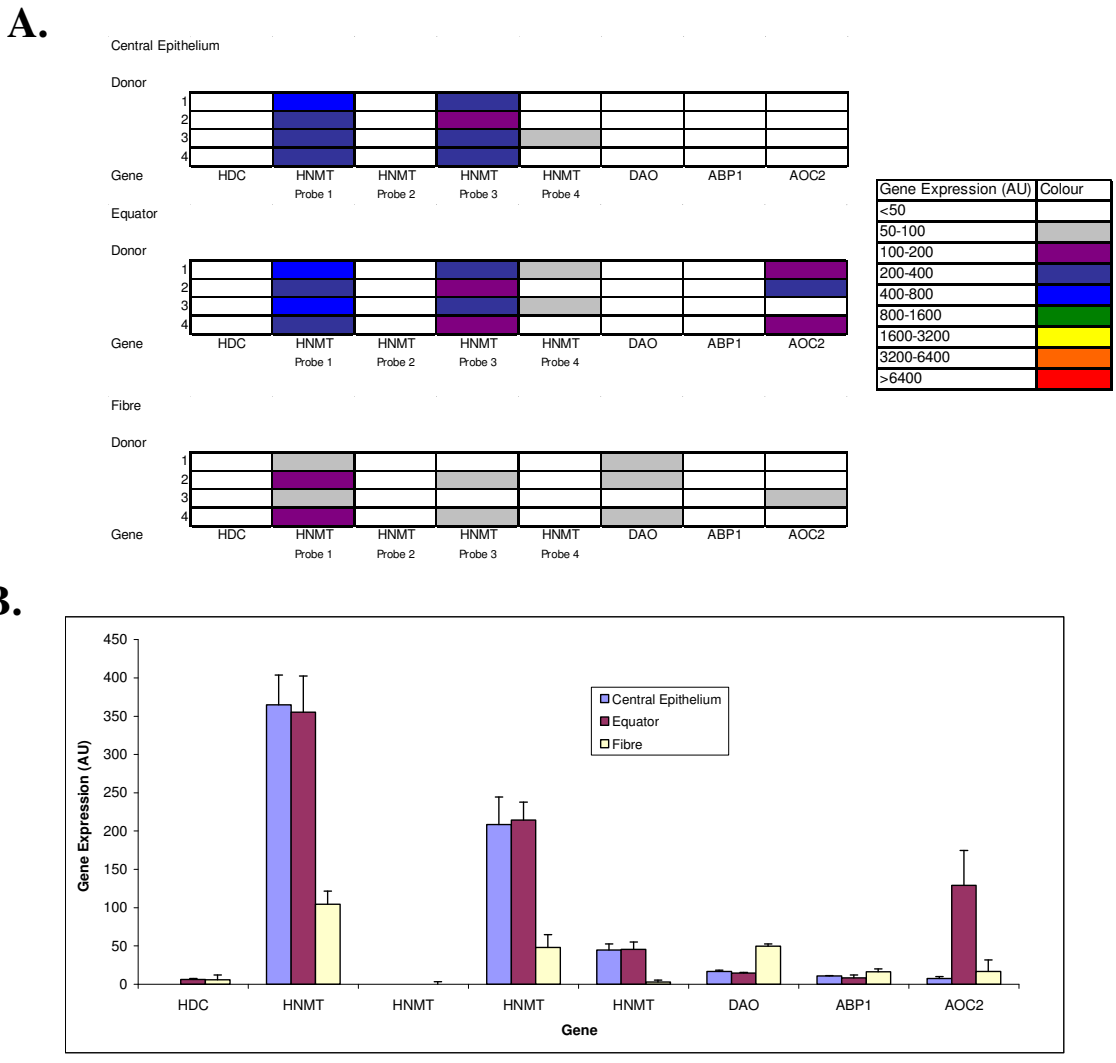
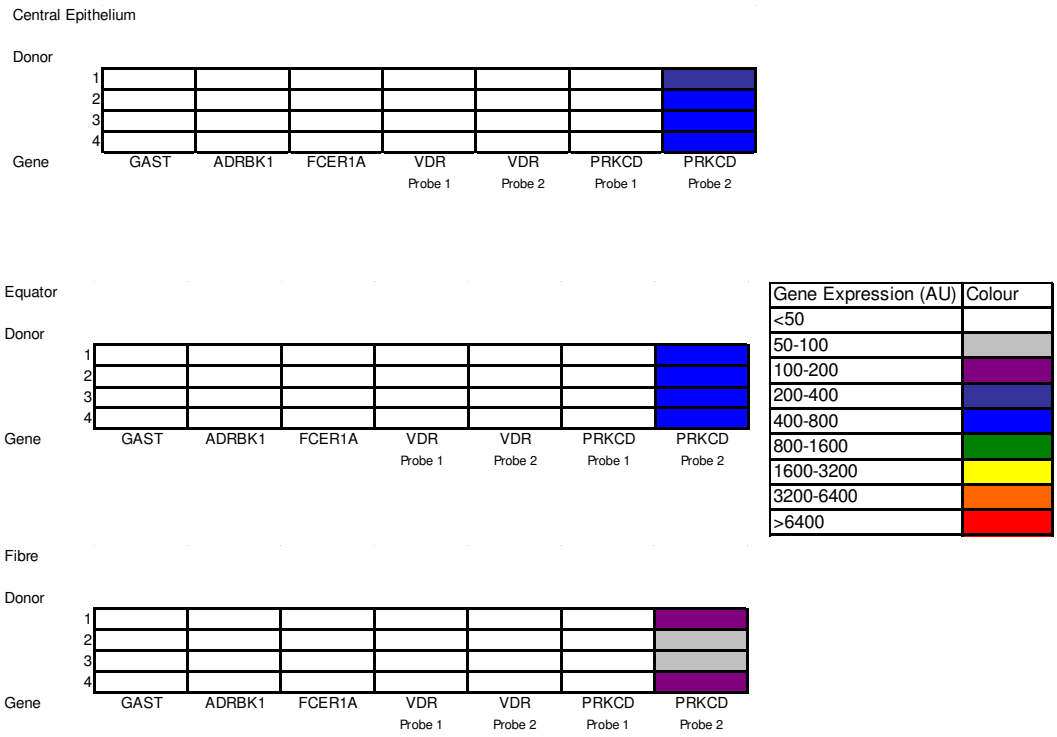


Fig. 3.2. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes regulating histamine synthesis and histamine breakdown in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

Of the histamine signal regulators evaluated, only PRKCD (Protein Kinase C) was detected at a significant level. Gastrin, ADRBK, FCER1A and VDR were not detected (Figure 3.3).

A.



B.

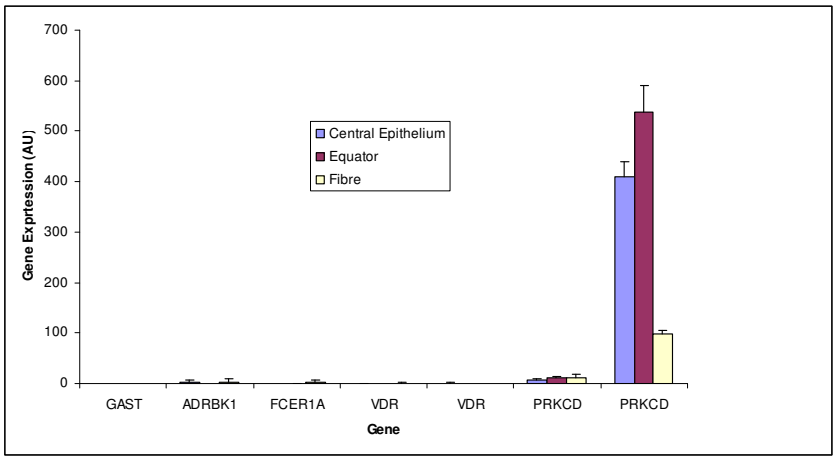


Fig. 3.3. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes associated with Histamine signalling regulation in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

Cytokines that have a well established association with histamine were analysed; these include the genes encoding IL3, IL4, IL6, IL8, IL13, and CSF2. Interestingly, no message for these cytokines was detected in any regions of the lens (Figure 3.4).

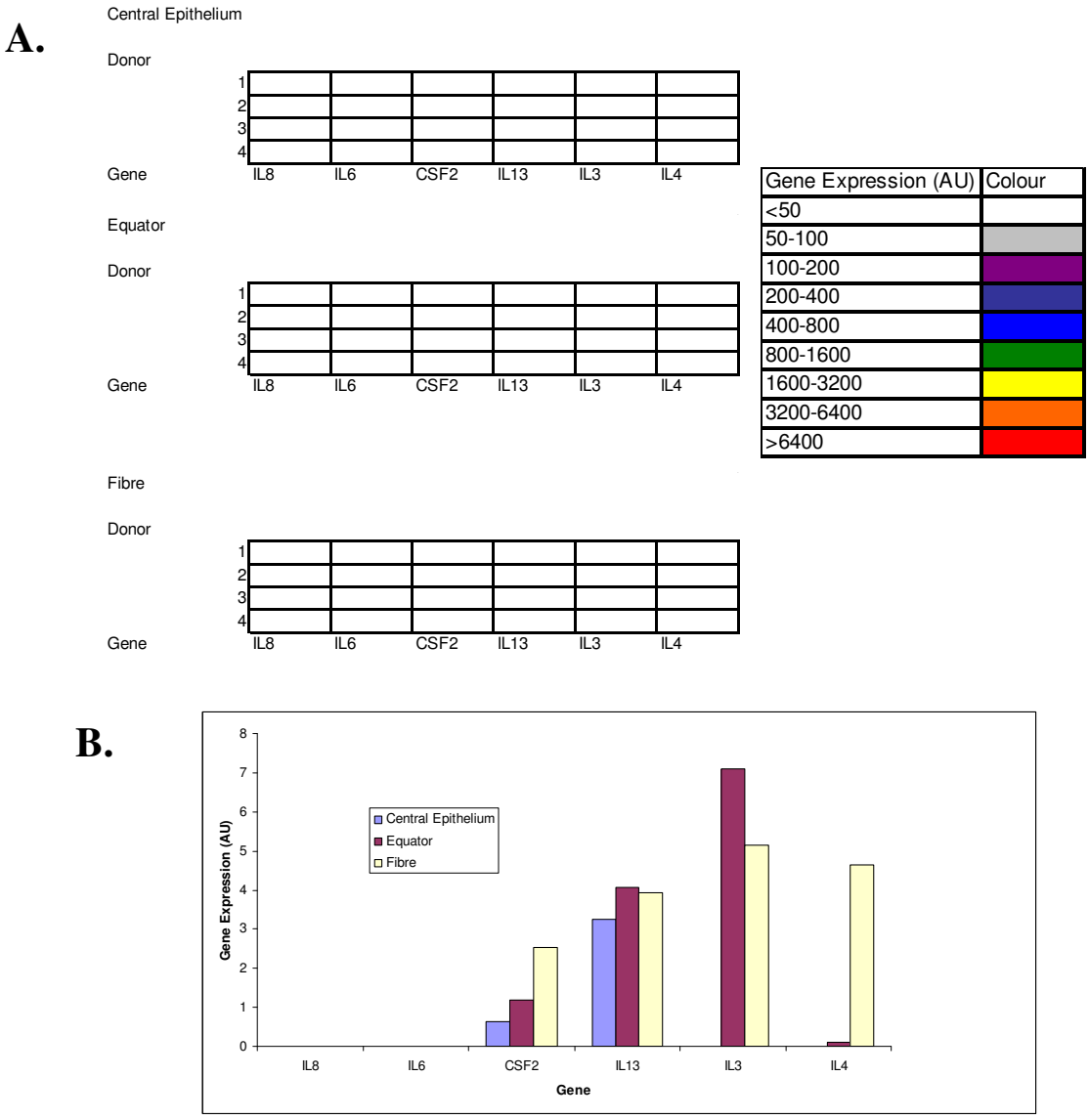
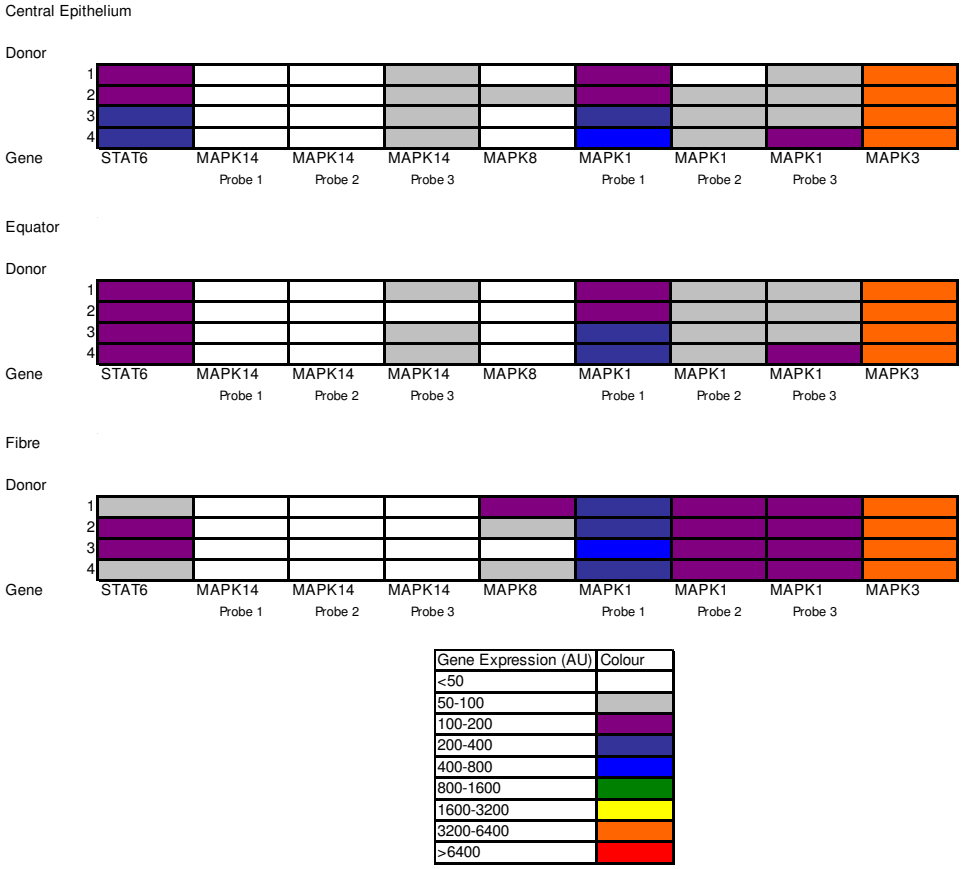


Fig. 3.4. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes encoding cytokines associate with Histamine in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

MAPK3 (ERK1), encoding a secondary messenger in cell signalling, was observed at significantly higher levels than other secondary messengers in all regions of the lens

(Figure 3.5). *MAPK1(ERK2)* and *STAT6* were also detected at moderate levels compared to other second messengers in all three regions; Detected levels of *MAPK1* were significant in fibre cells relative to the epithelial populations.

A.



B.

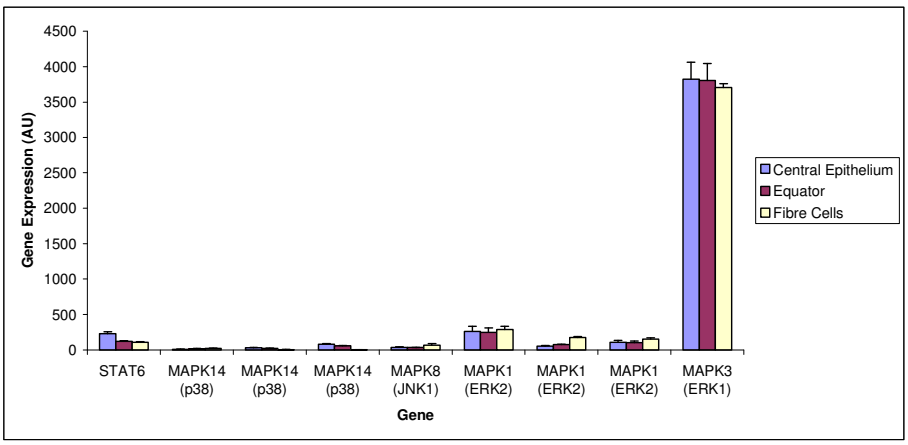


Fig. 3.5. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes associated with histamine and cell signalling in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

Three further genes analysed that are associated with the inflammatory response, namely von Willebrand factor (*VWF*), Defensin beta 4 (*DEFB4*) and prostaglandin-endoperoxide synthase 2 (*PTGS2*) were studied (Figure 3.6). Of these three, only VWF was detectable in the lens; however levels were relatively low and only significant in the equatorial region.

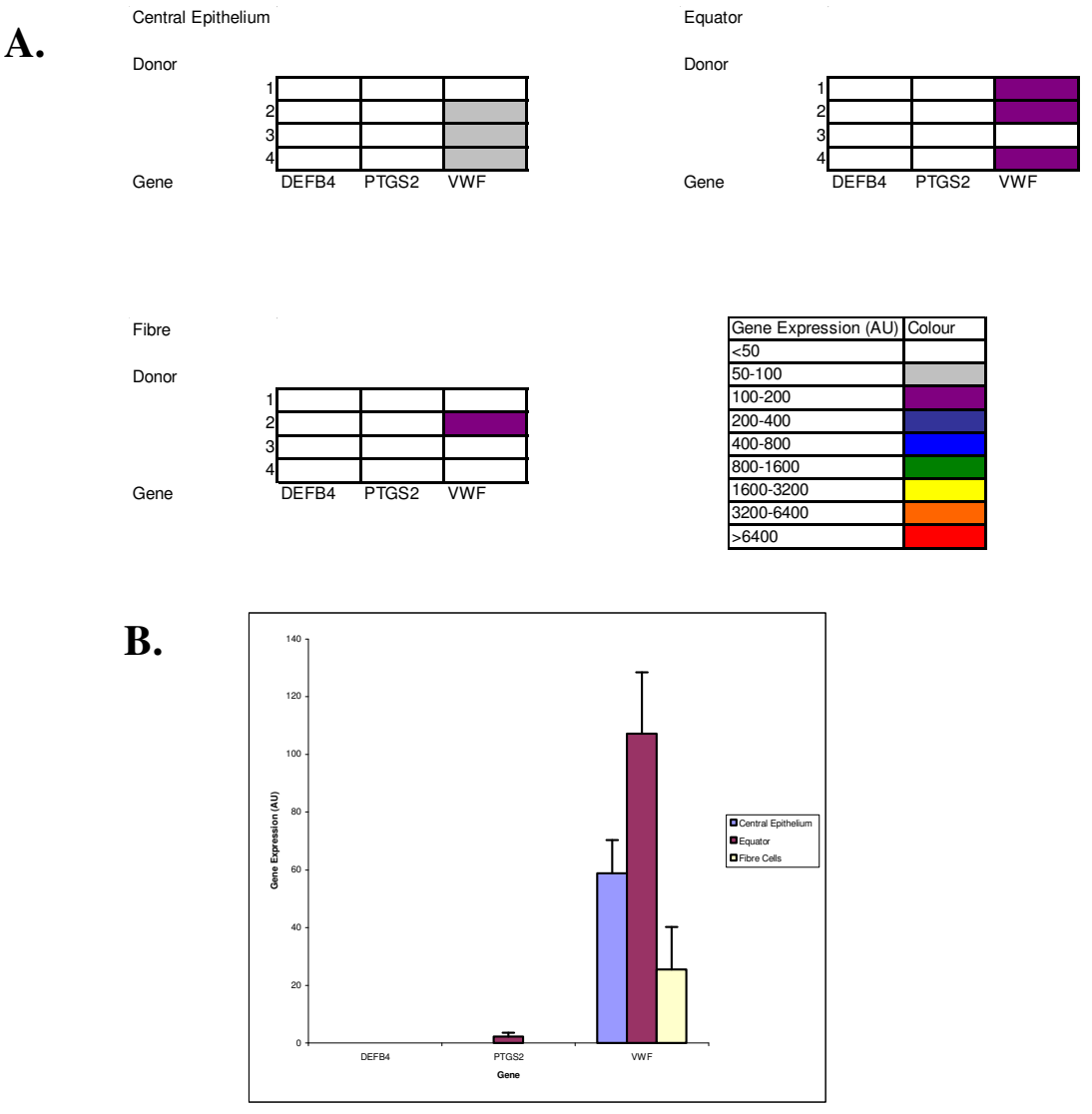


Fig. 3.6. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes associated with an inflammatory response and histamine in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

CTNNB1 (Catenin) encodes a protein that forms adherin junctions and anchors the

A.

cytoskeleton. It was detected at significant levels in all regions of the lens with very little difference in the expression pattern between these regions (Figure 3.7). Significantly high expression levels of *LASP1*, encoding an actin binding protein, were observed in the central epithelium and equatorial regions; expression in the fibre cells was lower but still significant. *PPP1R14A*, encoding a smooth muscle myosin phosphatase inhibitor, was detected at significantly higher levels in the fibre cells compared with the other two regions where it was detected at relatively low levels (Figure 3.7).

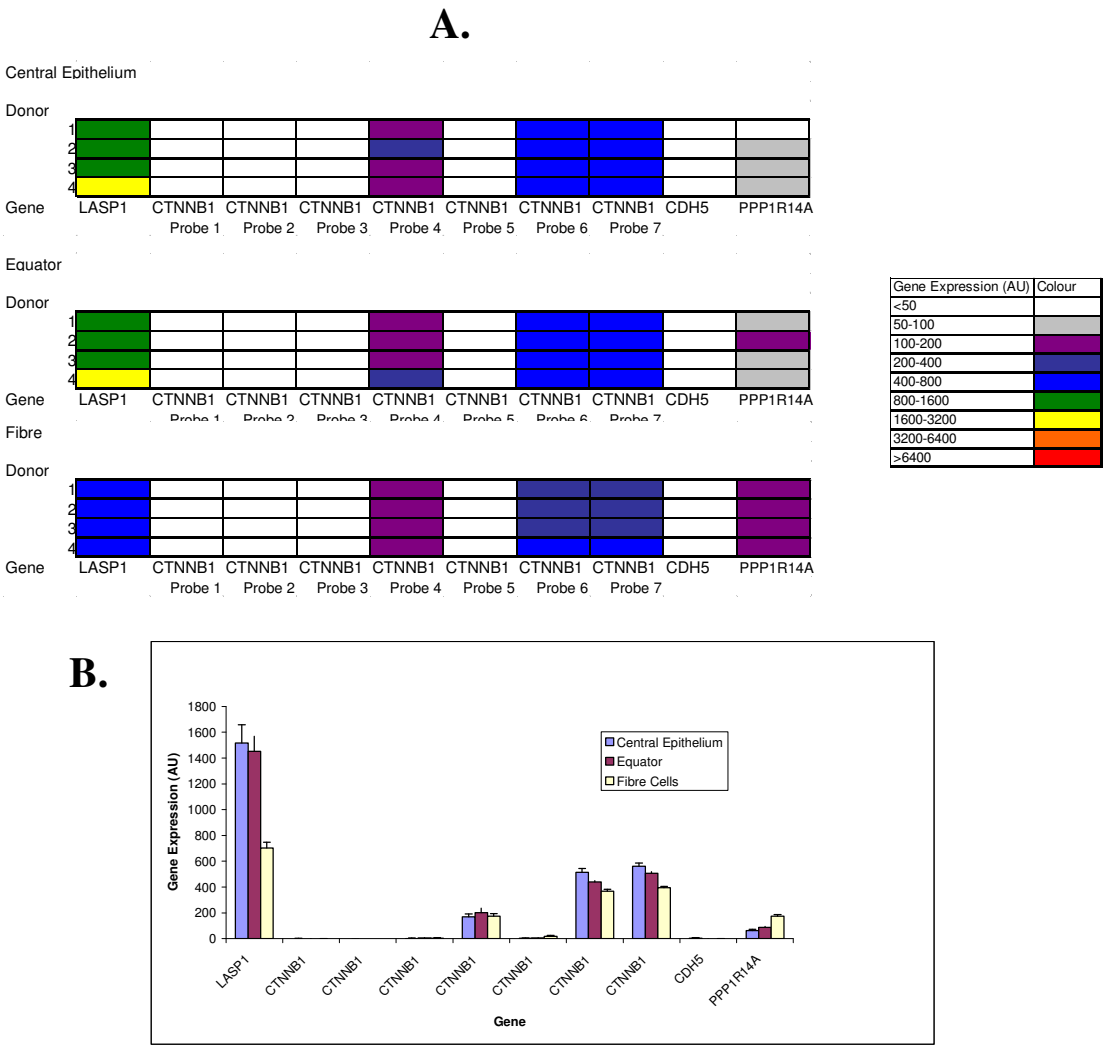


Fig. 3.7. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes associated with cellular junctions, cytoskeleton that are linked to histamine regulation in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

Cell Receptors that are activated by histamine induced mediators were also assessed. No significant level of expression was detected for any of these genes. *GABRB3* was the only gene showing an indication of expression, but this was not significant (Figure 3.8).

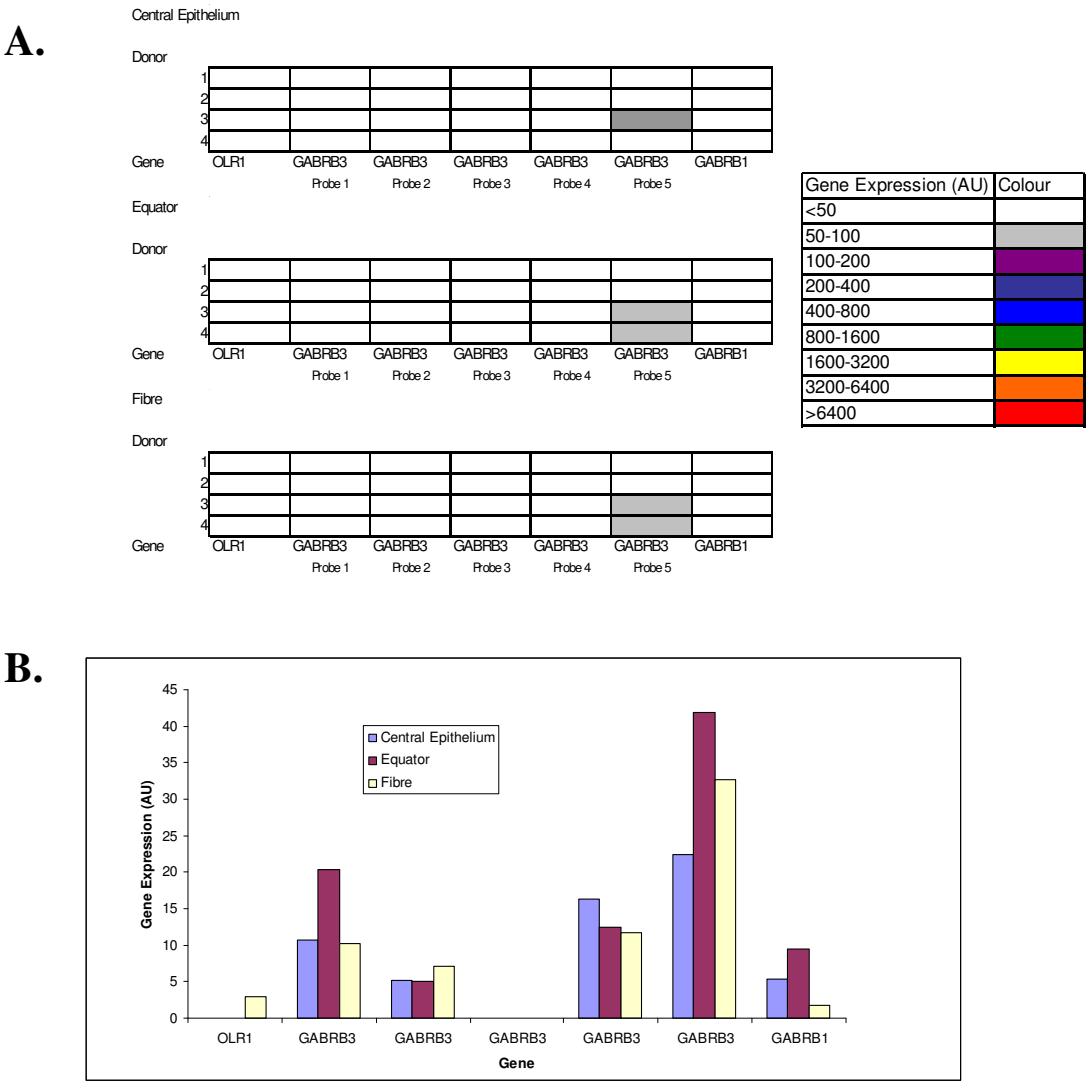


Fig. 3.8. Heat maps of gene expression of genes in three distinct regions of the human lens. All the genes were expressed at low levels in all three regions of the lens. Expression of genes and gene fragments encoding receptors influenced by Histamine, in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

3.1.4 Inhibitors and Inducers of Histamine Release

All the inhibitors analysed were not detected at significant levels in any region of the lens (Figure 3.9).

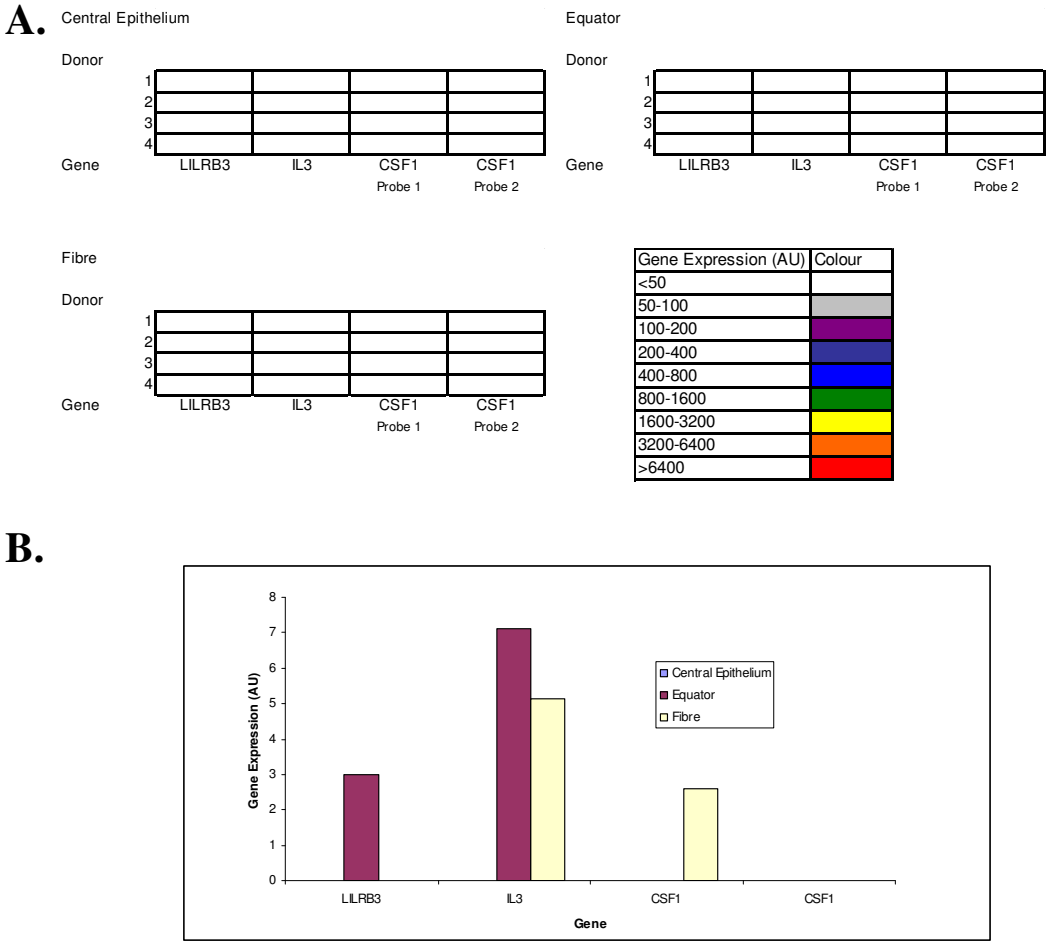


Fig. 3.9. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of gene fragments associated with the inhibition of Histamine release, in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

In contrast the inducers of histamine release were detected at significant levels. Tumour protein translational controlled 1 also known as histamine releasing factor (*TPT1* or *HRF*) was detected at significantly high levels in all three regions of the lens. *ABL1*, a tyrosine kinase receptor, was also detected at significant levels in epithelial regions but not the fibre cells. *SYK*, another tyrosine kinase, was observed at in the central epithelium at a higher level than in the equatorial region, but not significantly, and was not detected in the fibre cells. *BCR* was only observed at significant levels in the central epithelium and only detected at low levels in the equatorial region and fibre cells (Figure 3.10).

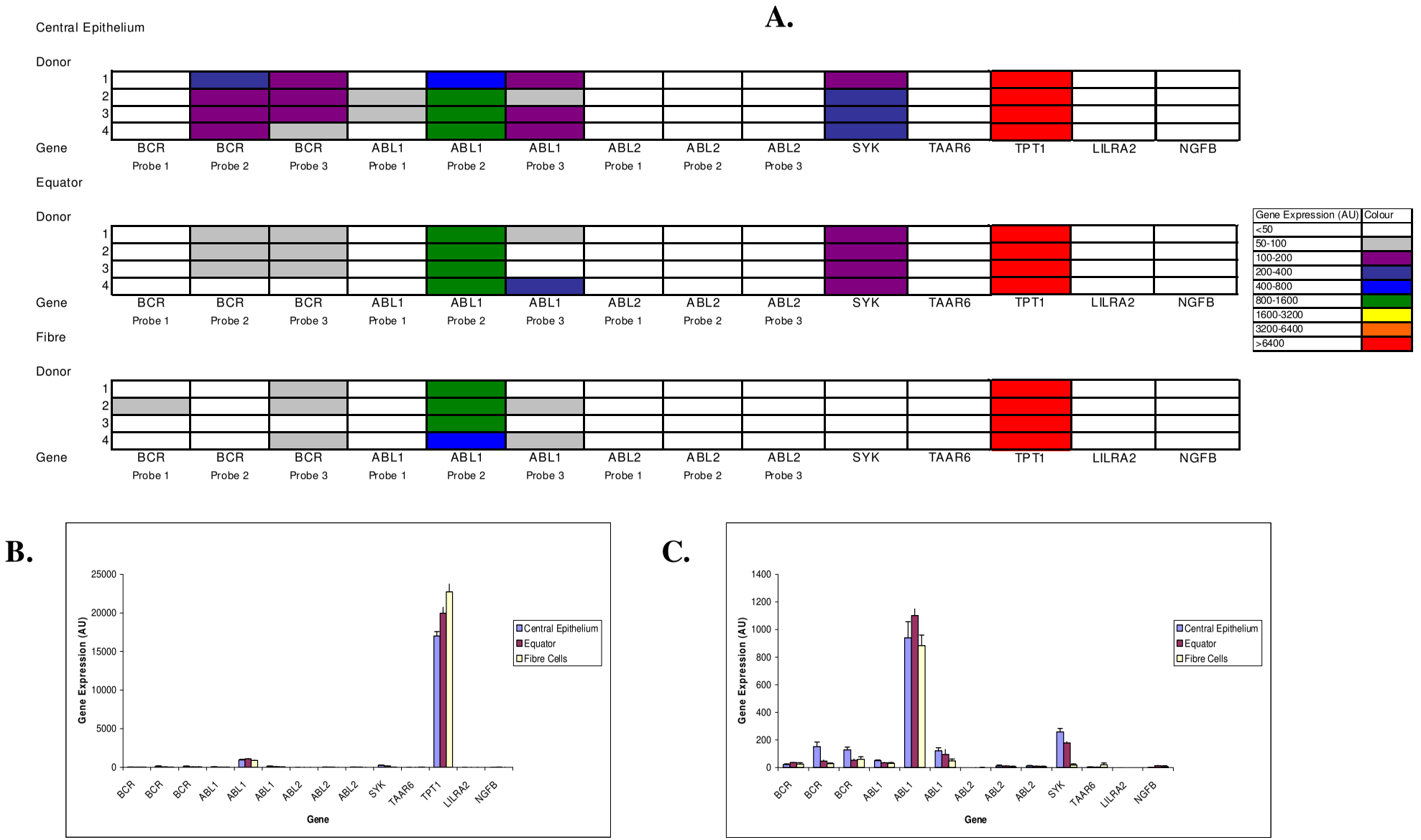


Fig. 3.10. Heat maps of gene expression of genes in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. . **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values including TPT1 and **C.** a bar chart not containing TPT1. (Mean ± SEM) from all four donors.

CDC25B was detected at significantly high levels in both the central epithelium and equatorial region (Figure 3.11). It was also detected at lower levels in the fibre cells but this was still significant. No other gene associated with cell cycle control was detected at a significant level, though *CDC25C* was detected at in the fibre cells albeit at a very low level (Figure 3.11).

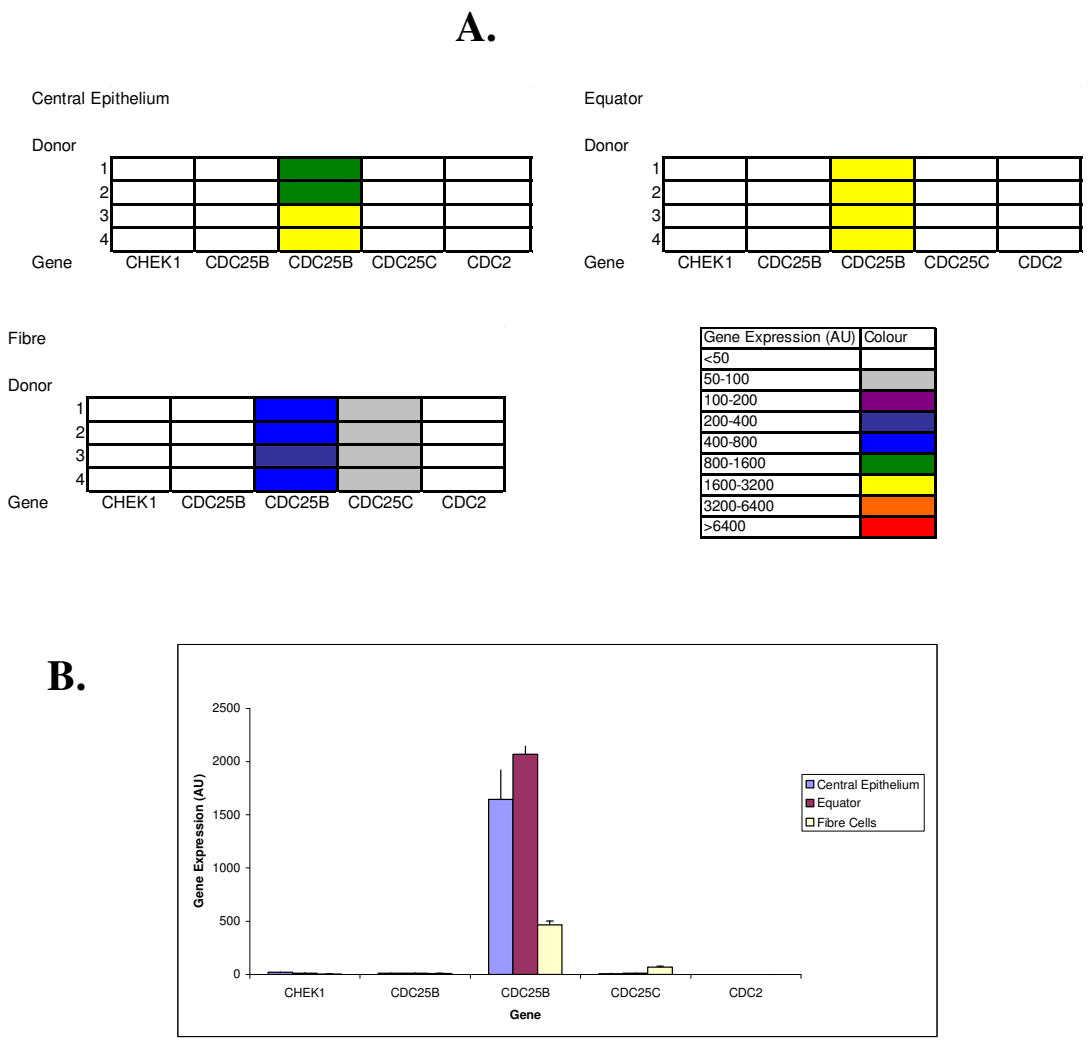


Fig. 3.11. Heat maps of gene expression of genes in three distinct regions of the human lens. Specifically expression of genes associated with histamine and cell cycle control in three distinct regions of the human lens. Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

Selected genes were used to show that the samples for each region are pure samples. When the samples for each region were analysed the expected pattern of expression was observed (Figure 3.12).

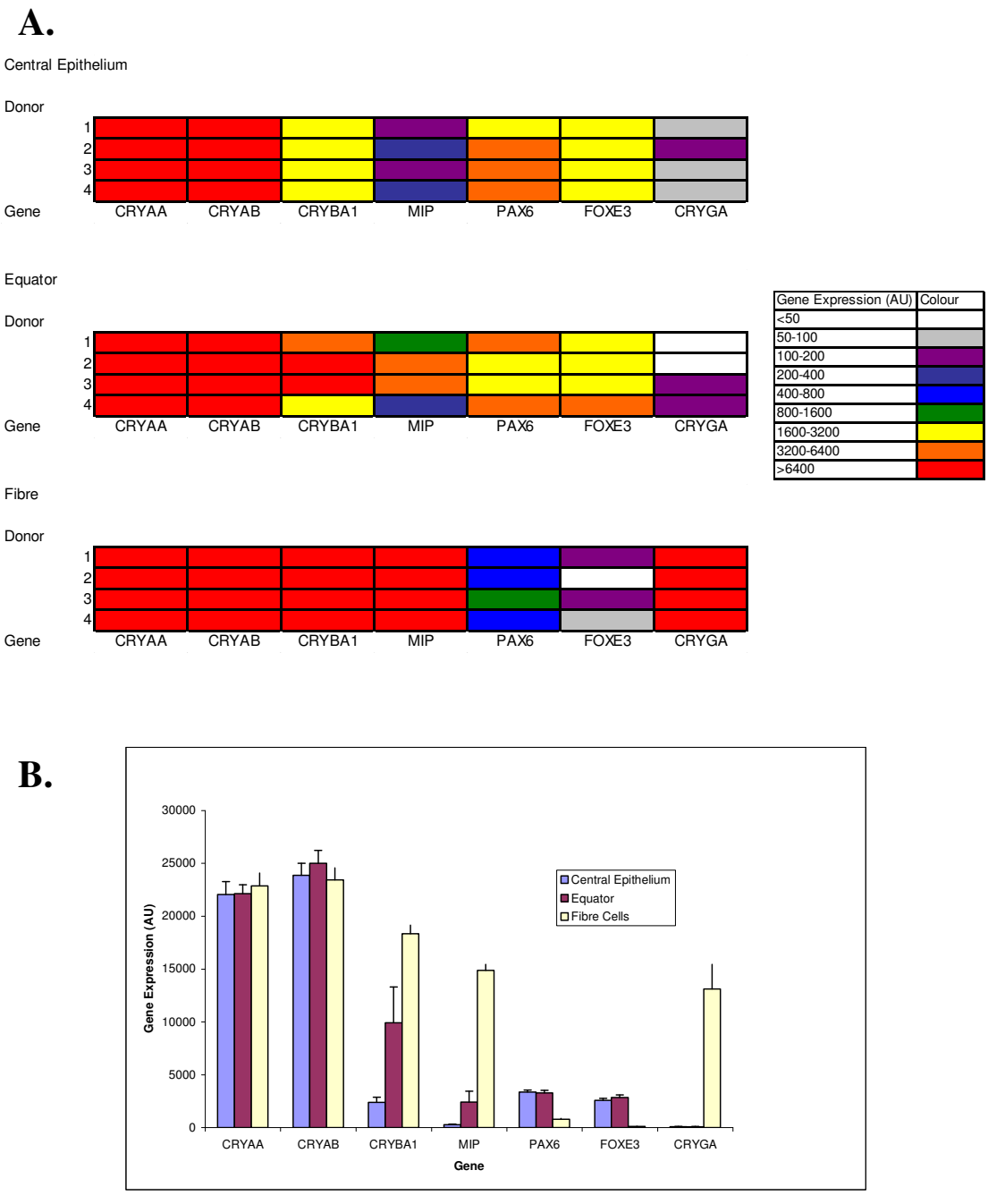


Fig. 3.12. Heat maps of the expression of genes. Expression of gene markers of lens cells in 3 regions of the human lens demonstrate . Expression was ascertained following analysis of Illumina gene array data sets. **A.** The data are presented as heat maps showing expression for each donor lens and **B.** as a bar chart showing pooled values (Mean ± SEM) from all four donors.

3.2 Growth

3.2.1 Histamine Induced Growth of FHL 124 Cells

Data obtained from patch growth assays revealed a concentration-dependent growth of FHL 124 cells in response to histamine. A promotion of growth was observed following addition of 100μM histamine, such that a 15.2 ± 5.8% increase in cell population was detected (Figure 3.13). Moreover, 100μM histamine also increased patch area by 12.4 ± 6% relative to the serum free control (Figure 3.14). To provide a more sensitive evaluation of histamine effects on cell proliferation, the 3H thymidine incorporation assays were employed. Using this system, the data again show a concentration-dependent response to histamine (Figure 3.15). Significant effects on proliferation were observed following the application of 30 or 100μM, such that observed levels were 155.2 ± 7.6% and 164.9 ± 14.1% respectively when compared to serum free controls.

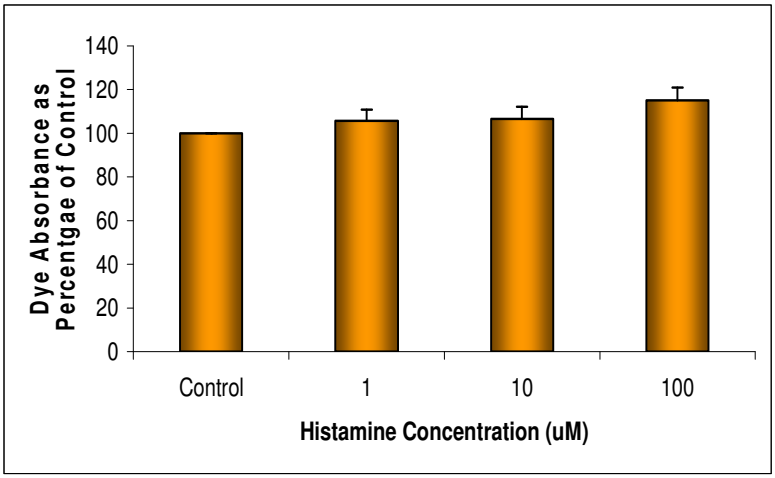


Fig. 3.13. Cell population determined by total protein levels following a 72 hour incubation period in the presence of 1, 10 and 100uM Histamine. There were no significant changes in cell population but there is a general trend that dye absorbance increases with histamine concentration. (N=11)

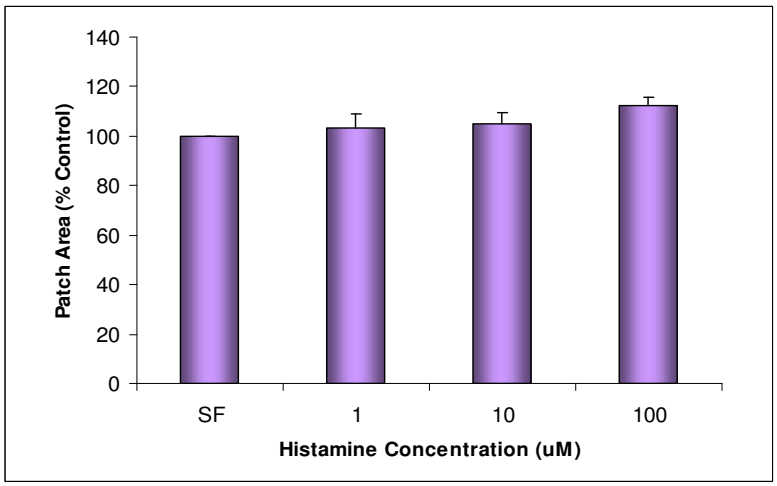


***Fig.* 3.14.** Cell growth/coverage assessed by changes in cell patch area following a 72 hour incubation period in the presence of 1, 10 and 100uM Histamine. There were no significant changes in cell population but there is a general trend that dye absorbance increases with histamine concentration. (N=11)

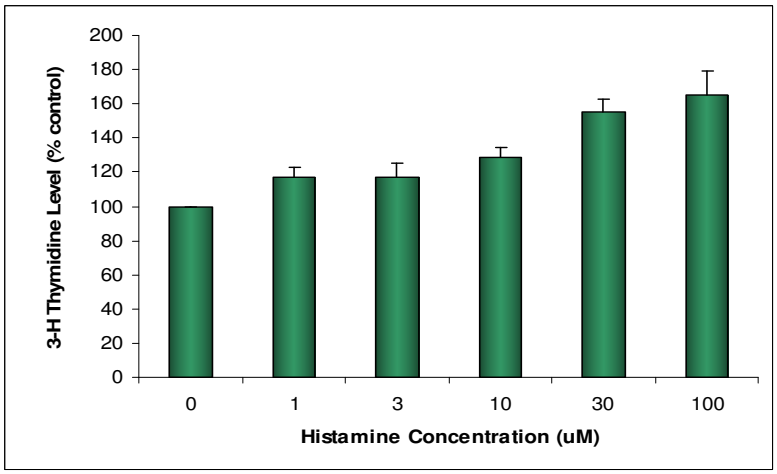


Fig. 3.15. 3H-Thymidine levels in FHL-124 cells, as percentage of control, following incubation with 0, 1, 3, 10, 30 and 100uM Histamine. There was a significant increase in 3H Thymidine levels when FHL-124 cells were incubated with 30 and 100uM Histamine. The other concentrations showed a dose dependant increase over control levels but were not significant increases. Data are expressed as mean ± SEM, n = 3. * indicates a significant difference between treated group and baseline (ANOVA with Dunnett's post-hoc analysis, $p \leq 0.05$).

3.2.2 Histamine Induced Growth is Histamine Receptor 1 Dependent

Cells maintained in the presence of 30μM histamine showed an increase in both cell population and patch area relative to the control group that were 45.1 ± 4.77% and 11.93 ± 3.32% respectively (Figure 3.16). Cells maintained in the Histamine receptor 1 antagonist, triprolidine at 100μM had a similar cell population and patch area to the

serum free control. Cells maintained in the presence of 100µM triprolidine and 30µM histamine had cell populations and patch area that significantly differed from the histamine treated group (Figure 3.16). Therefore, triprolidine suppressed the histamine induced response.

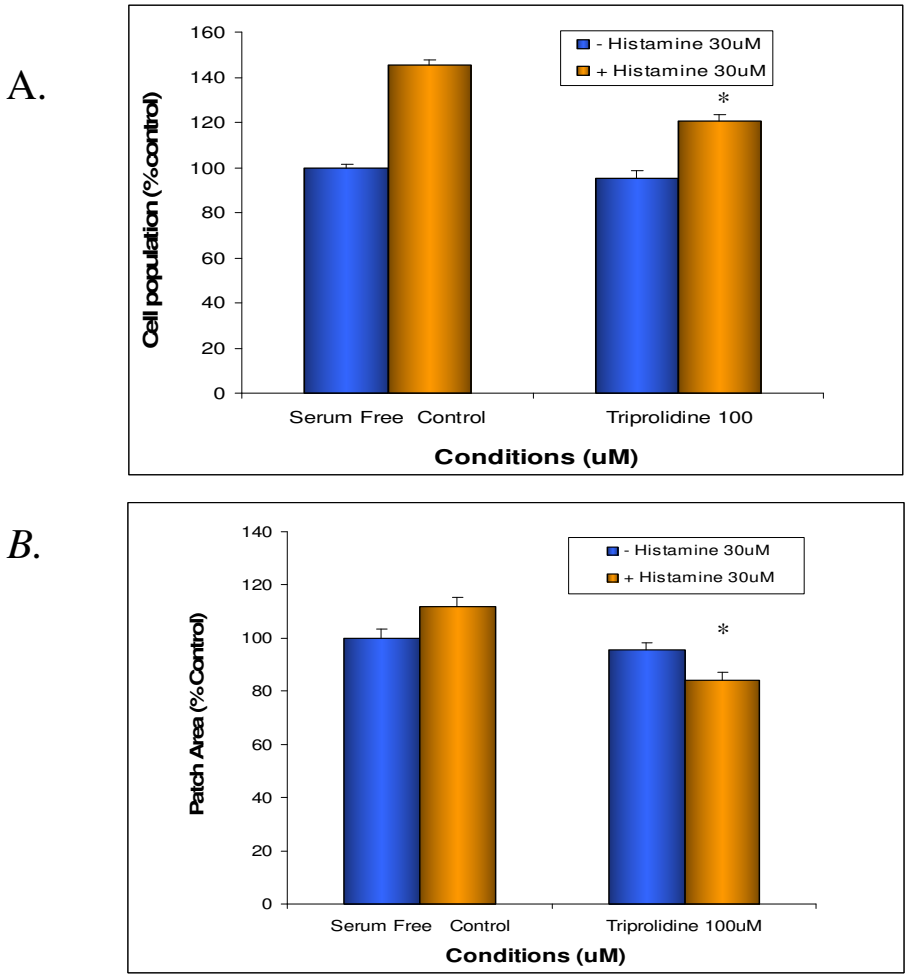


Fig. 3.16. HRH1 dependent histamine induced growth. (A) Cell population determined by total protein level and (B) growth assessed by changes in patch area in response to 30µM histamine in presence and absence of 100µM triprolidine. Data are expressed as mean ± SEM, n = 4. * indicates a significant difference between control cells and corresponding triprolidine treated group (ANOVA with Tukey's post-hoc analysis, $p \leq 0.05$).

3.2.3 Histamine Induced Cell Growth on Capsular Bags

Two sets of matched capsular bags were prepared as described in the methods section. One capsular bag was maintained in serum-free medium and the other supplemented

with100µM histamine. The preparations from different donors grew at different rates; however in both cases the capsular bag maintained in 100µM histamine covered the posterior capsule faster than the match-paired control (Figure 3.17).

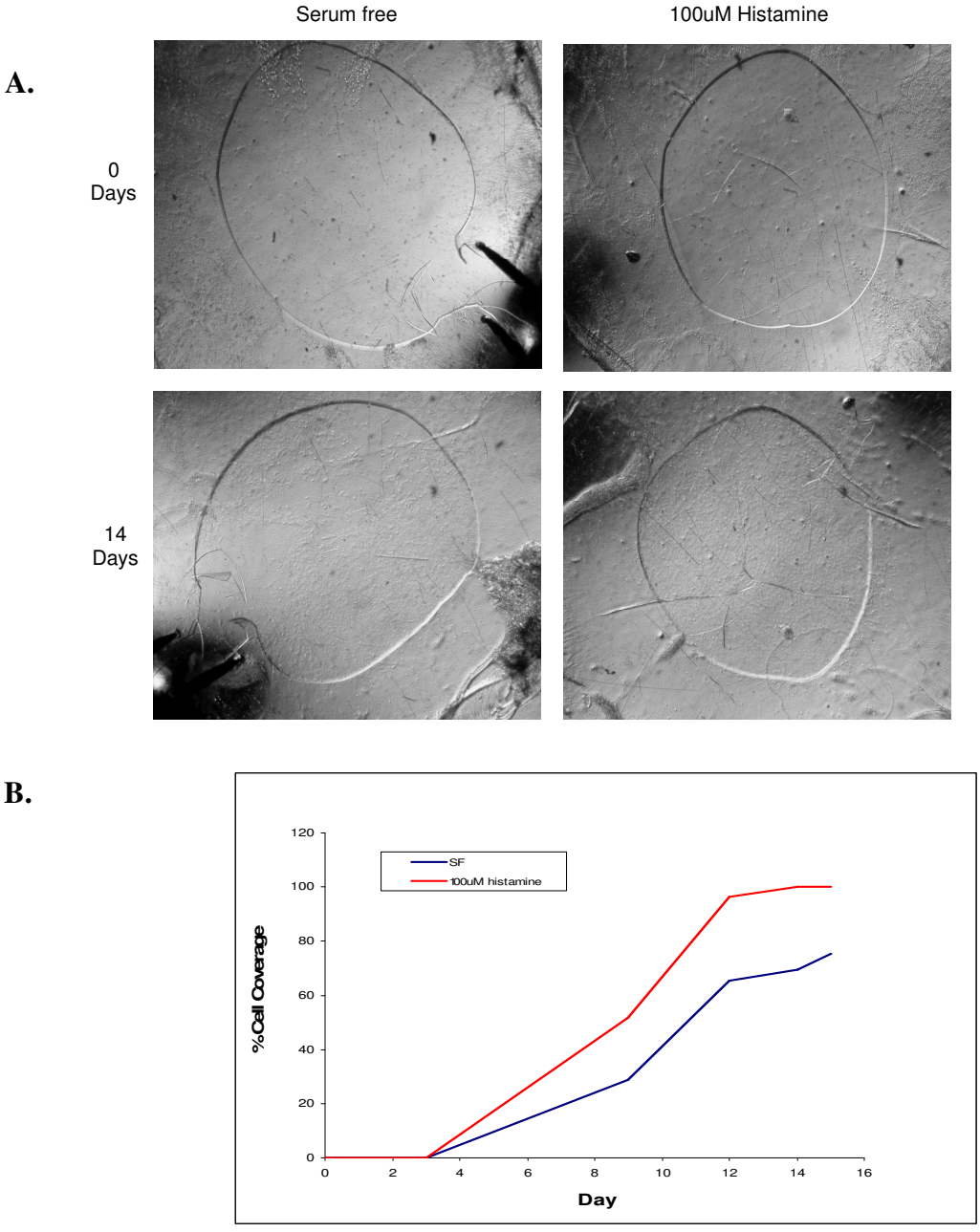


Fig. 3.17. A Low-power modified dark field images of capsular bags immediately following preparation and following 14 days of culture in the presence or absence of 100µM histamine. **B** The progression of growth across the posterior capsule of the illustrated preparations is presented above. This experiment was repeated on one further occasion and produced similar results.

3.3 Intracellular signalling

3.3.1 Histamine Induced Calcium Signalling is Mediated through H1 Receptors

No significant change in baseline intracellular calcium concentration was observed with 0.1µM histamine. Addition of histamine at 1µM and above showed a concentration dependant release of calcium from the intracellular store of FHL-124 cells (Figure 3.18).

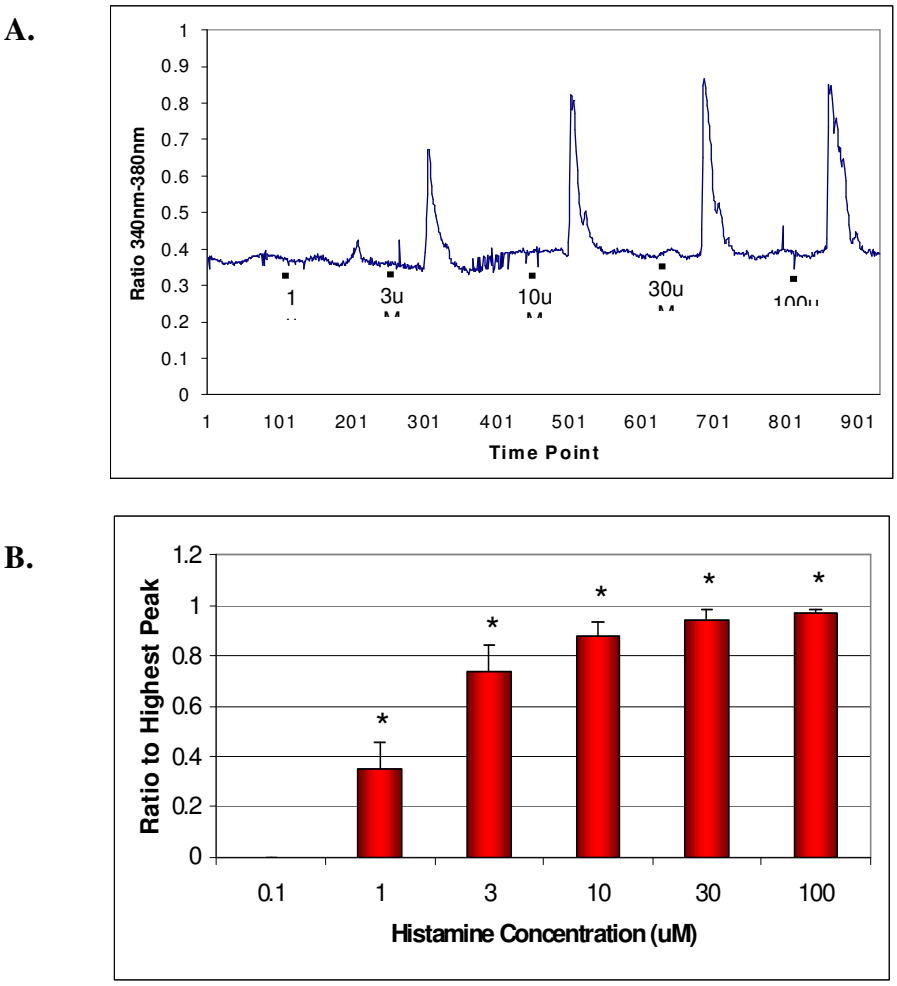


Fig. 3.18. Histamine modulates calcium mobilisation in FHL 124 cells in a concentration dependent manner. **A.** A typical trace, showing an incremental increase in the ratio of signal following excitation of FURA2 at 340 and 380nm; an increase in ratio corresponds to elevated intracellular calcium level. **B.** Pooled data showing the relative calcium response at different histamine concentrations; data are expressed as mean ± SEM, n = 4. * indicates a significant difference between treated group and baseline (ANOVA with Dunnett's post-hoc analysis, $p \leq 0.05$).

To determine which histamine receptors were responsible for the calcium response, molecular antagonists for the three main groups of histamine receptor were used (Figure 23). Pre-treatment with 100µM triprolidine, an HRH1 antagonist, completely negated calcium release typically associated with 100µM histamine When 100µM histamine was added following pre-treatment with 100µM Ranitidine, an HRH2 antagonist, or 10µM Thioperamide, an HRH3 antagonist, no significant inhibition was observed (Figure 3.19).

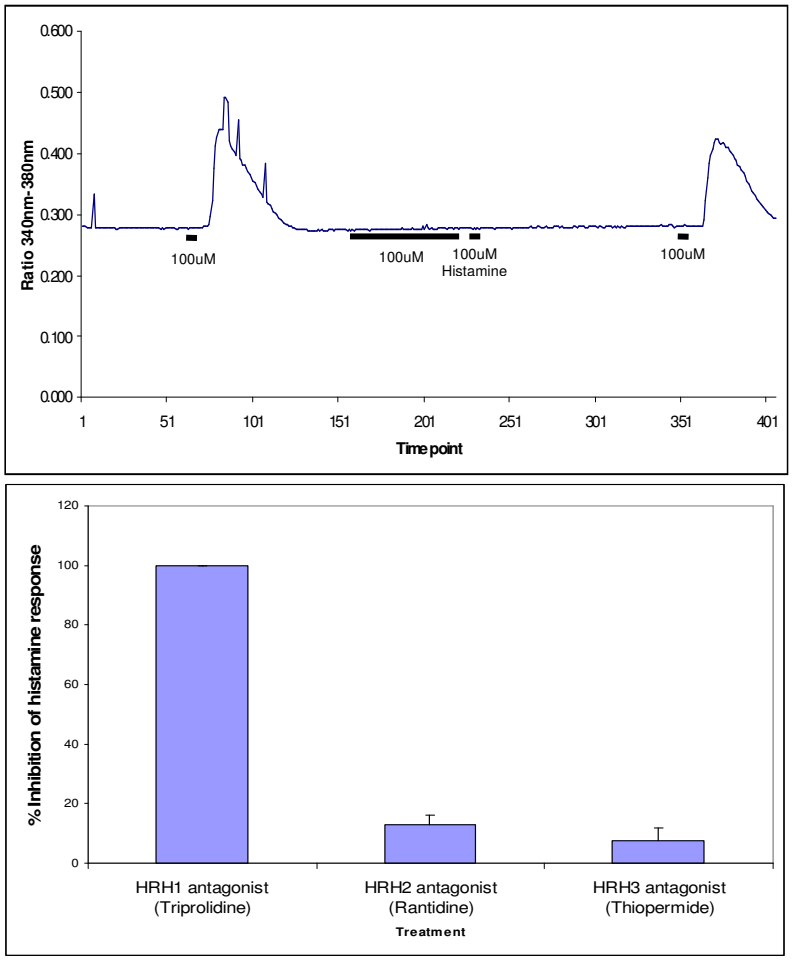


Fig. 3.19. Characterisation of histamine induced calcium mobilisation in FHL 124 cells. **(A)** Pre-incubation with the HRH1 antagonist Triprolidine (100µM), caused total inhibition of the histamine-induced response. Pre-incubation with HRH2 or HRH 3 antagonists did not significantly perturb histamine induced calcium mobilisation (data not shown). 100uM ATP was added to demonstrate that the calcium store was unaffected by the antagonists. (B) Pooled data showing the ability of HRH antagonists to inhibit a 100µM histamine induced calcium response. Data are expressed as SEM, n≥3. * indicates a significant difference between antagonist treated group and histamine treated control (students t-test, $p \leq 0.05$).

3.3.2 Histamine Activates Additional Signalling Pathways

A time course was performed to see the effects of 100μM histamine upon the expression of three signalling molecules using BIOplex analysis (Figure 3.20). The levels of phosphorylated P38 (p-P38) were seen to increase within minutes of histamine addition and continued to rise until levels peaked at the 10 minute time-point before gradually decreasing and returning to baseline one hour following initial stimulation (Figure 3.20). A similar sequence of events was observed with pERK levels and to a lesser extent pJNK. Peak values for p-P38 and pERK were significant relative to unstimulated controls, such that the change in level was 2.1 and 2.6 fold respectively (Figure 3.20). While pJNK in response to histamine was elevated 3.1 fold of control this was not significant due to variability in response (Figure 3.20).

3.3.3 ERK Mobilisation in Response to Histamine

Immunocytochemistry was used to evaluate the distribution of pERK following application of 100μM histamine (Figure 3.21). Following 5 minutes exposure to histamine a clear localisation of pERK is observed in the nucleus of FHL-124 cells. At the 10 minute time-point significant levels of pERK can be observed, which appears to be largely cytoplasmic in distribution. The level of pERK then progressively declines, such that levels are similar to unstimulated controls following 60 minute incubation. This pattern is similar to that seen in the BIOplex data for p-ERK.

3.3.4 Histamine Regulation of Cytokine Release.

The relative protein level of cytokines in FHL-124 cells were analysed using BIOplex suspended bead array analysis (Figure 3.22). This included a large number of

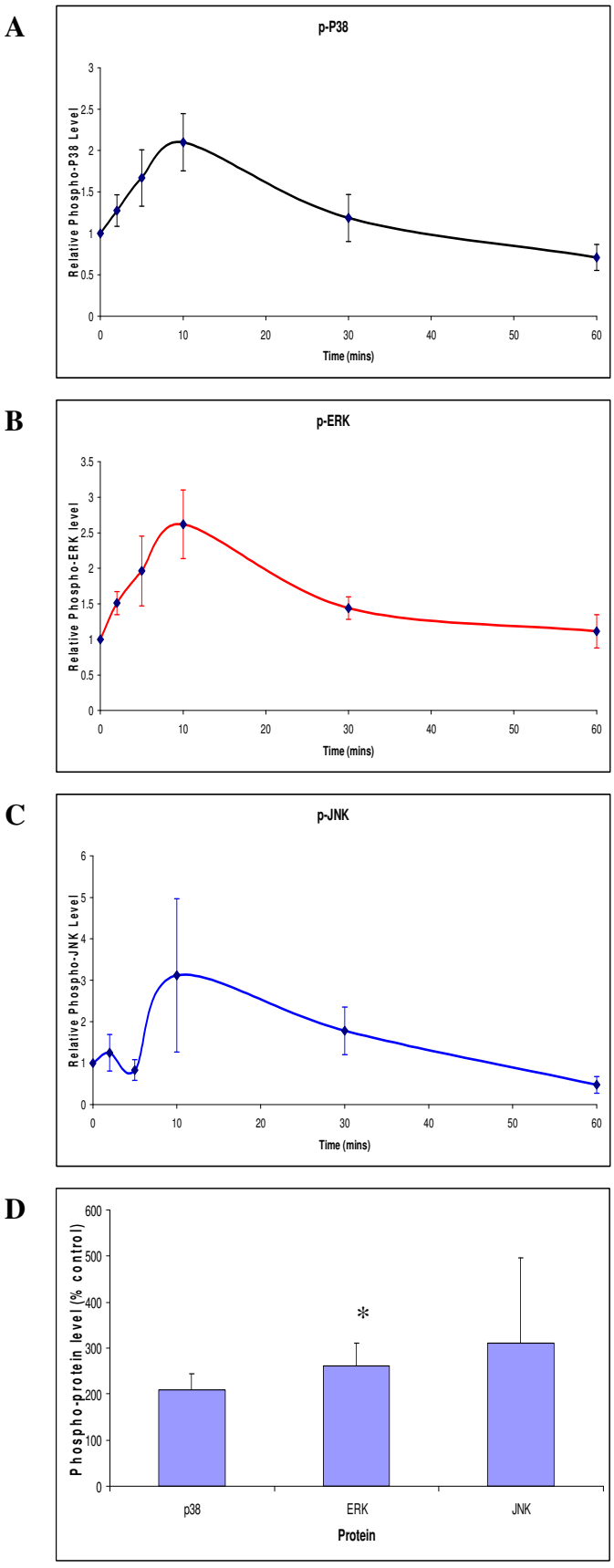


Fig. 3.20. Histamine-induced signaling detected by suspended bead array assay. Changes in phosphorylation levels of (**A**) P38, (**B**) ERK1/2, and (**C**) JNK in response to 100μM histamine were detected over a 1-hour period. The data represent mean ±SEM, n = 4 normalized to the corresponding total of ERK1/2, P38, and JNK for each sample respectively. (**D**) Mean phosphorylation levels at the 10 minute time point for the three target proteins. *Significant difference between histamine-treated and unstimulated control ($P \leq 0.05$, 1 tailed t-test).

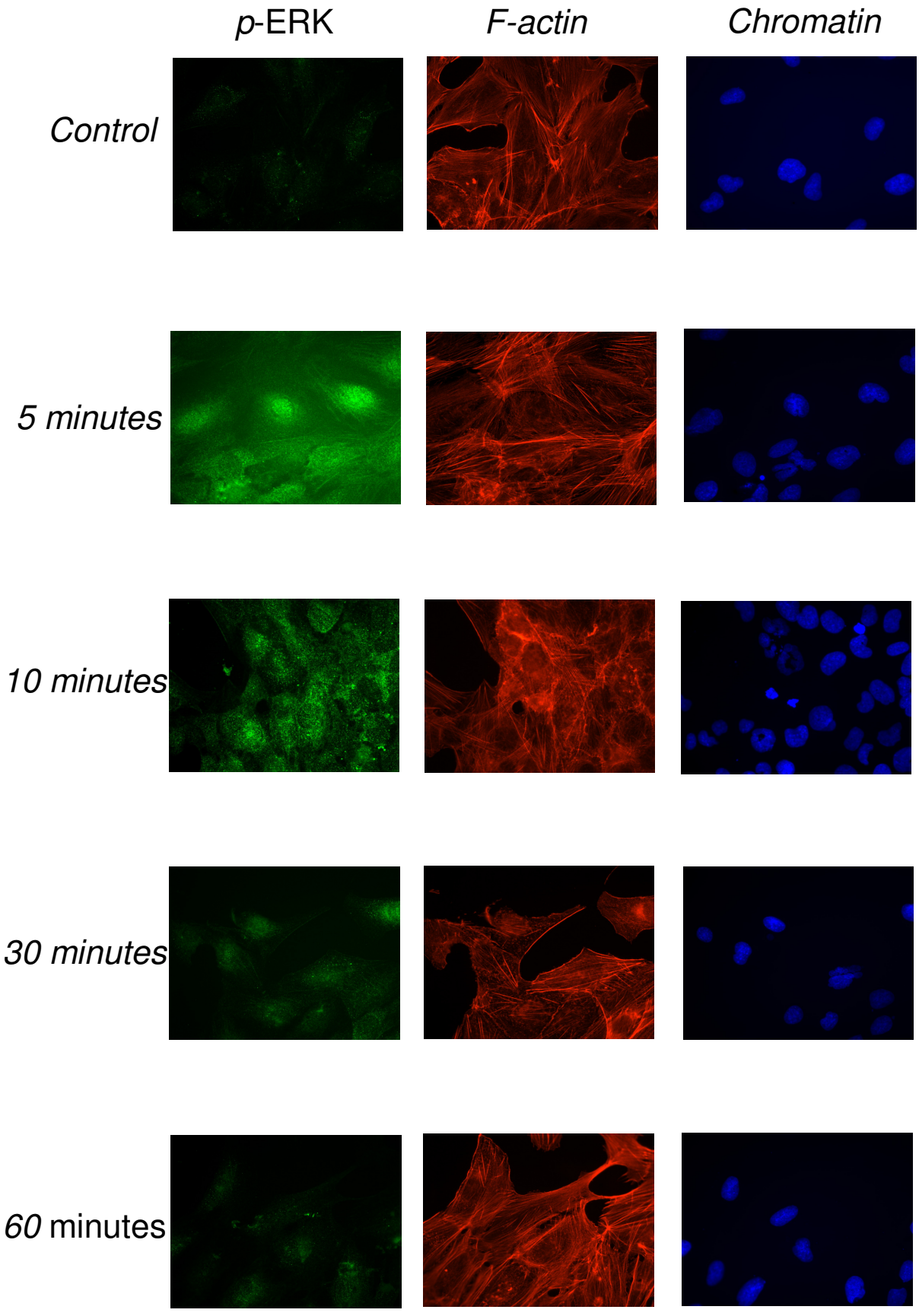
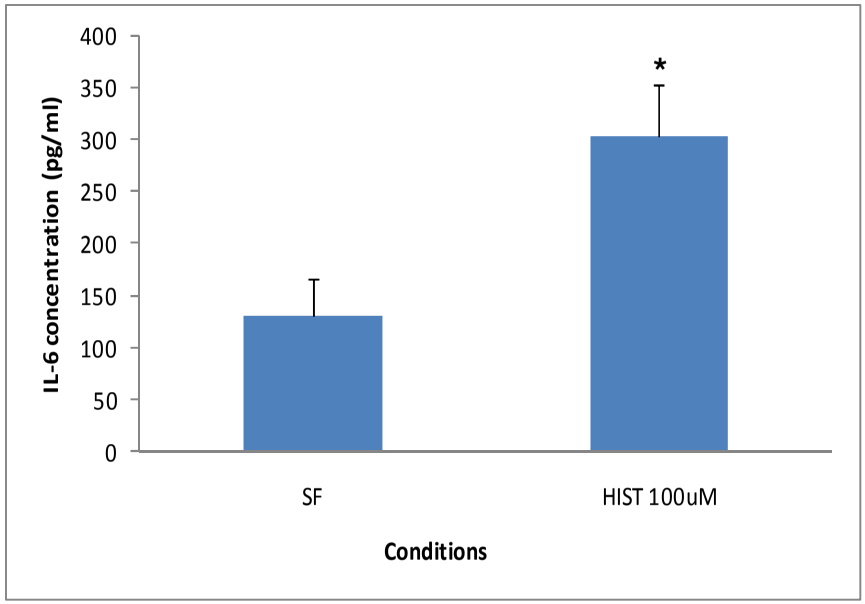


Fig. 3.21. Fluorescent micrographs showing p-ERK (Green) distribution in FHL-124 cells following exposure to 100uM Histamine. The actin (red) and chromatin (blue) stains are counterstained to show the cytoskeleton/overall cell shape and nuclear region of the cells respectively. This experiment was repeated on two further occasions with similar results obtained. The image represents a field of view 139 x 114µm.

Cytokine	IL-1b	IL-2	IL-4	IL-6	IL-8	IL-10	IL-5	IL-7	IL-12	IL-13	IL-17	IL-9	IL-15
SF	ND	ND	ND	131.4375	171.885	34.305	ND	3.7075	29.5725	27.7975	ND	58.06333	24.8525
HIST	ND	ND	ND	303.1875	321.418	46.88	ND	6.8225	45.8225	39.6625	ND	55.985	26.6725

Cytokine	GM-CSF	IFN-g	TNF-a	G-CSF	MCP1	MIP-1b	IL-1ra	Eotaxin	FGF	IP-10	MIP1a	PDGF bb	RANTES	VEGF
SF	15.22	ND	29.32	ND	798.58	78.24	126.47	109.02	112.19	3990.46	103.85	ND	375.14	413.11
HIST	ND	ND	ND	ND	994.99	53.80	126.47	ND	79.57	4363.34	133.11	92.78	556.54	499.17

Increase	
Decrease	
Not detected	



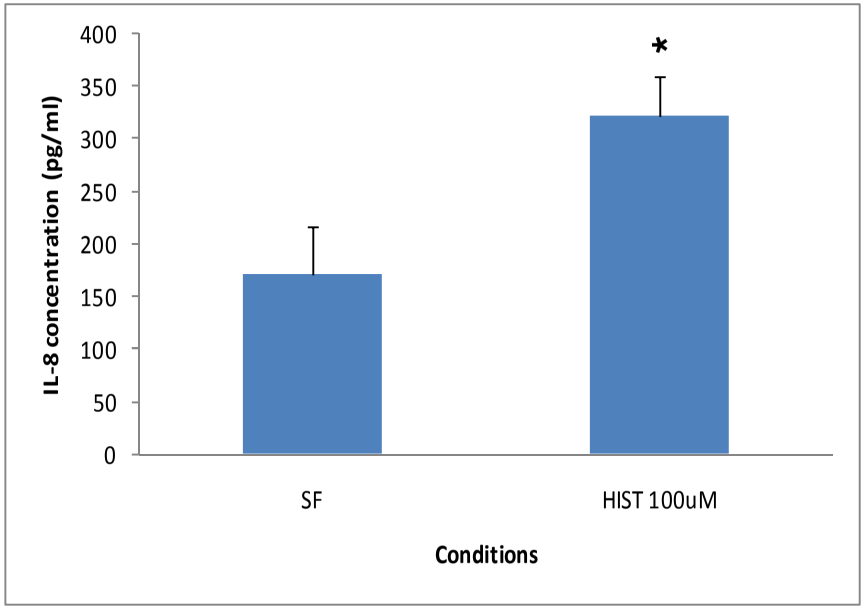


Fig. 3.22 Histamine-induced cytokine release detected by suspended bead array assay (A) Levels of detected in the culture medium of FHL 124 cells 24 hours following exposure to 100uM Histamine or maintenance in serum free (control) medium. (B) and (C) highlight changes in IL-6 and IL-8 levels in response to histamine. Data are expressed as mean ± SEM, n=4. * indicates a significant difference between groups (Students ttest, $p \leq 0.05$)

interleukins which are involved in inflammatory responses. The overall profile of interleukins expressed was not changed by the addition of 100μM histamine as no new interleukins were expressed and none were inhibited compared with serum free controls (Figure 3.22). However, interleukins IL-6 and IL-8 showed a significant increase in expression when cells were maintained in the presence of 100μM histamine compared to the serum free control (Figure 3.22). A change in expression pattern was also observed with the other cytokines analysed. Granulocyte macrophage colony-stimulating factor (GM-CSF), Tumour necrosis factor- alpha (TNF-α), and eotaxin were all expressed in serum free conditions but were then not detected following exposure to 100μM histamine (Figure 3.22). Platelet-derived Growth Factor-BB (PDGF-bb) was not detected in serum free conditions but was detected in response to 100μM histamine addition. Macrophage Inflammatory Protein-1 beta (MIP-1b) and fibroblast growth factor (FGF) showed decreases from the serum free levels when in the 100μM histamine conditions though not significant. Monocyte chemotactic protein-1 (MCP-1), interferon-inducible protein 10 (IP-10), Macrophage Inflammatory Protein- 1 alpha (MIP-1a), regulated upon activation, normal T cell expressed and secreted (RANTES), and Vascular endothelial growth factor (VEGF) all showed increase when in 100μM histamine conditions though none were significant increases (Figure 3.22).

4. Discussion

The current project revealed a number of interesting findings that have furthered our knowledge of histamine regulation of the human lens. The normal human lens does not express the machinery to synthesise histamine, but demonstrates high levels of releasing factors and histamine receptor. Stimulation of lens cells with histamine promotes multiple signalling pathways and increases growth rate. This information has relevance to the homeostasis of the normal lens and progression of lens pathologies.

4.1 Histamine and the Normal Function of the Lens

Gene array analysis of three regions in a normal clear lens was extremely informative. This gave insight into the possible functions for histamine in the lens by seeing which genes were expressed and importantly those that were not.

4.1.1 The Lens Can Respond to and Break Down Histamine but Does Not Contribute to the Histamine Stored within the Eye

If histamine is to play a role in the normal lens it is imperative that it can respond to the stimuli if available and with this regard HRH1 was detected in the lens epithelial cell compartments. This concurs with previous studies on the lens, which demonstrated a H1 receptor dependent calcium response in the native human lens {R. A. Riach, 1995 #6}. However, the current data suggest that histamine elicits all its actions through this receptor as HRH2-4 receptors were not detectable. HRH1 has primary roles in inducing smooth muscle contraction and mediating inflammatory responses but can promote a wide number of additional cell functions, which include

migration and proliferation typically through mobilisation of intracellular calcium stores {Hill, 1990 #11}{S. J. Hill, 1997 #44}{Stark, 2007 #45}.

As the lens has the ability to respond to histamine signalling it is logical that breakdown systems are present to regulate the level of signalling. With regards to this the lens expresses histamine N-methyltransferase (*HNMT*) in all three regions and Amine oxidase, copper containing 2 (*AOC2)* in the equatorial region.

The lens however is unlikely to contribute to the histamine pool within the eye. This is perhaps predictable as histamine is traditionally stored in specialised cells such as mast cells that are not located within the lens {M. E. Parsons, 2006 #54}. Nevertheless the array data confirm this as histidine decarboxylase (HDC), the gene involved in histamine production, was not detected. Therefore, if histamine is to stimulate the lens it must become available through non-lenticular sources, many of which are known to exist in the eye. The Iris and choroidal tissue for example both contain mast cells and can store histamine {McMenamin, 1997 #55}{May, 1999 #34} {T. Nakagami, 1999 #35}. There are also conditions such as uveitis and pterygia when normal histamine function alters leading to eye pathologies. {N. Girolamoa, 2004 #15} {C. H. Lee, 1993 #56}

The most widely understood process for histamine release in the eye is as a consequence of an inflammatory response. It is reported that a release of histamine is required to initiate uveitis, a chronic inflammation of the eye, {K. Yamashiro, 2001 #57} and is also implicated in conditions such as pterygium {R. Maini, 2002 #60}. However, it is interesting to note that the eye is not generally inflamed and thus

sources of histamine are present within the eye at all times and not just during pathological states. It is therefore possible that histamine plays a role in the normal functioning of the eye and histamine is regulated and released through processes other than an inflammatory cascade; processes that are not well understood. One possible regulator of histamine in the eye is histamine releasing factor, which is encoded by the gene TPT1.

TPT1, also known as histamine releasing factor (HRF), is expressed at very high level in all three regions of the lens. HRF was first discovered as a promoter of tumour growth in mice and no other function was found at that point. It has since been shown to be a heat shock protein and have anti-apoptotic properties. It is now known to be ubiquitously expressed with both intracellular and extra cellular functions. It was also discovered that HRF was identical to proteins that had histamine releasing properties {U. Bommer, 2004 #40}. Its ability to cause histamine release from mast cells and basophils has been widely investigated and were originally thought to be Ig-E dependent but further research has given weight to the argument that HRF mediates responses independently of Ig-E independent properties {J. M. Langdon, 2004 #18} {J. M. Langdon, 2008 #19}. It can also induce the release of several cytokines from basophils, T-cells and B-cells {U. Bommer, 2004 #40}. These cytokines can then prime basophils for histamine release through phosphorylation of several signalling molecules. The properties of histamine release from basophils in humans changes depending on the sub population of basophil donors. To the extent that individuals with allergic disorders have different molecular properties than those that don't {B. M. Vonakis, 2008 #20}.

HRF has been demonstrated to release histamine from multiple cell types found within different tissues of the eye {S. M. MacDonald, 1995 #58} {B. M. Vonakis, 2008 #20} {J. M. Langdon, 2008 #19}. If the HRF released from the lens can reach the histamine stores in surrounding tissue, such as the ciliary body and iris, then histamine could be released into the aqueous humour and be available to the lens. If this mechanism does exist then it opens up the question of why histamine is regulated in this way and what function does histamine play in the normal lens and surrounding tissue?

The lens is often put under mechanical stress to change the refractive power during the process of accommodation. Accommodation is characterised by changes in lens shape in order to focus on objects at different distances. It is not beyond the realms of possibility that mechanical events can cause the release of HRF into the surrounding tissue such as the iris where there are mast cells and basophils {May, 1999 #34}. The HRF would induce the release of histamine from these cells into the surrounding tissue including the lens. The cells in the lens would then respond to histamine through the HRH1 receptor inducing cellular changes that allow the lens cells to respond to mechanical stimulation. As the lens has to change its shape from a rounder to flatter configuration or vice versa during accommodation it also has to change its volume and therefore fluids have to be able to leave and re-enter the lens {R. Gerometta, 2007 #33}. Histamine could therefore play a role in changing the osmotic potential of the aqueous humour causing changes in fluid flow of the lens. Antihistamines cause side effects such as blurred vision especially of distance focusing {Pearson, 1950 #10} {J. M. White, 1988 #39}. However, it is not fully understood how antihistamines cause blurred vision but the fact that it does lends

support to the idea that histamine plays a role somewhere within the visual axis. One possibility is that changes in lens hydration cause the lens to thicken {M. A. Spiteri, 1983 #17}. This may result because histamine is reported to affect vascular permeability {G. Majno, 1961 #59} and therefore could play a role in changes to osmotic potential of the surrounding aqueous and vitreous humours and thus influence a number of tissues including the lens. However, the current body of data allows us to speculate that anti-histamine drugs could have direct actions on the lens that could influence accommodative change. This theory however, would require a number of questions to be answered to provide supporting evidence. These questions include: Can HRF be released from the lens in response to mechanical stimulation? If HRF is released can this cause histamine release from local ocular tissues e.g. ciliary or iris? What are the diffusion rates following histamine release from ciliary and iris? What level of histamine is predicted to become available to lens cells following release?

HRF can perform a number of biological roles; however its close association to histamine, the presence of histamine sources in the eye and the lens ability to respond to histamine make for an interesting model for histamine regulation of the lens.

4.1.2 Other genes associated with histamine in the normal lens

The gene array analysis also flagged up many genes associated with histamine with a wide range of functions. These included genes for cytokines that are involved in the inflammation cascade, genes encoding proteins involved with the cytoskeleton, cell-cell interactions and cell cycle control. The expression or lack of expression of these genes does not give a huge amount of information on its own. They do however give an idea of the areas that should be investigated further. The genes for the histamine

receptors immediately show that HRH1 is the most likely receptor to be responsible for the functions of histamine within the lens. HRH1 has also been shown to be the main receptor in the native human lens and that it invokes a calcium response {R. A. Riach, 1995 #6}. This means that histamine can initiate downstream responses through calcium signalling as HRH1 is a G-protein coupled receptor that causes a release of intra-cellular calcium {Stark, 2007 #45} {S. J. Hill, 1997 #44}. However other signalling pathways such as the MAP kinase pathway and the STAT pathway are also linked with histamine {A. J. Robinson, 2001 #61} {Lipnik-Stangelj, 2006 #62} {F. Hao, 2008 #14}, though this has not been previously demonstrated in the lens. This means that any response resulting from histamine stimulation is likely to involve cross talk between signalling pathways. This is supported by the fact that calcium signalling has been demonstrated many times to be able to activate a wide range of signalling pathways {M. J. Berridge, 2000 #3}. Regional differences across the lens epithelium have suggested this is possible. Histamine stimulates the human lens at both the central and equatorial region with similar potency {D. J. Collison, 2001 #68} when using calcium mobilisation as the output. However, other studies {J. M. Maidment, 2004 #69} revealed differences in ERK level between the two regions. Therefore, activation of different signalling pathways by histamine in different compartments of the lens could in turn elicit different functions, such as cell migration and proliferation.

The normal lens has the capacity to respond to histamine and it is interesting to consider an active role in the homeostasis and function of the lens. However, at present such ideas are formative and speculative requiring further experiments to test these concepts. There is little doubt, however, that histamine is available to the lens

during inflammation and under these circumstances is likely to affect the function of lens cells.

Inflammation is an important stage in the immune response but can be very damaging to the tissue/organ it is triggered in {P. M. Henson, 1987 #70} {Nathan, 2002 #71}. This would be particularly dangerous in the eye as it is an intricate structure where the smallest amount of damage to the visual axis can be detrimental to vision. This is why the eye has what is known as ocular immune privilege {Streilein, 2003 #36}. This means that the eye contains a different immunological environment to that of other organs of the body. This privilege takes the form of blood-tissue barriers, a lack of lymphatic drainage and tolerance-promoting Antigen-presenting cells. All this is a trade off so that the eye may have less ability to fight off infection or tumours but there is less chance of an autoimmune or allergic response damaging the eye {Streilein, 1996 #72}. When it comes to ocular immune privilege there has been little research on the consequences of histamine stimulation in the lens.

4.2 Histamine and pathologies

The current study demonstrates that histamine can induce a significant increase in growth rate of lens cells. While this may have implications for growth in the normal lens, it is also likely to play an important role in progression of PCO. Histamine is likely to be available to lens cells following the ocular immune privilege response following cataract surgery and therefore the observed responses are likely to play out *in vivo*. This is the first study to demonstrate that histamine can promote growth of human lens cells. A previous study using the rabbit lens cell line NN1003A did not

demonstrate a stimulation of growth in response to histamine {G. Duncan, 1996 #73}. This contrasting outcome however could result from species differences, which could involve the level of histamine receptors, the histamine receptor sub-type or the activation of different signalling pathways. The influence on growth by histamine has been reported in non-lenticular cells and tissues. For example, Leonardi et al demonstrated that proliferation of sub-conjunctival fibroblasts, from Vernal Keratoconjunctivitus patients, was promoted by histamine and this was regulated by HRH1 {A. Leonardi, 1999 #74}. Again, in the present study, HRH1 was found to be the key receptor mediator.

4.2.1 Histamine Induced Signalling

It has already been shown that histamine can induce calcium mobilisation in human native lens cells {D. J. Collison, 2001 #68} {R. A. Riach, 1995 #6}. Therefore it was logical to determine the effect of histamine on calcium mobilisation within the FHL 124 cell line. A concentration-dependant response to histamine was observed with a peak calcium response between 30μM and 100μM. The response pattern observed in the cell line was comparable to the cells of the native human lens. In general, we can observe histamine responses in the cell line and native lens. However, it did appear that the cell line was approximately 10 fold less sensitive to histamine than the native tissue {R. A. Riach, 1995 #6}. Nevertheless, this is an important validation of the use of the FHL-124 cell line in making comparisons to the native human lens and making predictions from in vitro results to processes that occur in the normal and pathological lens. It will be of great interest in the future to determine sensitivity to histamine of cells growing on the lens capsular bag, which would serve as a model of PCO. The

gene array data showed that the histamine receptor HRH1 was by far the most highly expressed in lens cells. This receptor is therefore the most likely to mediate this histamine induced calcium response and any possible downstream cellular functions. This was validated, when Triprolidine, a selective HRH1 antagonist was shown to ablate the calcium response from 100μM histamine. Calcium signalling has been shown to play a key role in cell survival and growth of lens cells {G. Duncan, 1997 #53} {G. Duncan, 1999 #75} {L. Wang, 2005 #76}. Application of the Ca ATPase inhibitor thapsigargin depletes the ER calcium store and suppresses calcium signalling. Using this method to disrupt calcium signalling reduced proliferation detected using 3H thymidine incorporation of both NN1003A {G. Duncan, 1996 #73} rabbit lens epithelial cells and FHL 124 human lens epithelial cells {L. Wang, 2005 #77}.

It is apparent from a number of studies that histamine can also invoke a number of signalling pathways in addition to classical calcium signalling {Lipnik-Stangelj, 2006 #62} {F. Hao, 2008 #14} {A. J. Robinson, 2001 #61}. Therefore it was important to determine if this were true in the lens. Using the suspended bead array method (BIO-PLEX) histamine was shown to phosphorylate P38 and ERK relative to unstimulated controls; however JNK was not altered to a significant degree despite showing a similar expression profile. These three molecules represent proteins from the three known mammalian MAP kinase pathways {M. J. Robinson, 1997 #63} {G. L.Johnson, 2002 #64}. The change in MAPK pathways secondary messengers shows that histamine is able to induce multiple pathways. Signalling through these pathways will have an effect on gene expression and may also play a role in histamine induced growth and other as yet undetermined cellular outputs. P38 MAP kinase cascades

have been shown to have a wide array of functions in the immune system but also in stress response signalling. It has also been shown to mediator cell proliferation and apoptosis {Martin-Blanco, 2000 #37}. JNK MAPK signalling pathways are known to be activated by cytokines and in response to environmental stress. They are also known to play roles in cell proliferation and apoptosis {Davis, 2000 #30} {C. R. Weston, 2002 #31}. ERK MAPK signalling pathways were the first to be discovered and also play roles in cell proliferation and apoptosis {C. Peyssonnaux, 2001 #7} {M. M. McKay, 2007 #8}. Previous work has shown that lens cell growth can be inhibited by disruption of the ras-raf-MEK-ERK pathway using the MEK inhibitor U0126. When applied to a human capsular bag, a marked inhibition but not ablation was seen {J. M. Maidment, 2004 #69}. Activation of ERK is therefore an important signalling mechanism for lens cells and histamines is likely to influence growth through this system

Therefore, once it was determined that histamine could induce a calcium response that was caused through HRH1 and in addition stimulate MAPK signalling pathways, it was logical to test whether this receptor regulated proliferation. If histamine induced proliferation was prevented through the use of an HRH1 antagonist then it would be known that histamine induced proliferation through HRH1 receptor signalling should be a key area of future investigation. Using this approach, it was found that HRH1 was indeed responsible for growth. As the MAPK pathways studied are known to induce cell proliferation a selective analysis of their role in histamine induced lens cell proliferation is merited. This could be tested by application of specific inhibitors of each pathway individually in FHL-124 cells in the presence of histamine. This would be best done with the more sensitive 3H-thymidine insertion assay. If any of

the inhibitors block growth then it will prove that it is that signalling pathway that is inducing proliferation. However, if none of the antagonists are shown to block growth then a combination of antagonists would need to be used. It could be that all three pathways are required to elicit a growth response or that two of them do. Therefore to find out which pathways do and which don't different combinations may need to be used. This would involve using two antagonists and not the other. If there is still a growth response after using all three antagonists then these pathways will be shown not to be involved in histamine induced growth. Another interesting experiment would be to use an inhibitor of calcium signalling Such as thapsigargin. If it can be shown that growth is caused while calcium signalling is inhibited then the Histamine must be inducing growth through a different pathway directly rather than through the mobilisation of intracellular calcium.

4.2.2 Histamine causes a change in cytokine expression in FHL-124 cells

Having established a number of signalling pathways in the lens that are activated by histamine and a growth function, the involvement of histamine in regulating cytokine expression was investigated using suspended bead array methods (BIO-PLEX). These studies revealed an ability of histamine to alter interleukin expression, which could contribute to the functional responses observed with histamine stimulation. Histamine is known to cause the release/production of a wide variety of cytokines, which are often associated with inflammation, from a number of different cell types {A. Mazzoni, 2001 #78} {M. Matsubaraa, 2005 #79} {J. Sirois, 2000 #80} {Falus, 1993 #66}. A Number of cytokines were identified in the gene array analysis to be linked

with histamine. A significant increase in the levels of IL-6, IL-8 and IL-13 was observed in response to histamine. IL-8 has been shown to cause cell migration and proliferation in a number of cell lines. IL-8 caused migration and proliferation in a cancer cell line through 'activation of a disintegrin and metalloprotease' (ADAM), epidermal growth factor receptor EGFR and the MAPK pathway {Y. Itoh, 2005 #26} {A. Li, 2003 #48}. IL-6 signals through gp130 which is linked to activation of both the JAK/STAT pathway and the MAPK pathway {T. Taga, 1997 #22}. Both these pathways play a role in cell survival and proliferation {P. C. Heinrich, 2003 #65} {G. L.Johnson, 2002 #64}. IL-6 could therefore be a candidate for effecting histamine induced FHL-124 proliferation through activation of the JAK/STAT and MAPK pathways. IL-8 could also be a candidate through activation of PLC {D. Wu, 1993 #49}.

IL-13 has a wide range of roles throughout the body mainly being involved with inflammation and immune system regulation and is known to have functions in inhibiting cytokine release. Kaviratne et al showed that IL-13 could induce fibrosis in *Schistosoma mansoni* infected cells in mice through a TGF-β independent mechanism {M. Kaviratne, 2004 #28}. However other models have shown IL-13 can stimulate and activate TGF-β to induce fibrosis {C. G. Lee, 2001 #51}. Further work has also shown that IL-13 can modulate collagen production in human skin and keloid fibroblasts {A. Oriente, 2000 #52}. IL-13 could therefore play a role in the fibrotic processes of PCO and therefore may warrant further investigation.

4.2.3 A potential role for antihistamines in the treatment of PCO

The aim of this work was to analyse histamine within the normal lens but also to research a possible role for histamine in the development of PCO. It was also already known that the eye can generate histamine {J. Z. Nowak , 1987 #82} and that following surgery an increase in proteins is found in the aqueous humour providing the lens with a possible source of histamine {Wormstone, 2002 #50}. What has been found with these experiments is that histamine can induce growth in both FHL-124 cells and human lens cells. As cellular proliferation is a major process in the development of PCO histamine could play a role in its development {I. M. Wormstone, 2009 #81}. This could be very important as if histamine can be conclusively proven to have a role in the development of PCO then antihistamines could be used to manage this development. Application of HRH1 antagonists at the time of surgery could be used to reduce the contribution of histamine in PCO progression and thus have a benefit to the patient.

PCO is thought to be caused through introduction of proteins from the breach of the blood aqueous barrier in to the aqueous humour, ocular immune privilege and sustained autocrine activity of lens epithelial cells {Wormstone, 2002 #50}. Even though the increase in proteins in the aqueous humour returns to pre-operative levels within weeks laser corrective surgery is not required for PCO until years later. Histamine is unlikely to be involved in the autocrine stage of PCO as the gene array showed no ability of the lens to generate endogenous histamine. However if histamine is shown to play a major role in the early phase of PCO through elevation in the aqueous humour and HRH1 activation then antihistamines that target HRH1

signalling could be used to prevent or diminish the induction of these autocrine systems. This would require treatment with antihistamines for a short time after surgery until the protein levels have returned to normal and the inflammatory response to surgery had ceased.

There are advantages to using antihistamines over newly developed cytotoxic agents. Antihistamines are already proven therapeutic drugs, widely available, and relatively cheap. They would require limited clinical testing as they could be used 'off the shelf'. This is a very important prospect that means the role of histamine in the development of PCO warrants further investigation.

4.3 Summary

This project set out to investigate any possible role for histamine in the development of PCO. To this end it was shown that histamine can induce proliferation, a major stage in PCO development, in FHL-124 cells and in capsular bag models. It was found that histamine could induce this proliferation through various pathways. It can cause calcium mobilisation through the HRH1 receptor and cause an increase in ERK, P-38 and JNK. It was also shown that histamine can cause an increase in cytokines IL-6 and IL-8. All these open up areas of further investigation for histamines possible role in PCO. Do the MAPK pathways play a role in histamine proliferation? What affect does an increase in IL-6 and IL-8 have on the lens?

Outside of this main focus analysis of gene array data showed that the lens has histamine receptors, HRH1 being the most abundant. It also showed that the lens can

not produce histamine as it lacks HDC but can break down histamine. This also raises questions about what processes may release histamine into the lens and what function this may play, some of which have been speculated on earlier in the discussion.

Any future work should endeavour to investigate further the mechanisms through which histamine can induce proliferation but also focus on the role histamine might play in the non-pathological lens.

5. References

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