

SIGNALLING PATHWAYS ACTIVATED
IN TYPE 1 MYOTONIC DYSTROPHY
LENS EPITHELIAL CELLS

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ABSTRACT

Purpose: Cataracts are a key feature of type 1 myotonic dystrophy (DM1), however, little is known about the mechanisms which underlie their development. This project aims to verify the suitability of lens cell lines derived from DM1 patients as a model for the disease and to use these to investigate the mechanisms which lead to cataract development.

Methods: Experiments were conducted using four DM1 and four control cell lines derived by SV40 transformation of human lens epithelial cells (LECs). The DM1 cell lines were characterised by measuring gene expression using QRT-PCR and identifying foci formation of triplet repeat RNA via FISH. Activation of signalling pathways was analysed using Western blotting. Active signalling pathways were inhibited to examine their roles in cell growth, death and apoptosis which were measured using BCA, LDH and TUNEL assays respectively.

Results: Human LECs expressed *DMPK* and transcripts formed foci in the nuclei of DM1 cells; however, no evidence of alternative splicing was found. DM1 LECs had significantly longer population doubling times and a shorter lifespan compared to controls. Serum deprivation led to increased levels of apoptotic cell death in DM1 LECs. DM1 LECs released autocrine signalling factors which activated the Akt pathway in the non-virally transformed lens cell line FHL124. Levels of pAkt and pJNK were subsequently shown to be elevated in the DM1 cell line, DMCat1. However, levels of Akt activity declined during the culture period. Inhibition of the PI3K/Akt pathway in DMCat1 led to increased levels of apoptotic cell death.

Conclusions: DM1 LECs require signalling via the PI3K/Akt pathway for survival. Sustained activation of JNK and decreasing activation of Akt could be responsible for the longer population doubling times and shorter lifespan of DM1 LECs. Increased LEC death could underlie cataract formation through a loss of homeostatic control in DM1 lenses.

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ABBREVIATIONS

ACC	Anterior cortical cataract
Akt	v-akt murine thymoma viral oncogene homolog 1
ANOVA	Analysis of variance
ASK1	Apoptosis signal-regulating kinase 1
ATP1A1	Na ⁺ /K ⁺ ATPase α 1 subunit
BAD	Bcl-2-antagonist of death
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCD	Charge-coupled device
CELF	CUG-BP1 and ETR-3-like factors
CM	Conditioned medium
CNS	Central nervous system
C _T	Threshold cycle
CTG	Cytosine-thymine-guanine
CUG	Cytosine-uracil-guanine
CUG-BP	CUG triplet repeat, RNA binding protein
DAG	Diacylglycerol
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DEPC	Diethylpyrocarbonate
DM	Myotonic dystrophy
DMPK	Myotonic dystrophy protein kinase
DMSO	Dimethyl sulfoxide
DMWD	Myotonic dystrophy WD repeat containing protein
DPBS	Dulbecco's phosphate buffered saline
dsRNA	Double-stranded RNA
EC	Excitation-contraction
ECG	Electrocardiography
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

eIF2 α	Eukaryotic initiation factor 2 α
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
EMG	Electromyography
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FISH	Fluorescent <i>in situ</i> hybridisation
FRS2	Fibroblast growth factor receptor substrate 2
FSH	Follicle-stimulating hormone
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GPCR	G protein-coupled receptor
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
HSA	Human skeletal actin
HSPG	Heparan sulphate proteoglycans
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding proteins
IGFR-1	Insulin-like growth factor receptor 1
IgG	Immunoglobulin G
IOL	Intraocular lens
IP ₃	Inositol 1,4,5-trisphosphate
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LEC	Lens epithelial cell

LEDGF	Lens epithelium-derived growth factor
MAPK	Mitogen-activated protein kinase
MBNL	Muscleblind-like
MEK	Mitogen-activated protein kinase kinase
MIP26	Major intrinsic protein of lens fibre 26
MMP	Matrix metalloproteinase
MPER	Mammalian protein extraction reagent
mTOR	Mammalian target of rapamycin
mTORC	Mammalian target of rapamycin complex
NC	Nuclear cataract
NCX	Na ⁺ /Ca ²⁺ exchangers
NHS	National health service
p70S6K	p70 S6 Kinase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween [®] 20
PCC	Posterior cortical cataract
PCO	Posterior capsule opacification
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK	Phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol 3-kinase
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PIP ₃	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PKR	dsRNA-activated protein kinase
PLC	Phospholipase C
PMCA	Plasma membrane Ca ²⁺ -ATPase
PSC	Posterior subcapsular cataract
PTEN	Phosphatase and tensin homologue deleted on chromosome ten
PVDF	Polyvinylidene difluoride
QRT-PCR	Quantitative real time-polymerase chain reaction
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium

RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase-polymerase chain reaction
RYR	Ryanodine receptor
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
SF	Serum-free
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homology domain containing protein
siRNA	Small interfering RNA
SIX5	Sine oculis homeobox homolog 5
SOS	Son of sevenless
SSC	Saline-sodium citrate
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
SV40	Simian virus 40
TdT	Terminal deoxynucleotidyl transferase
TGF- β 2	Transforming growth factor β 2
TSC	Tuberous sclerosis complex
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
UTR	Untranslated region
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 The human eye

The eye is a highly specialised organ which detects and interprets photic stimuli to allow us to perceive vision. The eye detects light energy and converts it into electrical signals which are sent to the brain and processed so that we can visualise our surrounding environment. The individual structures within the eye (see figure 1.1) are either involved in the transmission and focusing of light and generation of signals, or in the nourishment and support of the tissues within the eye (Forrester *et al.*, 2002).

The adult human eye is roughly spherical with a diameter of around 24 mm; it has an average volume of 6.5 ml and a mass of 7.5 g and is separated into anterior and posterior segments. The smaller anterior segment consists of the conjunctiva, cornea, iris, lens and aqueous humour. This segment is separated into the anterior and posterior chambers by the iris, but the chambers remain connected via the pupil. The anterior chamber is the space between the cornea and iris and the posterior chamber is the space between the iris and the lens; both chambers are filled with aqueous humour. The larger posterior segment consists of the sclera, choroid, retina and vitreous humour (Forrester *et al.*, 2002).

The outermost layer of the posterior segment is the sclera and this joins the transparent cornea and conjunctiva which form the outermost layer of the anterior segment. Together these form a layer called the fibrous coat which protects the eye. Beneath the fibrous coat are two further layers, the first of which is known as the vascular coat and is made up of the choroid, ciliary body and iris. The choroid is highly vascularised to nourish the outer layers of the retina and is pigmented to reduce light scatter within the eye. The ciliary body holds the lens in place and produces aqueous humour, which bathes the lens and fills the anterior segment. The iris is the coloured part of the eye and contains sphincter and dilator muscles that control the size of the pupil and therefore the amount of light that enters the eye. The innermost layer of the posterior segment is known as the neural coat

which consists of the retina. The retina has a highly complex, layered structure which contains the photoreceptor cells known as rods and cones. The region of the retina known as the macula is responsible for high visual acuity and contains a pit called the fovea where light rays are focused. The retina is responsible for turning light into electrical signals that are passed to the brain via neurones in the optic nerve which exits the retina at the blind spot (Forrester *et al.*, 2002).

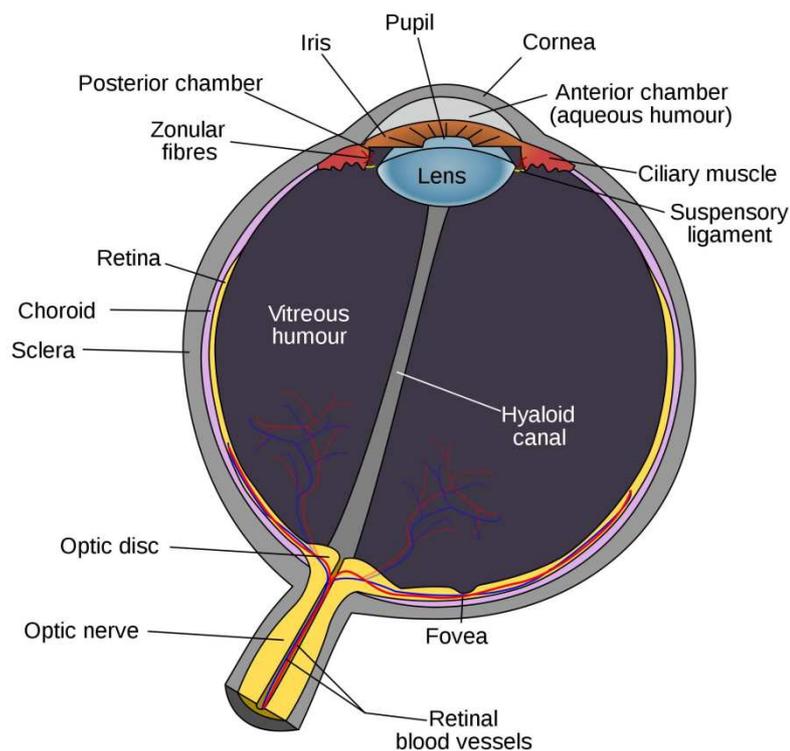


Figure 1.1 – A diagrammatic representation of the human eye, showing the main structures and their relative positions within the eye. The eye consists of three outer coats: the fibrous coat (sclera and cornea); the vascular coat (choroid and iris); and neural coat (retina), which encase the remaining structures of the eye, including the lens. The vitreous and aqueous humours provide nourishment and support and maintain the shape of the eye (image from <http://en.wikipedia.org/wiki/Eye>).

The three layers surround and house the remaining components that make up the eye. These include the lens, which is responsible for focusing light on to

the retina; the aqueous humour, which supports and nourishes the structures of the anterior segment of the eye; and the vitreous humour, which supports the structures of the posterior segment of the eye.

1.1.2 The passage of light through the eye

Light of wavelengths that are visible to the human eye is found in a relatively small region of the electromagnetic spectrum between 400 and 700 nm and corresponds to a colour range of violet to red. Light with wavelengths below 400 nm, such as ultraviolet (UV) light, is filtered out by the cornea and lens as its high energy would be damaging to the retina (Yanoff & Duker, 2009). Light enters the eye through the conjunctiva and cornea which are the transparent coverings of the eye. The cornea is responsible for two-thirds of the eye's refractive power, but unlike the lens its focus is fixed (Forrester *et al.*, 2002). The light passes through the clear aqueous humour and the amount of light that can pass through the pupil is determined by the muscles of the iris. The iris contracts or dilates to enable more or less light to pass towards the back of the eye depending on lighting conditions. In low light the iris dilates to allow more light to enter the eye and in bright light the iris contracts to restrict the amount of light that enters the eye.

Light passes through the lens which is a transparent structure responsible for the remaining refractive power of the eye. Unlike the cornea, the lens is capable of altering its shape, and therefore refractive power, in order to bring light from varying distances to focus on the same point of the retina. The light then passes into the vitreous humour, a clear gelatinous material, which fills the space between the lens and the retina. The vitreous humour consists mainly of water (over 98%) and a matrix of type II collagen fibres and hyaluronic acid, which increases its viscosity to 2-4 times that of water alone (Forrester *et al.*, 2002). This enables it to fulfil its role of maintaining the shape of the eye.

When focused light reaches the retina it is directed to the macula region and particularly the pit of the fovea. The retina is the photosensitive part of the eye and consists of ten distinct layers containing many different cell types (see figure 1.2). The retina is responsible for turning light energy into electrical signals which can be interpreted by the brain. Light passes through several layers of retinal cells before reaching the photoreceptor cells at the back of the retina. The

outermost layer of the retina (situated nearer the back of the eye) consists of retinal pigment epithelial (RPE) cells which contain pigment (melanin) and are responsible for capturing stray light to avoid light scatter within the eye. Other roles of the RPE cells include: the phagocytosis of outer segments of the photoreceptor cells; the turnover of vitamin A required for the production of photopigment; and the provision of a barrier between the blood vessels of the choroid and the retina.

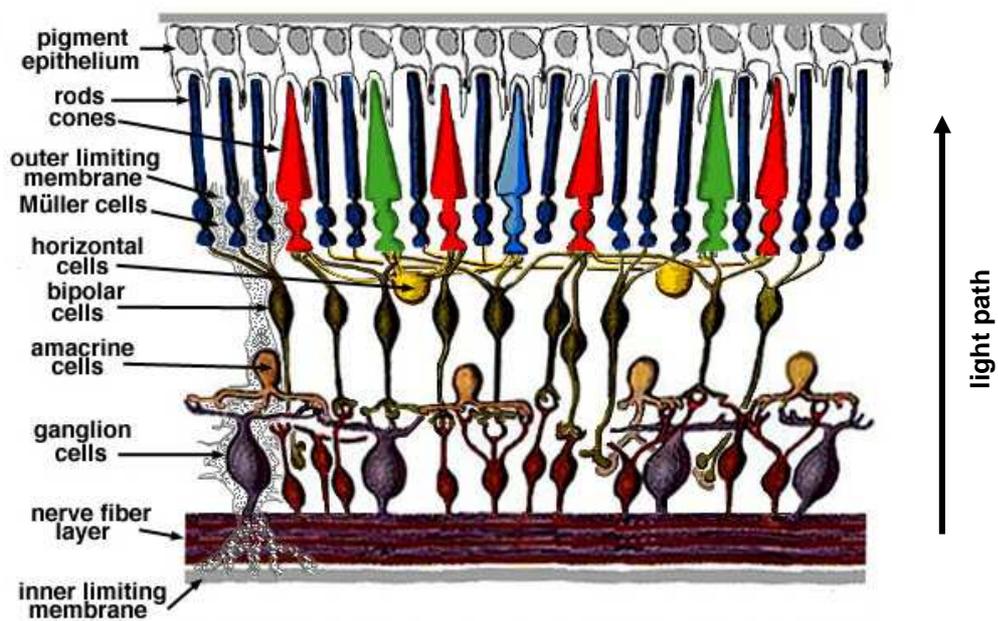


Figure 1.2 – A diagrammatic representation of the structure of the retina. The retina is made up of a pigmented epithelial cell layer and several layers of neuronal cells connected by synapses. The cells convert light energy into electrical signals which are passed to the brain via the ganglion cell axons which exit the eye at the blind spot and form the optic nerve (image from <http://psych.hanover.edu/Presentations/VoiceFound/images/retina2.jpg>).

Most of the light that passes through the layers of the retina is captured by the two types of photoreceptor cell known as rods and cones. Rod cells are highly sensitive and can be activated by a single photon of light. For this reason they are

mainly used for vision in low light intensity. Cone cells are less sensitive to light but allow the perception of colour. There are three types of cone cell, each being sensitive to a different part of the visible light spectrum. The photoreceptor cells contain photopigments which are activated by different wavelengths of light, causing the closure of Na^+ channels, hyperpolarisation and the reduction of tonic release of the neurotransmitter glutamate onto bipolar and horizontal cells. The bipolar cells pass the signal via a graded potential to either amacrine cells or directly to ganglion cells which fire action potentials when stimulated. The ganglion cells have long axons which exit the eye at the optic disk and form the optic nerve which goes to the lateral geniculate nucleus situated in the thalamus of the brain. The lateral geniculate nucleus is the processing centre for visual information and sends the signals via the optic radiation to the visual cortex where further analysis and interpretation occurs.

1.2 The human lens

The lens is derived from surface ectoderm which overlies the optic vesicle and forms the lens placode during development. The mature lens is a transparent, avascular tissue situated behind the iris and in front of the vitreous body in the anterior segment of the eye. The lens has an ellipsoid, biconvex shape with the anterior surface being less curved than the posterior. The adult human lens is approximately 10 mm in diameter and has a width of around 4 mm (Forrester *et al.*, 2002). It is held in place by the zonule fibres which are attached to the lens capsule (just in front of the equatorial line) at one end and the ciliary body at the other. The lens is nourished by vitreous humour on its posterior surface and aqueous humour on its anterior surface. As the lens does not have its own blood supply, all of the nutrients it receives come from the surrounding humours.

The function of the lens is to focus light onto the retina. In order to focus light from varying distances at the same point the lens is able to alter its shape by a process known as accommodation. When the ciliary muscle contracts, tension in the zonules is reduced and the lens relaxes to a more spherical shape to focus light from near objects. At rest (relaxed), the lens has a refractive power of approximately 15 dioptres, which is altered when it changes shape. When the ciliary muscle relaxes the converse is true and tension in the zonules increases to

flatten the lens enabling light from distant objects to be focused. Ciliary muscle contraction is activated by the parasympathetic nervous system via the oculomotor nerve and relaxation is under the control of the sympathetic nervous system.

Structurally the lens has three main components: the capsule; the anterior epithelium; and the fibres (see figure 1.3). All three components have a structure that has evolved to maintain transparency of the lens.

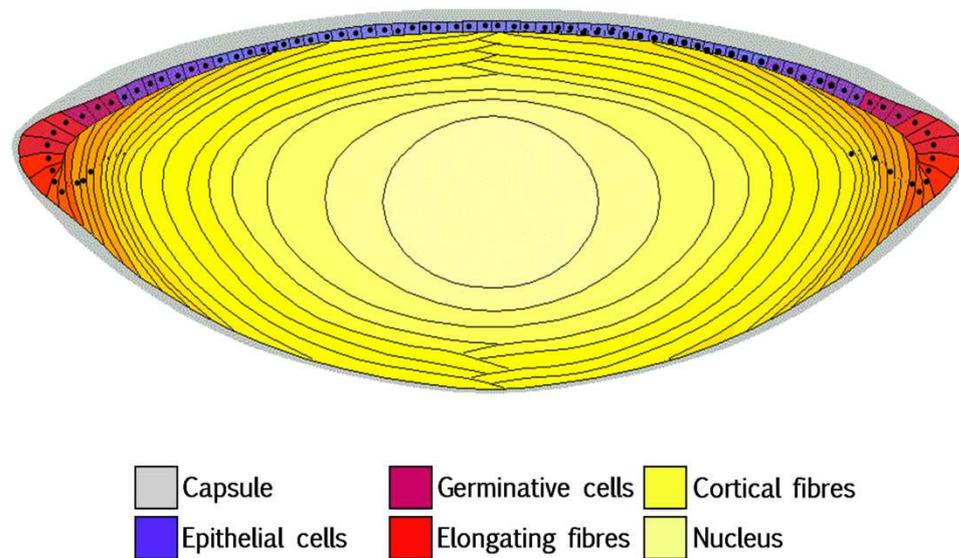


Figure 1.3 – A diagrammatic representation of the human lens. A layer of metabolically active epithelial cells (blue) are found on the anterior surface of the lens. Some of these cells, known as the germinative cells (pink), continually divide throughout life. They elongate (red) and migrate, forming new fibre cells (yellow) that become part of the fibre mass. The original embryonic fibres form the nucleus (pale yellow) of the lens. The lens is surrounded by the lens capsule (grey) (image from Maidment *et al.* (2004)).

1.2.1 The lens capsule

The lens capsule is a transparent, smooth, basement membrane that surrounds the entire lens and protects it from infectious viruses and bacteria. The lens capsule acts as a reservoir of growth factors and provides epitopes for lens cell surface receptors which encourage cell survival, growth and migration (Danysh & Duncan, 2009). The membrane also acts as a selectively permeable

barrier to allow the movement of metabolic substrates and waste between the lens and the humours of the eye due to the lack of blood vessels that would usually perform this task. The lens epithelial cells slowly produce and turnover the matrix material of the thick, elastic lens capsule. The capsule is mainly composed of laminin and type IV collagen, but also contains nidogen and several heparan sulphate proteoglycans (Danysh & Duncan, 2009). The thickness of the adult lens capsule varies from around 2 μm at the posterior pole to around 30 μm at the anterior pole.

1.2.2 The lens epithelium

The lens epithelium is a monolayer of tightly packed cuboidal epithelial cells which cover the anterior surface of the lens. The epithelium is divided into three parts: the non-dividing epithelial cells situated in the central anterior region; the germinative dividing cells which surround them; and the differentiating cells which elongate into new fibre cells at the equatorial region. The cells in the germinative zone grow throughout life, resulting in new fibre cells being added to the fibre cell mass which increases in size with age.

The lens epithelial cells (LECs) are responsible for maintenance of homeostatic control of the lens. The LECs regulate levels of ions, nutrients and water and maintain the osmolarity and volume of the lens. A gradient of sodium ions (Na^+) and potassium ions (K^+) exists across the lens with high levels of Na^+ at the posterior where it enters by diffusion and high K^+ at the anterior. The LECs contain Na^+/K^+ ATPase pumps which pump Na^+ out into the aqueous humour and K^+ ions in. This results in a negative resting potential which enables the cells to maintain their volume (Bhat, 2001). A concentration gradient of calcium ions (Ca^{2+}) is also necessary for normal lens cell function with the intracellular levels being over 1000-fold lower than extracellular levels (Paterson & Delamere, 2004). The gradient is maintained via plasma membrane Ca^{2+} -ATPase (PMCA) and $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX), which pump Ca^{2+} out of the cell and sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pumps, which pump Ca^{2+} into the endoplasmic reticulum (ER) calcium store (Rhodes & Sanderson, 2009).

LECs produce the antioxidant glutathione to protect against oxidative insults which can inhibit the pumps that maintain the ion gradients and therefore

lead to a loss of homeostasis within the lens (Delamere & Tamiya, 2004; Marian *et al.*, 2007).

1.2.3 The lens fibres

The lens fibres constitute the overwhelming bulk of the lens. Mature fibre cells are long and thin in shape, stretching from the anterior to the posterior pole of the lens, reaching up to 12 mm in length, but with a diameter of only 4-7 μm . The ends of mature fibre cells meet anteriorly at the lens epithelium or posteriorly at the capsule where they overlap at the anterior and posterior sutures, forming a new growth shell. The fibre cells are tightly packed in layers, called laminae, and linked to each other laterally by many gap junctions and channels formed by connexins and major intrinsic protein 26 (MIP26). The gap junctions allow rapid communication between the cells of the lens, allowing solute and ionic transfer from cell to cell. Neighbouring fibre cells are also joined by ball-and-socket junctions and membranous interdigitations along their lateral edges which provide stability and structure (Forrester *et al.*, 2002). MIP26 acts as a water channel and also plays a role in the formation of gap junctions, the organisation of the lens protein γ -crystallin and in cell to cell adhesion to limit inter-fibre space (Golestaneh *et al.*, 2004). The highly ordered arrangement of the fibre cells and the lack of extracellular space ensures the transparency of the lens (Forrester *et al.*, 2002).

Fibre cells differentiate and elongate from the dividing LECs at the equator of the lens. The equatorial cells continue to divide and new fibres are continually added to the fibre mass, leading to an increase in lens size throughout life. Since all cells are retained by the lens, fibre cells laid down in the embryo and foetus are still found in the adult lens and form the central, denser nucleus.

As the fibre cells differentiate and mature they synthesise crystallins, water channel and gap junctional proteins and gradually dismantle their organelles (Rao & Maddala, 2006). The lens fibres become packed with crystallins, which are water soluble proteins that increase the refractive index of the lens and maintain transparency. The main crystallins found in the human eye are α -, β -, and γ -crystallins. The α -crystallins are members of the small heat shock protein family and also have roles as molecular chaperones, which may be of importance

in protecting lens proteins and preventing their precipitation during ageing (Andley, 2007).

The organisation of the proteins that maintain transparency is dependent on the fibre cell cytoskeleton, which performs a scaffolding role. Further roles of the cytoskeleton include regulation of fibre cell shape, elongation and migration. The cytoskeleton consists of actin, vimentin and spectrin microfilaments, microtubules and intermediate filaments, as well as lens specific beaded filaments (Forrester *et al.*, 2002; Rao & Maddala, 2006).

The highly ordered structure of the components of the lens is crucial to its transparency. Loss of homeostasis in levels of ions and water and the loss of the tightly packed structure of the lens fibres can lead to a loss of transparency due to light scatter.

1.2.4 The ageing lens

As the human lens ages, several changes occur which can impact on vision. The most common changes that occur in the ageing human lens are increases in stiffness, colouring and light scatter (Vrensen, 2009). The refractive power of the lens decreases with age from 15 dioptres during youth to 6-8 at around 40 years of age and 2-0 at around 60 years of age (Forrester *et al.*, 2002; Yanoff & Duker, 2009).

The condition presbyopia is the inability to focus on near objects, which arises due to a loss of elasticity of the lens with age; in particular the hardening of the nucleus of the lens. The decrease in lens elasticity is also accompanied by atrophy of the ciliary muscle fibres (Forrester *et al.*, 2002). Reading glasses are then required in order to view close objects in focus. The level of α -crystallin also decreases with age, which coincides with an increase in stiffness. During ageing, the level of free α -crystallin decreases as it binds to degraded proteins to prevent them from precipitating. At around 40 years of age no free α -crystallin is found in the lens and after this point the level of stiffness in the lens increases dramatically (Truscott, 2009). With no free α -crystallin to act as a molecular chaperone, levels of insoluble proteins that cause light scatter begin to increase.

The lens becomes increasingly yellow with age due to increased absorption of light at the blue end of the spectrum and increased light scattering as the cells begin to lose their highly ordered structure. The UV light filter, 3-

hydroxykynurenine-3-O- β -glucoside (3-HKG), becomes covalently bound to lens proteins during ageing, producing a yellow pigment which contributes to colouration and also to the development of age-related nuclear cataract (Yanoff & Duker, 2009).

The formation of both nuclear and cortical cataracts has been linked to ageing. The hardening of the lens could alter the flow of water, ions and antioxidants through the fluid cell membranes by affecting the activity of membrane bound proteins involved in their transport. This could result in a loss of homeostasis and an increase in reactive oxygen species, leading to nuclear cataract. Also the hardening of the lens nucleus could result in shearing and ruptures between the hard nucleus and the softer cortex during attempts to focus and accommodate, leading to cortical cataract (Truscott, 2009).

The density of the LEC layer decreases with age as the surface of the lens increases and the proliferative capacity of the germinative cells decreases. As a result the epithelial cells flatten to maintain coverage of the lens (Yanoff & Duker, 2009). The LEC layer is responsible for the maintenance of homeostasis of ion and water levels within the lens. A thinning epithelial cell layer is likely to contribute to cataract formation due to a loss of homeostasis (Li *et al.*, 1995).

1.3 Cataract

Cataract is an opacity in the lens which obstructs the passage of light and causes light scattering (see figure 1.4). The lens becomes cloudy and opaque, resulting in a loss of visual acuity and contrast sensitivity. Cataract can result in varying degrees of visual impairment, ranging from blurred vision to blindness.

Cataract is the leading cause of blindness worldwide, responsible for almost 50% of cases, and it is estimated that 17 million people are currently rendered blind due to the condition (Javitt *et al.*, 1996). In developed countries the prevalence of blindness due to cataract is low. In most developing countries, however, prevalence is much higher, as treatment is not readily available. The incidence of cataract increases with age and studies have shown that the risk of developing cataracts increases with every decade of life after 40 years of age (McCarty *et al.*, 1999; Klein *et al.*, 2002). A study by McCarty *et al.* (1999) showed that 2-3% of people tested had cataract between the ages of 40-49, which

increased to 100% in people tested over the age of 90. A similar study by Klein *et al.* (2002) showed that the incidence of cataract over a 10-year period between the ages of 43-54 was between 14-19%, compared to 84-90% of those studied over the age of 75. The impact of this data becomes apparent when the continual increase in life expectancy, and therefore an ageing population, is taken into account.

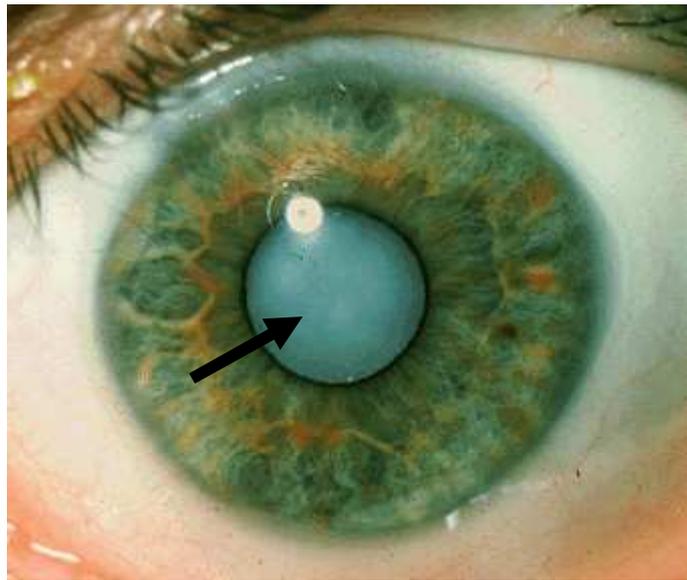


Figure 1.4 – Image of a cataractous lens. Cataract is an opacity of the lens, which obstructs the passage of light, causing scattering. This results in a cloudy appearance to the lens which can be seen behind the pupil of the eye (indicated by the arrow) (image from http://www.aapos.org/client_data/files/182_cataract1.jpg).

Although the greatest risk factor for developing cataract is age, many others have been identified including: environmental stresses, such as exposure to UV light, oxidative stress and radiation; genetic changes, such as mutations in crystallin genes; physical trauma; toxic substances, such as selenite; and certain medical conditions, such as diabetes. With an ageing population the prevalence of cataract is increasing and with no current cure, the only treatment is surgical removal.

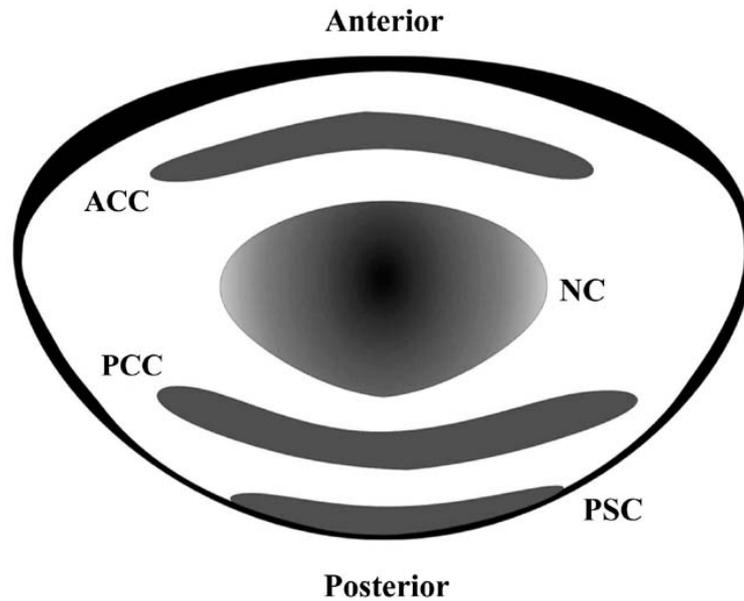


Figure 1.5 – A schematic diagram of the human lens, showing the relative positions of the various types of cataract. Nuclear cataracts (NC) are located in the nucleus of the lens. Cortical cataracts can either be anterior (ACC) or posterior (PCC). Posterior subcapsular cataracts (PSC) are located at the posterior of the lens (image from Das Gupta *et al.* (2004)).

Cataracts are classified by their position in the lens and are normally regarded as either nuclear, cortical, posterior subcapsular, or a mixture of these (see figure 1.5). Nuclear cataract affects the nucleus of the lens and is most often associated with oxidative damage and age-related changes, such as the accumulation of large high molecular weight protein aggregates. Cortical cataracts are also most commonly age-related and occur in the cortical fibres of the lens as the result of degeneration and liquefaction of the fibres. A breakdown in ion homeostasis leads to an influx of Ca^{2+} and Na^{+} ions, with the increase in intracellular Ca^{2+} resulting in activation of Ca-activated proteases (calpains) which break down the fibre cell protein. Cortical spokes form when groups of fibre cells are affected (Naumann *et al.*, 2008). Posterior subcapsular cataracts (PSC) form near the posterior edge of the lens and lead to high levels of glare. PSCs are the result of the appearance of granular material and vacuoles in the lens, but are often associated with other types of cataract, leading to a classification of mixed cataract. The development of PSCs has been linked to age, diabetes

mellitus, intraocular inflammation, smoking and use of corticosteroids (Hodge *et al.*, 1995; Delcourt *et al.*, 2000; Hennis *et al.*, 2004).

1.3.1 Cataract surgery

Surgical removal of cataracts is the most common operation performed by the National Health Service (NHS) in the UK. Around 300 000 are performed every year, and with each operation costing around £700 to £1200, this is an incredible financial burden on the NHS.

Cataract surgery lasts around 15 minutes and is performed under local anaesthetic. The most common method is extra-capsular cataract extraction (ECCE), which removes the fibre mass, but leaves the capsular bag (lens capsule) in place to house an artificial intraocular lens (IOL) (see figure 1.6a and b). The cataract operation can be briefly described as follows: small incisions are made in the front of the eye to enable access to the lens and a viscoelastic substance is injected into the anterior chamber to maintain space and pressure during the procedure. A capsulorhexis is performed on the lens, where a circular piece of tissue is removed from the anterior surface, which contains the anterior epithelial cells attached to the lens capsule. A balanced salt solution is injected into the lens to separate the capsule from the fibres and to separate the nucleus from the cortex in a process known as hydrodissection and hydrolineation. Phacoemulsification is then performed on the fibre mass. An ultrasonic device is inserted into the capsular bag to breakdown the fibres which are then aspirated. The majority of the lens is removed during surgery, leaving only the posterior capsule and a small amount of the anterior capsule. This is referred to as the capsular bag. A folded IOL is then inserted into the capsular bag, where it unfolds and assumes its position in the visual axis (Benjamin & Little, 2007).

1.3.2 Posterior capsule opacification

A common complication of cataract surgery is the secondary loss of vision that occurs in a significant proportion of patients. During cataract surgery a portion of the anterior capsule of the lens is left as part of the capsular bag (see figure 1.6b). LECs attached to the lens capsule are difficult to remove during surgery and subsequently mount a wound healing response where they attempt to recover the denuded areas of the anterior capsule by multiplying and migrating. In

doing so, some migrate onto the surface of the IOL and also onto the previously cell free posterior capsule where they encroach onto the visual axis and cause fibrotic changes to the matrix (see figure 1.6c). Contraction of the capsule forms wrinkles which scatter light (Wormstone *et al.*, 2009).

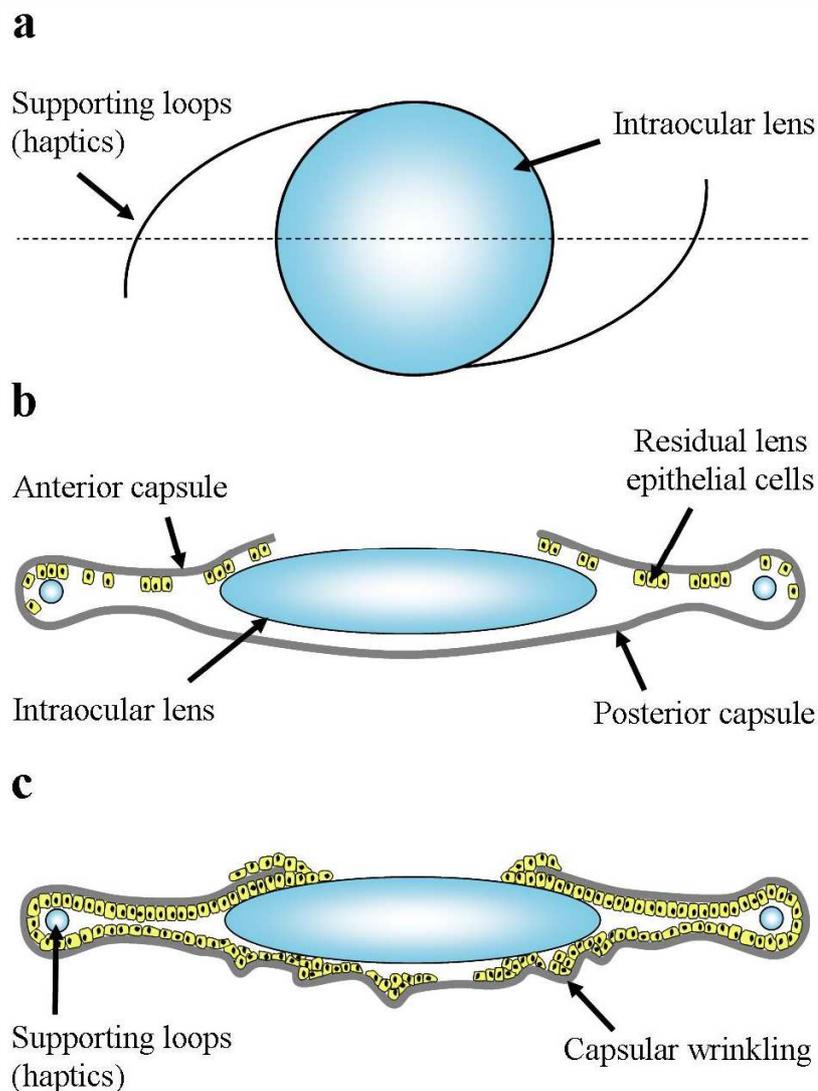


Figure 1.6 – A diagrammatic representation of a capsular bag and the development of posterior capsule opacification (PCO) (a) An intraocular lens (IOL) with the supporting loops (haptics) which hold it in place following implantation. The dotted line indicates the area where the cross section seen in images b and c was taken (b) Modern cataract surgery forms a capsular bag which comprises of the posterior capsule and a portion of the anterior capsule. The capsular bag is used to house an IOL. (c) The residual LECs multiply following surgery and migrate onto the previously cell free posterior capsule, resulting in wrinkling and light scatter in a condition known as PCO (image adapted and modified from Wormstone (2002)).

The incidence of PCO is significantly higher in younger patient groups, most likely due to the increased growth capacity of their cells. The incidence of PCO in patients under 40 years of age is as high as 70%, compared to less than 40% in patients over 60 years old (Wormstone, 2002).

PCO is treated using a neodymium-yttrium-aluminum-garnet (Nd-YAG) laser which is used to remove a section of the posterior capsule to clear the visual axis. Nd-YAG treatment is not without risk (e.g. retinal detachment) and also costs the NHS a considerable amount each year. Prevention of PCO by more effective removal of LECs and better IOL design is the focus of current research.

1.4 Myotonic Dystrophy

Myotonic dystrophy (DM, also known as dystrophia myotonica) is the most common form of adult muscular dystrophy, affecting around 1 in 8000 people worldwide. It is an autosomal, dominantly inherited, neuromuscular disorder. DM is characterised by myotonia (a difficulty in relaxing the muscles following contraction), muscle weakness and atrophy (a wasting of the muscles), cardiac conduction defects and the development of pre-senile cataracts. Other symptoms include mental retardation, testicular atrophy, frontal balding and insulin resistance. DM is a multisystemic disease, affecting many tissues and organs within the body.

DM is a highly variable disease with the range of symptoms, age of onset and severity of symptoms varying from person to person (Harper, 2001). Symptoms can present at any time from birth to old age and vary from mild to severe. In its mildest form, the disease may present with only pre-senile cataract. In its most severe form the disease may be present at birth, causing mental retardation, muscle weakness and respiratory problems. DM can shorten life expectancy, but this is most significant in patients affected from a young age. DM patients have an increased risk of respiratory and cardiac diseases and of sudden death from heart failure (Meola, 2000).

1.4.1 The history of DM

DM was first described as an independent disorder, distinct from other myotonic diseases in 1909. Hans Steinert reported nine cases of the disease, where

he observed a similar distribution of muscle weakness and atrophy and weak speech. He also described testicular atrophy in four of the cases (referred to in Harper, 2001). The disease was described independently in the same year by Frederick Batten and H.P. Gibb (Batten & Gibb, 1909).

In 1911 J.G. Greenfield recognised cataract as a symptom of DM after observing a high prevalence of cataract in a large family affected by the disease. The systemic nature of DM was first noted in 1912 by H. Curschmann, who considered cataract and testicular atrophy to be a sign of generalised endocrine disturbance. In 1918 B. Fleischer described cataracts in previous generations of patients who had otherwise appeared unaffected by the disease and was the first to describe the phenomenon called anticipation, where the severity of the disease increases from generation to generation (referred to in Harper, 2001).

Mental retardation and cardiac conduction defects had been linked to DM in numerous studies by 1950. In 1960 the congenital form of the disease and its maternal transmission were described by T.M. Vanier (referred to in Harper, 2001). By this point the clinical features of DM had been well described, although the genetic mutation underlying the disease was still a mystery. The genetic mutation behind DM was discovered in 1992, however, some of the patients suffering from the disease were not shown to carry this mutation (Aslanidis *et al.*, 1992; Brook *et al.*, 1992; Buxton *et al.*, 1992; Mahadevan *et al.*, 1992). In 2001, a rarer second mutation resulting in DM symptoms was discovered (Liquori *et al.*, 2001). The two forms of the disease were renamed as type 1 (DM1) (also known as Steinert's disease) and type 2 (DM2) (also known as proximal myotonic myopathy).

1.4.2 Clinical features of DM

The multisystemic nature of DM means that a patient can present with any of the symptoms associated with the disease, which can lead to difficulty in making a diagnosis as specialists in particular areas, such as ophthalmologists and cardiologists, may not be aware of the condition. A diverse range of clinical features are associated with DM. Many of the symptoms observed are common to both forms of the disease, however, some present in only one form of DM and many symptoms vary in severity or location.

Skeletal muscle weakness, wasting, myotonia and pain are common in DM, although the pattern of muscle involvement varies between the two forms. In DM1 the facial and distal limb muscles are most affected, with proximal skeletal muscle weakness occurring as the disease progresses. In contrast, weakness of proximal skeletal muscles is more prominent than distal muscles in DM2 (Machuca-Tzili *et al.*, 2005). In both forms, muscles of the neck and hands are affected and facial muscles show signs of weakness, including ptosis (drooping of the eyelid), albeit milder in DM2. Muscle biopsies from both DM1 and DM2 patients show similar histological features such as fibre atrophy (some with pyknotic nuclear clumps), central nucleation (sometimes arranged in chains) and extreme variations in fibre size. Replacement of necrotic muscle tissue with fibrotic and adipose tissue can also be observed. In DM1 it is mainly type 1 muscle fibres affected, compared to type 2 muscle fibres in DM2 (Wells & Ashizawa, 2006). Involvement of the smooth muscle is seen in DM1 but not in DM2 and includes dysmotility of the gastrointestinal tract resulting in, for example, diarrhoea, constipation and gallstones; and hypotension (Wells & Ashizawa, 2006). Muscle pain is also observed in both forms of the disease. Myotonia, which is a difficulty in relaxing the muscles due to an increase in the frequency of action potentials, is present in both forms of DM; however, it is more severe and symptomatic in DM1 patients. Myotonia is caused by spontaneous electrical discharges in the muscles that wax and wane (mostly waning in DM2) in frequency and amplitude, which is detected by electromyography (EMG) (Logigian *et al.*, 2007). The abnormal electrical activity results in the inability to relax the muscles, causing for example the sustained grip following a handshake. Myotonia affects distal muscles more than proximal and can also affect facial muscles, tongue and bulbar muscles (which control talking, chewing and swallowing) (Machuca-Tzili *et al.*, 2005; Logigian *et al.*, 2007). Myotonia often improves during repetitive exercise of the affected muscles, known as the 'warm up' effect. The severity of myotonia is positively correlated with muscle weakness in DM1, but this is not the case in DM2 (Logigian *et al.*, 2007).

The cardiac muscle is also affected by DM, resulting in cardiac arrhythmias, cardiomyopathy and conduction defects, which can all cause sudden death in DM patients. Cardiac conduction defects are diagnosed by electrocardiography (ECG) and are common to both forms of the disease,

however, around 75% of DM1 patients suffer conduction blocks, compared to only 20% of DM2 patients (Wells & Ashizawa, 2006).

A key feature of DM is cataract formation, which is observed in both forms of the disease and in mildly affected patients it can be the only sign or symptom of DM. The cataract that forms is distinct in its early stages from other types of cataract and can be easily seen under slit-lamp examination.

Central nervous system (CNS) abnormalities are more prominent in DM1, with degenerative CNS changes leading to mental retardation, depression, hypersomnia and anxiety disorders (Wells & Ashizawa, 2006).

Endocrine abnormalities are common in DM. Abnormal glucose tolerance is present in both forms of the disease and can lead to diabetes mellitus, which is more common in DM2. Hypotestosteronism with elevated levels of follicle-stimulating hormone (FSH), frontal balding and testicular atrophy are also common to both forms. Hyperhidrosis (excessive levels of perspiration) is found mainly in DM2 (Wells & Ashizawa, 2006). The occurrence of pilomatricomas, which are benign calcifying epitheliomas of the hair follicle, appear to be more common in DM1 patients than in the general public. It has also been suggested that DM1 is linked to certain types of cancer with cases of thymoma and neoplasms of the parotid gland, parathyroid and thyroid being the most commonly reported (Harper, 2001; Mueller *et al.*, 2009).

A congenital form of DM, where development of the foetus is affected and signs and symptoms are present from birth, is only seen in DM1. During pregnancy, mothers may notice reduced foetal movement and polyhydramnios (excess of amniotic fluid). Following birth, clinical characteristics of this form of the disease include hypotonia (low muscle tone resulting in being 'floppy' at birth); talipes; a tented upper lip; a high-arched palate; bilateral facial weakness; problems with swallowing, breathing and suckling; faecal incontinence and mental retardation (Machuca-Tzili *et al.*, 2005; Schara & Schoser, 2006). Interestingly, neither myotonia nor cataracts are present at birth but both develop after the first decade of life.

Diagnosis of DM is made after clinical examinations and tests have confirmed the presence of many of the clinical features described above (See table 1.1 for a summary of the major clinical features associated with the two forms of the disease). Genetic tests which can identify the underlying genetic mutations are

also available and are commonly used to confirm the findings and differentiate between the two forms as variability in the presenting symptoms make a positive diagnosis almost impossible.

Feature	DM1	DM2
Muscle		
Distal muscles affected initially	+	-
Proximal muscles affected initially	-	+
Myotonia	+	±
Weakness	+	+
Atrophy	+	±
Pain	±	+
Type 1 muscle fibres affected	+	-
Type 2 muscle fibres affected	-	+
Heart		
Cardiac conduction defects	+	+
Eye		
Cataract	+	+
Central nervous system		
Cognitive dysfunction	+	±
Hypersomnia	+	±
Mental retardation	+	-
Endocrine system		
Insulin resistance	+	+
Testicular atrophy	+	+
Frontal balding	+	+
Hyperhidrosis	-	+
Thyroid dysfunction	+	±
Genetic features		
Congenitive form	+	-
Anticipation	+	-

Table 1.1 – A summary of the major clinical features associated with the two forms of DM.
 - = absent, ± = present to a mild degree, + = present.

1.4.3 The genetic mutations underlying DM

Two distinct mutations are known to cause DM, resulting in two forms of the disease. DM1 is the most prevalent form (responsible for around 98% of cases), although in some countries, such as Germany and Poland, the incidence of the two forms is approximately equal. The mutations that underlie DM are a type of mutation known as a repeat expansion, which are repeat sequences found within DNA that are unstable and of variable length. There are over 20 disorders caused by repeat expansions, including fragile X syndrome and the cerebellar ataxias. DM1 is caused by the expansion of a cytosine-thymine-guanine (CTG) triplet repeat located in the 3' untranslated region (UTR) of the *DMPK* gene on chromosome 19 (Brook *et al.*, 1992). DM2, however, results from a CCTG repeat located in the first intron of the *ZNF9* gene on chromosome 3 (Liquori *et al.*, 2001). Therefore, the mutations underlying both forms of DM are located in non-coding regions of genes, which are transcribed into RNA but are not translated into protein. Despite the differences in the sequence and location of the repeats that cause the two forms of DM, they share many of the same symptoms (summarised in table 1.1). Those of DM1, however, appear to be more severe than those of DM2.

The inheritance of both mutations is autosomal dominant, which means an affected individual will have one normal copy of the gene and one containing the repeat mutation. There is, therefore, a 50% chance of an affected individual passing the disease to their offspring, with males and females having an equal chance of inheriting the disease.

A small, stable CTG repeat in the *DMPK* gene is present in normal individuals, varying in length from 5-37 repeats. DM1 patients, however, have unstable repeats with lengths starting at 50 and ranging to over 1000. Repeats in the region of 38-50 do not result in DM1, but are considered a premutation which could lead to the development of the disease in future generations (Wells & Ashizawa, 2006). Repeat numbers in DM2 can be much larger and range from 75 to 11 000. In normal individuals the repeat tract within the *ZNF9* gene is shorter (≤ 26 repeats) and interrupted with GCTG and/or TCTG motifs (Liquori *et al.*, 2001).

The repeat regions that cause DM are unstable and biased towards expansion. The repeats expand in the somatic cells of an individual during their

lifetime and also between generations, particularly when passed maternally. The number of repeats also varies depending on cell and tissue type. For example, cells in skeletal muscle, heart and brain have significantly larger repeat expansions than leukocytes (Thornton *et al.*, 1994). This is known as somatic mosaicism. Expansions during the lifetime of an individual and somatic mosaicism are more pronounced in DM2 (Wells & Ashizawa, 2006).

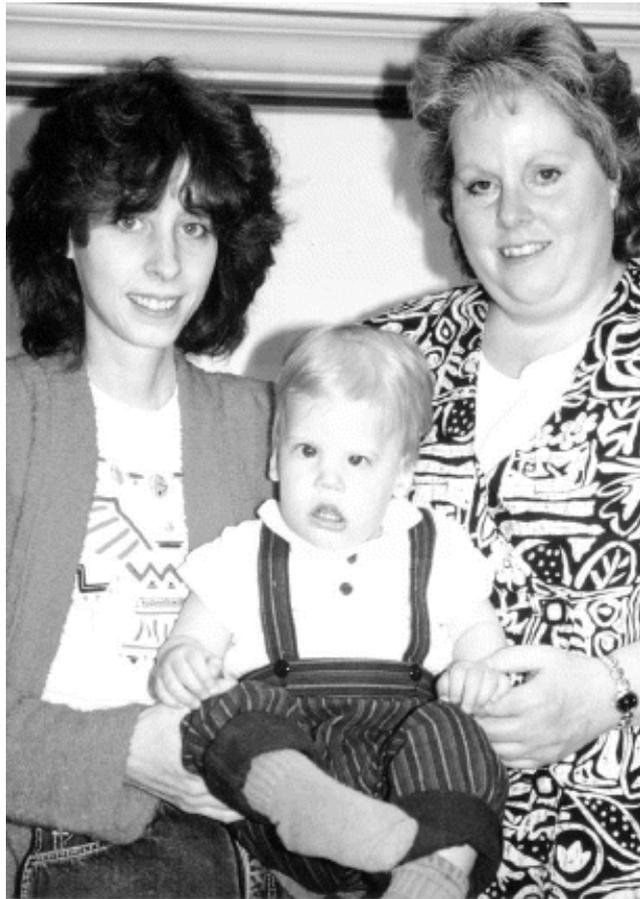


Figure 1.7 – A family affected by DM1 showing anticipation where the severity of the disease increases in subsequent generations. The grandmother (right) is only mildly affected, whereas the mother (left) shows signs of classic adult onset DM1, such as weakened facial muscles. The congenitally affected child has a characteristic tented upper lip and talipes (image from Jorde *et al.* (2000)).

In DM1 the expansion of repeats between generations results in a phenomenon known as anticipation, where the severity of the disease increases

from generation to generation due to increasing repeat numbers. There is little evidence of this in DM2, despite the much larger repeat numbers associated with this form of the disease. Anticipation results in more severe symptoms and an earlier onset with successive generations leading to three different forms of DM1: mild; adult onset; and congenital (see figure 1.7). Individuals with the mild form are born with between 50 and 100 repeats, those with the adult onset form have 200 to 500 and those with the severe congenital form have over 1000 repeats (Harper *et al.*, 2004).

In DM1, the transmission of repeats from generation to generation is different when passed maternally or paternally. Repeats in the range of 50 to 80 are most likely to expand when passed paternally, whereas larger repeats of more than 100 are most likely to expand when passed maternally (Harper *et al.*, 2004). The congenital form of DM1 is almost exclusively passed on by the mother, suggesting that oocytes with large triplet repeats remain viable, but sperm with the same sized repeats are unable to produce offspring (Meola, 2000).

1.4.4 Molecular diagnosis of DM

Variability between the two forms of DM results in the requirement of genetic testing for an accurate diagnosis. DNA tests involve amplification across the repeat expansion at the DM1 or DM2 locus by polymerase chain reaction (PCR), which reveals either two normal sized alleles in unaffected individuals, or a single allele in affected individuals as alleles containing large repeats do not amplify. To distinguish between affected individuals and unaffected homozygotes (15% of the population in DM2) which also present with a single band on PCR, Southern blot analysis is used. Somatic mosaicism in DM2 may cause a smear from the expanded allele using this method and therefore requires an additional repeat assay where the area containing the repeat is amplified by PCR with reverse primers at various sites within the CCTG repeat tract and the product is probed with specific internal oligonucleotide probes (Wells & Ashizawa, 2006).

1.5 Myotonic dystrophy type 1

DM1 is the most prevalent form of DM and has been estimated to be the cause of 98% of cases. The following chapters of this thesis are aimed solely at

describing DM1 and at elucidating the mechanisms behind the effects of the CTG triplet repeat expansion.

1.5.1 The DM1 gene locus

The DM1 locus (see figure 1.8) is located in a gene rich region of chromosome 19q13.3 within the *DMPK* (dystrophia myotonica protein kinase) gene, which is flanked upstream by the *DMWD* (dystrophia myotonica-containing WD repeat motif) gene (formally known as *gene 59*) and downstream by the *SIX5* (sine oculis homeobox homolog 5) gene (formally known as *DMAHP*). All three genes are located within a region spanning only 40 kb.

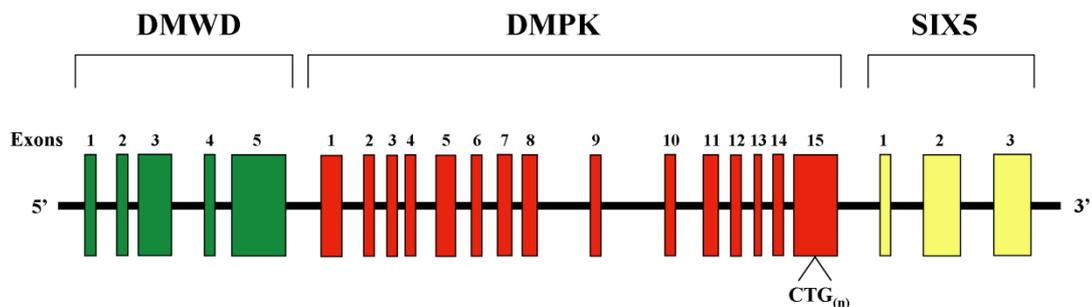


Figure 1.8 – A diagrammatic representation of the DM1 locus. The CTG triplet repeat is located within exon 15 of the *DMPK* gene.

The *DMPK* gene is around 13 kb in length and contains 15 exons. The mutation is found within the final exon which encodes the 3' UTR of the *DMPK* mRNA. The *DMPK* gene encodes a serine/threonine protein kinase, which belongs to the AGC kinase family (the collective name for cAMP-dependent protein kinase A, cGMP-dependent protein kinase G, and phospholipid-dependent protein kinase C) and is closely related to other kinases that interact with members of the Rho family of small GTPases (van Herpen *et al.*, 2005). There are 6 major isoforms and 1 minor isoform of DMPK resulting from a combination of three alternative splicing events (see figure 1.9). Of the major isoforms, four encode proteins of around 74 kDa and the final two, which are predominantly found in

smooth muscle, encode proteins of around 68 kDa (Elkins *et al.*, 2009). The seventh minor isoform is only found in humans and does not contain the triplet repeat in its 3' UTR. The predicted sequence of DMPK shows five distinct domains, three of which are conserved between isoforms: a leucine-rich N-terminus; a serine/threonine protein kinase domain; and a coiled-coil region. Alternative splicing controls the inclusion of the final two domains which are isoform specific. These are the VSGGG motif and the C terminal tail, of which there are three forms (the seventh isoform contains a further unique C terminal tail) (Elkins *et al.*, 2009). The C terminal domain controls the subcellular localisation of the DMPK protein (Wansink *et al.*, 2003; van Herpen *et al.*, 2005). The smooth muscle isoforms have a short C terminal tail of only 2 amino acids, which targets them to the cytoplasm. Of the other two tail forms, one is hydrophobic which targets to the ER, and one is hydrophilic which binds to the mitochondrial outer membrane (Kaliman & Llagostera, 2008).

DMPK has been shown to be expressed in detectable amounts in the eye and in skeletal, smooth and cardiac muscle in humans (Dunne *et al.*, 1996; Winchester *et al.*, 1999; Lam *et al.*, 2000). More sensitive methods used to analyse expression in mice, have shown *DMPK* to also be present in brain, testis, thymus, pituitary, skin, lung and liver (Sarkar *et al.*, 2004a). The function of *DMPK* is not certain but overexpression of *DMPK* in undifferentiated mouse muscle cells resulted in expression of skeletal muscle-specific genes suggesting a role for *DMPK* in the myogenic pathway (Bush *et al.*, 1996). A study conducted on skeletal muscle cells from *dmpk* knockout mice also revealed a possible role for *DMPK* in the modulation of Ca^{2+} homeostasis and events of excitation-contraction coupling (Benders *et al.*, 1997). Overexpression studies carried out in human lens cells resulted in blebbing of the plasma membrane, suggesting a further role in reorganisation of the actin cytoskeleton and plasma membrane (Jin *et al.*, 2000). The reorganisation of the cytoskeleton is an important factor in synaptic plasticity and studies have shown that *DMPK* may also play a role in this, which could be relevant to the cognitive impairment observed in DM1 (Schulz *et al.*, 2003). Further studies have identified possible substrates for *DMPK* which include phospholemman, phospholamban and the targeting subunit of myosin phosphatase (Mounsey *et al.*, 2000; Muranyi *et al.*, 2001; Kaliman *et al.*, 2005). *DMPK* has also been shown to phosphorylate histone H1 in vitro (Dunne *et al.*,

1994). A novel heat shock protein, designated myotonic dystrophy protein kinase binding protein (MKBP), was shown to bind and activate DMPK in vitro. MKBP was found to be highly expressed in human heart and skeletal muscle (Suzuki *et al.*, 1998). Recently, DMPK has been shown to bind to other heat shock proteins including α B-crystallin, which is predominantly expressed in the lens, heart and skeletal muscle, which are all tissues affected by DM1 (Forner *et al.*, 2010).

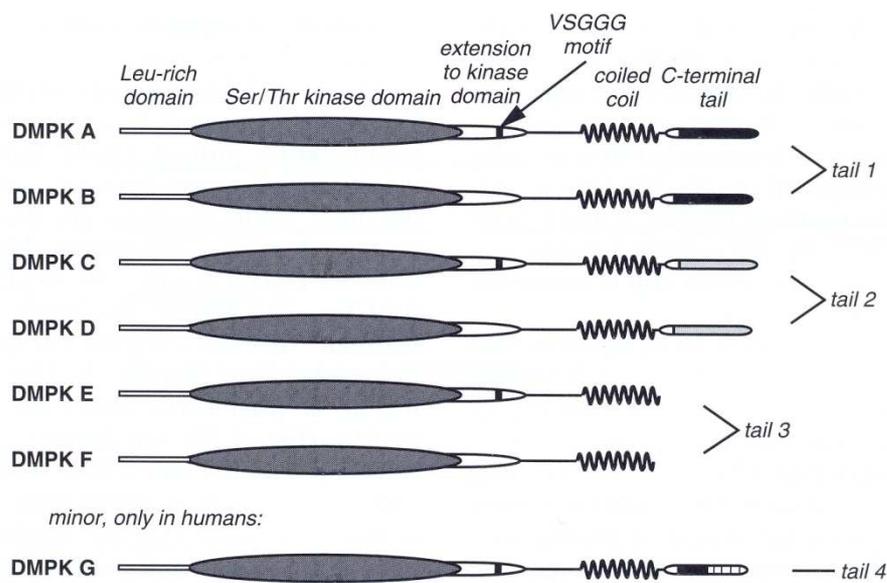


Figure 1.9 – A diagrammatic representation of the protein domain organisation of DMPK isoforms produced by alternative splicing of the *DMPK* gene. DMPK A-F are the major isoforms, whereas DMPK G is a minor isoform only found in humans. All isoforms share a leucine-rich N-terminal domain, a serine/threonine protein kinase domain and a coiled-coil region. Alternative splicing events lead to the presence or absence of a VSGGG motif within the kinase domain and the production of three different C-terminal tails, with a fourth unique tail exclusive to the minor G isoform (image from Wells & Ashizawa (2006)).

Immediately downstream of the *DMPK* gene is the gene *SIX5* which contains 3 exons and encodes a homeodomain containing transcription factor. *SIX5* contains a Six domain (SD) which lies immediately N-terminal to a Six-type homeodomain (HD), which are both required for binding to specific DNA

sequences and also to transcriptional coactivators (Kawakami *et al.*, 2000). SIX5 can also act as a transcriptional repressor through interactions between the SD and Groucho family corepressors (Sato *et al.*, 2002). SIX5 has been shown to bind to the Na⁺/K⁺ ATPase α 1 subunit gene (*ATP1A1*) regulatory element (ARE) and the myogenic enhancing factor 3 (MEF3) consensus sequence, which regulate expression of genes such as *ATP1A1*, myogenin, aldolase A and cardiac troponin C (Harris *et al.*, 2000). *SIX5* is expressed in human skeletal and heart muscle, eye and brain (Thornton *et al.*, 1997; Winchester *et al.*, 1999). The mouse homologue has also been shown to be expressed in the testis where it is proposed to play a role in spermatogenic cell survival and spermiogenesis (Sarkar *et al.*, 2004b).

Upstream of *DMPK* is the gene *DMWD* which is around 11 kb in length and contains 5 exons (Jansen *et al.*, 1995). The protein encoded by this gene has a proline rich N-terminal domain and contains four WD repeat units, but its function is unknown. *DMWD* is most prominently expressed in the brain and testis, but also in the heart, liver, kidney and spleen (Shaw *et al.*, 1993; Westerlaken *et al.*, 2003). High levels of the protein DMWD have been found in maturing and mature neurones, especially in the dendritic projections which contain many synapses, leading to speculation that it is involved in neuronal development (Westerlaken *et al.*, 2003).

1.5.2 How does the DNA triplet repeat cause DM1?

Despite the discovery of the mutation that underlies DM1, the pathological mechanisms that lead to the wide array of symptoms seen in the disease is the subject of much debate. Many theories exist to explain how the expansion of a DNA triplet repeat could cause the multisystemic effects observed in DM1 but there are currently three main hypotheses to explain this: a reduction in *DMPK* expression; a reduction in expression of the neighbouring genes, *SIX5* and *DMWD*; or a gain of function by the mutant *DMPK* mRNA. There is support for all three hypotheses but whether the effects seen in the patients can be attributed to only one or a combination of these is unknown.

1.5.2.1 *DMPK* haploinsufficiency

As the repeat is found within the *DMPK* gene, the most obvious theory for the cause of DM1 is an effect in *cis* on the *DMPK* gene resulting in a change in

the DMPK protein. As the repeat is located in a non-coding region of the *DMPK* gene, the disease is unlikely to be the result of a gain of function through the production of a mutant protein. However, it could be the result of a reduction in the expression of *DMPK* or a reduction in the translation of *DMPK* mRNA into protein.

Studies have shown that mutant *DMPK* mRNA forms highly stable foci which are retained in the nucleus, however, a threshold of around 150 repeats has to be reached before this is observed (Taneja *et al.*, 1995; Davis *et al.*, 1997; Hamshere *et al.*, 1997). The retention of transcripts only occurs from the affected allele, whereas those from the normal allele are able to pass into the cytoplasm, making them available for translation. Some mutant transcripts have been found within the cytoplasm but only as the result of the breakdown of the nuclear envelope during cell division, however, many of the tissues affected by DM1 are predominantly composed of postmitotic cells, therefore the retention of transcripts most likely results in haploinsufficiency of *DMPK* (Davis *et al.*, 1997).

Despite the evidence for this hypothesis it is unlikely to be sufficient to cause DM1 alone as no case of DM1 has ever been shown to be the result of a point mutation in the *DMPK* gene (Day & Ranum, 2005). Studies carried out in mice have shown that knockouts of one or both alleles of *Dmpk* result in only mild effects and development seemed unaffected (Jansen *et al.*, 1996; Reddy *et al.*, 1996; Berul *et al.*, 1999; Llagostera *et al.*, 2007). The study by Jansen *et al.* (1996) showed only minor muscle fibre size changes in adult nullizygous (*Dmpk*^{-/-}) mice, whereas Reddy *et al.* (1996) showed a progressive, late onset, skeletal myopathy, which was compared to the adult onset form of DM1. Berul *et al.* (1999) showed that *Dmpk*^{-/-} mice develop cardiac conduction defects which are also characteristic of the human form of the disease. Subsequently, cardiac myocytes isolated from *Dmpk*^{+/-} and *Dmpk*^{-/-} mice were shown to display abnormalities in Na⁺ channel gating and Ca²⁺ cycling, both of which were implicated in the cardiac dysfunction observed in DM1 (Lee *et al.*, 2003; Pall *et al.*, 2003). A more recent study by Llagostera *et al.* (2007) showed that *Dmpk*^{-/-} mice exhibit impaired insulin signalling and have an abnormal glucose tolerance which could be linked to the insulin resistance which leads to diabetes in DM1. Despite the features seen in the mouse models, none of the studies have shown the additional signs and symptoms characteristic of DM1, such as myotonia and cataract. It is therefore unlikely that

all of the effects seen in DM1 are the result of haploinsufficiency of *DMPK*, but it is possible that this contributes to the effects.

1.5.2.2 Reduced expression of *SIX5* and *DMWD*

The second hypothesis aimed at describing the cause of DM1 is a *cis*-acting effect on the surrounding genes at the DM1 locus. The genes at the locus are so close that the 3' terminus of the *DMWD* gene overlaps with the promoter of the *DMPK* gene and the 3' terminus of the *DMPK* gene overlaps with the promoter of the *SIX5* gene (Shaw *et al.*, 1993; Boucher *et al.*, 1995; Frisch *et al.*, 2001). The expansion of the CTG repeat has been suggested to result in changes to the higher-order structure of the DNA, resulting in changes in expression levels of the surrounding genes, known as the 'field-effect'. The extended CTG repeats seen in DM1 have been shown to create the strongest known natural nucleosome positioning signal which attracts nucleosome assembly *in vitro* (Wang *et al.*, 1994). The wrapping of the CTG repeat around nucleosomes results in alterations to the local chromatin structure which limits the accessibility and formation of transcriptional complexes and could therefore limit the expression of all three genes at the DM1 locus.

The promoter region of *SIX5* contains an exceptionally large (around 3.5 kb) CpG island (cytosine and guanine nucleotides joined by phosphodiester bonds), which is interrupted by the CTG repeat (Boucher *et al.*, 1995). The triplet repeat expansion has been shown to alter the chromatin structure around the site, making the DNA in the region less accessible to nuclear proteins, such as DNase I (Otten & Tapscott, 1995). Many studies have looked at the expression levels of *SIX5* in DM1 and the majority have shown a reduction in expression (Hamshere *et al.*, 1997; Klesert *et al.*, 1997; Thornton *et al.*, 1997; Eriksson *et al.*, 1999; Inukai *et al.*, 2000; Frisch *et al.*, 2001; Rhodes *et al.*, 2006). Thornton *et al.* (1997) showed that expression of the DM1-linked allele in myoblasts of DM1 patients was reduced and in some cases approached complete inactivation, however, expression was not reduced in a DM1 patient with a small CTG expansion (< 80 repeats) and no muscle weakness. Surprisingly, Klesert *et al.* (1997) and Inukai *et al.* (2000) showed that expression of *SIX5* was actually reduced by more than half, suggesting that expression from the normal allele was also somehow affected. Conflicting studies by Hamshere *et al.* (1997) and Eriksson *et al.* (1999) showed

no difference in levels of *SIX5* expression in DM1 cell lines and skeletal muscle samples compared to controls.

Knock-out studies performed in mice have shown that partial (*Six5*^{+/-}) or complete (*Six5*^{-/-}) loss of *Six5* results in the development of some of the signs and symptoms characteristic of DM1. Both *Six5*^{+/-} and *Six5*^{-/-} mice develop cataracts and the incidence and severity of lens opacities were shown to increase with decreasing *Six5* dosage (Klesert *et al.*, 2000; Sarkar *et al.*, 2000). A further study has shown that male *Six5*^{-/-} mice are sterile and suffer from testicular atrophy and both *Six5*^{+/-} and *Six5*^{-/-} mice were shown to have increased FSH levels (Sarkar *et al.*, 2004b). These are all characteristic symptoms of DM1; however, it is unlikely that a loss of *SIX5* alone could cause DM1 due to the lack of any abnormalities in skeletal muscle function.

In the field-effect model, levels of *DMWD* expression are also predicted to be affected by the change in local chromatin structure. Again, a number of studies have looked at expression levels of *DMWD* and have had conflicting results (Hamshere *et al.*, 1997; Alwazzan *et al.*, 1999; Eriksson *et al.*, 1999; Frisch *et al.*, 2001). Hamshere *et al.* (1997) and Frisch *et al.* (2001) showed that there was no difference in levels of *DMWD* expression between DM1 and control cell lines and Eriksson *et al.* (1999) showed considerable overlap in expression levels in skeletal muscle biopsies from DM1 patients and control individuals. However, Eriksson *et al.* (1999) did show that there was a strong inverse correlation between repeat size and *DMWD* expression in the patient samples. Alwazzan *et al.* (1999) performed allele-specific analysis in both nuclear and cytoplasmic compartments of DM1 and control fibroblast cell lines. No difference was seen in levels of transcripts in the nuclear fraction, but a reduction in expression of 20-50% from the DM1-linked allele was seen in the cytoplasmic fraction. No correlation was seen in this study between repeat size and expression levels in the cell lines.

CNS abnormalities are common in DM1 with features such as variable cognitive dysfunction (including memory loss, lower IQ and mental retardation), somnolence, anxiety disorders and cerebral white matter lesions being associated with the disease (Harper, 2001). *DMWD* is found mainly in the brain and changes in *DMWD* expression levels could result in the CNS abnormalities observed. The inverse correlation between repeat size and *DMWD* expression in DM1 patients samples observed by Eriksson *et al.* (1999) could be used to describe why mental

retardation is such a prominent feature in congenital DM1 where repeat sizes are very large, compared to lower IQs which are seen in patients with adult onset DM1. There are at present no mouse models to elucidate any possible effects caused by a loss of *DMWD*.

The field-effect hypothesis aims to describe the multisystemic effects seen in DM1 by the loss of multiple genes at the DM1 locus; however, there are still symptoms of DM1 that have not been observed in mouse models. One of the characteristic features of DM1 is myotonia, which has not been described by this theory. The theory also fails to explain how a second form of DM, DM2, can present with almost identical symptoms such as myotonia, cataract and cardiac abnormalities, without having any link to the DM1 locus or the genes within it. It is clear that although the field-effect model could explain some of the multisystemic effects observed in DM1, a further pathogenic mechanism must be present to explain the remaining effects.

1.5.2.3 Toxic gain of function by *DMPK* mRNA

The final theory to describe the cause of DM1 is the *trans*-acting effect of the repeat at the mRNA level. The DNA triplet repeat is transcribed into RNA, resulting in a CUG_(n) repeat which is proposed to form stable secondary structures that sequester specific RNA binding proteins in a gain-of-function manner. The proteins sequestered by the CUG repeats have roles in splicing and could explain the altered splice patterns which are observed in DM1. The toxic gain of function hypothesis has the greatest support as the cause of DM and current research efforts are mostly focused in this area.

Evidence for this theory came from a study where expanded (around 250 repeats) and non-expanded (5 repeats) repeats were inserted into a genomic fragment containing the human skeletal actin (*HSA*) gene and were expressed in the muscle of transgenic mice (Mankodi *et al.*, 2000). The expanded repeats were inserted into the final exon of the *HSA* gene between the termination codon and polyadenylation site to mimic the situation seen in the *DMPK* gene, but without involving any of the genes that are normally associated with DM1. Mankodi *et al.* (2000) showed that the presence of long triplet repeats caused myotonia in mice as early as 4 weeks of age, which did not occur in short-repeat or wild-type mice. Myotonia was not observed in any of the previous mouse models for the disease

involving knock-outs of the genes at the DM1 locus. The transcripts were also observed to form foci and be retained in the nucleus, as is seen with the mutated *DMPK* transcripts in DM1.

The expanded CUG repeats have been shown to form stable hairpin structures where the repeat tract folds back on itself and results in double-stranded RNA (dsRNA) with G•C and C•G base pairs separated by U•U mismatches (Tian *et al.*, 2000). The dsRNA structures have been shown to bind to and activate PKR, which is the dsRNA-activated protein kinase. PKR is involved in the viral response pathway which aims to prevent or slow viral replication through initiating mechanisms such as increased mRNA degradation and apoptosis (Tian *et al.*, 2000). The CUG repeats have also been shown to bind to RNA-binding proteins from two families known as CUG-BP1 and ETR-3-like factors (CELF) and muscleblind-like (MBNL) proteins (Timchenko *et al.*, 1996; Miller *et al.*, 2000). Both families are involved in the regulation of alternative splicing events during mRNA processing. The misregulation of splicing events is a characteristic of DM1, where the splicing patterns for a subset of transcripts revert to those observed during embryonic development. The MBNL proteins, MBNL1, MBNL2 and MBNL3, bind to the dsRNA formed by the CUG repeats and have been shown to co-localise with the foci produced by mutant *DMPK* transcripts in DM1 cells (Fardaei *et al.*, 2001; Fardaei *et al.*, 2002). The MBNL proteins are sequestered by the mutant *DMPK* transcripts and are unable to perform their usual functions. MBNL proteins regulate alternative splicing of muscle-specific chloride channel (ClC-1), cardiac troponin T (TNNT2), skeletal muscle troponin T (TNNT3), insulin receptor (IR) and Tau (τ) (Kanadia *et al.*, 2003; Ho *et al.*, 2004; Jiang *et al.*, 2004; Dansithong *et al.*, 2005; Dhaenens *et al.*, 2008). Interestingly, the *Drosophila* MBNL homologue, *muscleblind*, is essential for terminal differentiation of muscle and the photoreceptors of the eye, which are two tissues affected by DM1 in humans (Begemann *et al.*, 1997; Artero *et al.*, 1998). In contrast to the MBNL proteins, CUG-binding proteins 1 and 2 (CUG-BP1/2) are not sequestered by the CUG repeats, although a small fraction may localise to foci as they have been shown to bind to single-stranded RNA (ssRNA) at the base of the CUG repeat hairpin (Timchenko *et al.*, 1996; Lu *et al.*, 1999; Michalowski *et al.*, 1999; Fardaei *et al.*, 2001). In DM1, CUG-BP1/2 are translocated to the nucleus of DM1 cells where they have been shown to be

hyperphosphorylated by PKC (Timchenko *et al.*, 1996; Roberts *et al.*, 1997; Philips *et al.*, 1998; Kuyumcu-Martinez *et al.*, 2007). Phosphorylation of CUG-BP1/2 increases their half life, resulting in their accumulation and therefore increased levels of activity. Despite the increase in activity, levels of CUG-BP1/2 mRNA were not affected in DM1 (Timchenko *et al.*, 2001a). CUG-BP1/2 act antagonistically to MBNL proteins in the splicing of TNNT2, IR and CIC-1 (Philips *et al.*, 1998; Savkur *et al.*, 2001; Charlet-B *et al.*, 2002; Ho *et al.*, 2004; Dansithong *et al.*, 2005; Paul *et al.*, 2006). Foetal splicing patterns of these transcripts are observed in DM1 and may contribute to the cardiac abnormalities, insulin resistance and myotonia which are common features of the disease.

Evidence for the toxic RNA theory comes from a knockout mouse model for *Mbnl1* which was shown to develop cataracts with dust-like opacities, myotonia and altered regulation of mRNA splicing of transcripts for CIC-1, TNNT3 and TNNT2 (Kanadia *et al.*, 2003). A knockout mouse model for *Mbnl2* has also been shown to develop myotonia, display histological features of muscle such as increased numbers of central nuclei and decreased fibre size and show altered regulation of mRNA splicing of transcripts for CIC-1 (Hao *et al.*, 2008). Down-regulation of *MBNL1/2* expression in normal human myoblasts using small interfering RNA (siRNA) resulted in abnormal IR splicing, as did overexpression of *CUG-BP1* (Dansithong *et al.*, 2005). In the same study it was also shown that *MBNL1* down-regulation caused disaggregation of 70% of the foci formed in DM1 cells, compared to 25% following *MBNL2* downregulation, suggesting that MBNL1 plays a key role in foci formation and maintenance (Dansithong *et al.*, 2005). Morpholino antisense oligonucleotides have also been used to block the interaction of Mbnl1 with CUG repeats in the transgenic mouse model of DM1 where CUG repeats were inserted into the HSA gene. Nuclear foci were reduced and Mbnl1 was redistributed, allowing the regulation of splicing events to be corrected (Wheeler *et al.*, 2009). MBNL proteins have also been shown to co-localise with mutant transcripts in nuclear foci and have been implicated in the abnormal splicing of CIC-1 and IR in DM2 (Mankodi *et al.*, 2001; Fardaei *et al.*, 2002; Mankodi *et al.*, 2002; Savkur *et al.*, 2004). The toxic gain of function model could therefore explain the similarities between the two forms of DM, as the production of foci and sequestration of MBNL proteins occur irrespective of the genes that are affected by the repeat mutations.

Interestingly, DM-like symptoms were induced in transgenic mice overexpressing *DMPK* containing a normal number of CTG repeats (11 repeats). *DMPK* transcripts were found to accumulate over time and symptoms became apparent in ageing mice between 11-15 months old. Symptoms included muscle myopathy and myotonia, hypertrophic cardiomyopathy and a reduction in the tolerated workload of the mice. The authors suggested that this was due to the accumulation of *DMPK* transcripts containing smaller repeats which mimicked the effects of those containing larger CUG repeats in DM1 (O'Coilain *et al.*, 2004). A separate group have also shown that the overexpression of a normal *DMPK* 3' UTR containing 5 CTG repeats leads to DM-like symptoms in transgenic mice, however, these effects were not age-dependent. This study showed that the transgenic mice developed myotonia as early as 2 days after the induction of transgene expression and sudden death due to cardiac conduction defects occurred after 4-6 weeks (Mahadevan *et al.*, 2006). A subsequent study by the same group showed that overexpression of the normal *DMPK* 3' UTR induced expression of the cardiac-specific transcription factor NKX2-5 which resulted in reduced levels of connexin 43 and connexin 40 (Yadava *et al.*, 2008). Connexins are important components of gap junctions which are required for the propagation of electrical impulses in the heart. In the same study the level of NKX2-5 was also found to be greater in the hearts of human DM1 patients and it was also present in DM1 skeletal muscle, but not in controls (Yadava *et al.*, 2008). Although the data described here appears to show that the CTG repeats are not responsible for the symptoms of DM1, it indicates that a gain of function by the *DMPK* transcripts is.

1.6 The eye and myotonic dystrophy

The eye is one of the many systems affected by DM1 and a wide range of ocular problems can occur. Symptoms in the eye include cataract, retinal degeneration, low intraocular pressure, enophthalmos, ptosis, epiphora, corneal lesions, extraocular myotonia and muscle weakness and abnormal central control of eye movement (Harper, 2001). Not all of the ocular abnormalities may be present and many of them go unnoticed by the sufferer, but a reliable symptom present in the majority of patients, and even in those unaffected by the more

general muscle symptoms, is cataract. Cataracts result in a loss of visual acuity and contrast sensitivity which can make everyday tasks very difficult.

1.6.1 Cataract and myotonic dystrophy

Cataracts were first linked to DM1 over one hundred years ago and since then have formed the basis of the phenomenon of anticipation in the disease. Cataract is a characteristic symptom of DM1 and even occurs in the adult onset form with minimally expanded CTG repeats, linking seemingly unaffected individuals to the disease (Harper *et al.*, 2004). Cataracts are therefore often found in older generations of affected individuals and can be used to identify the line by which the disease was inherited, enabling other family members who may be affected by the disease to be identified. DM1 cataracts normally present at an earlier age than is usually associated with senile cataracts and have been seen in DM1 patients from their second decade of life onwards.

The cataract that forms in DM1 is very distinctive in its early stages and cataract detection using slit-lamp examination was widely used as a diagnostic tool before genetic tests became available. The DM1 cataract is still used to identify new families with DM1 through minimally affected patients (Kidd *et al.*, 1995). In its early stages the DM1 cataract presents with white or multicoloured iridescent scattered dust-like opacities (see figure 1.10a), which are mainly found in the posterior subcapsular region of the lens (see figure 1.5). As the cataract develops the opacities increase in size and number and cortical spokes (see figure 1.10b) and star-shaped opacities form, which cloud the lens and eventually form a mature cataract. At this stage the cataract is indistinguishable from those of other causes as the iridescent opacities can no longer be seen (Harper, 2001).

Ultrastructural analysis of cataractous lenses from DM1 patients using electron microscopy revealed that the characteristic dust-like iridescent opacities are actually whorls of plasma membrane. The whorls of plasma membrane were found in fibre cells from about 10 fibres deep within the lens and contained cytoplasm. LECs had poorly delineated plasma membranes and were shown to contain nuclei with clumped chromatin, degenerating mitochondria and enlarged intracellular clefts or cristernae (Eshaghian *et al.*, 1978).

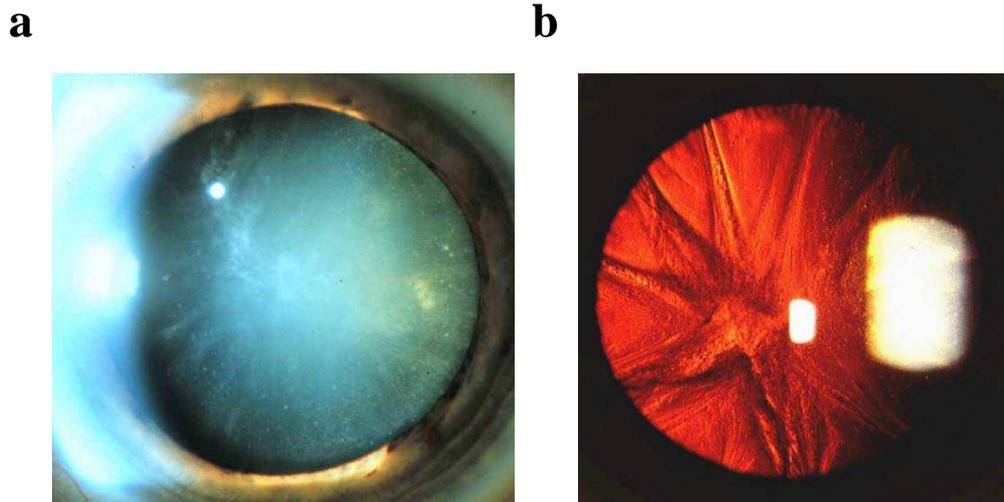


Figure 1.10 – Slit-lamp photographs of DM1 cataracts. (a) A DM1 cataract in its early stages, showing the white and iridescent scattered dust-like opacities which distinguish it from most other types of cataract (diffuse illumination). (b) A DM1 cataract at a slightly later stage where cortical spokes have begun to form and the scattered opacities become less conspicuous (retro-illumination) (images from <http://www.onjoph.com>).

The underlying cause of cataract formation in DM1 is unknown. At the molecular level, both *DMPK* and *SIX5* have been shown to be expressed in the eye and in particular in the lens. Conflicting results exist as to whether *DMPK* is actually expressed in the lens, but studies have shown it to be expressed in human, bovine and mouse lenses (Dunne *et al.*, 1996; Abe *et al.*, 1999; Winchester *et al.*, 1999; Sarkar *et al.*, 2004a; Rhodes *et al.*, 2006). *SIX5* has been implicated in the development of DM1 cataracts due to its role as a transcription factor in regulating *ATP1A1* expression. If *ATP1A1* expression is altered it could affect the ion balance in the lens and lead to cataract (Winchester *et al.*, 1999). Two different studies conducted at the same time showed that *Six5* knockout mouse models developed cataracts, however, the cataracts which formed were nuclear and not posterior subcapsular as is seen in DM1 in humans (Klesert *et al.*, 2000; Sarkar *et al.*, 2000). RNA extracted from the mouse models was used to analyse steady-state *Atp1a1* mRNA levels and had conflicting results. Klesert *et al.* (2000) showed that there was no difference in steady-state levels, whereas Sarkar *et al.* (2000) showed that steady-state levels increased as a function of decreasing *Six5*

dosage. The role of *SIX5* and *ATP1A1* in DM cataract is therefore still the subject of debate. Overexpression of *DMPK* in lens cells has been shown to result in apoptotic-like blebbing and actin cytoskeleton reorganisation, resulting in speculation that *DMPK* may play a role in the breakdown of organelles during lens fibre differentiation (Jin *et al.*, 2000). If this was the case then it has been proposed that reduced levels of *DMPK* could result in cataract formation due to the decreased removal of intracellular membranes, leading to the retention of organelles which can obscure vision (Jin *et al.*, 2000). Loss of *DMPK*, if its predicted role is true, could therefore account for the observations from the ultrastructural studies which showed that the iridescent particles were light reflecting membranous structures derived from the plasma membrane (Eshaghian *et al.*, 1978). If *DMPK* is indeed expressed in the lens then mutant RNA could also accumulate and sequester MBNL proteins as shown in other tissues. Interestingly, a *Mbnl1* knockout mouse model developed cataracts which showed the distinctive iridescent opacities normally observed in DM (Kanadia *et al.*, 2003). The *MBNL* homologue in *Drosophila*, known as *muscleblind*, is essential for muscle and eye differentiation, which would indicate a role for MBNL in the vertebrate eye.

At the cellular level, studies on cataracts in general have pointed at a loss of cells from the lens epithelium in the development of cataract due to the resulting loss of homeostasis in levels of ions and water (Konofsky *et al.*, 1987; Li *et al.*, 1995). Capsulorhexis samples from cataract patients were shown to have a significantly greater percentage of apoptotic cells compared to normal controls and LEC death was shown to precede cataract development in cultured rat lenses (Li *et al.*, 1995). Loss of cells from the LEC layer was shown to be particularly relevant in the development of subcapsular cataracts as the cell densities were much lower when compared to those of nuclear cataract (Konofsky *et al.*, 1987). A study in DM1 lenses by Abe *et al.* (1999) has indeed shown that DM1 cataract patients have reduced LEC densities and this was more noticeable in a patient with an earlier onset of the disease. The LECs in DM1 patients were also shown to be larger and were therefore able to maintain coverage of the anterior surface of the lens, however, it is likely that a reduced number of LECs are unable to maintain the balance of ions and water which is vital to lens transparency (Abe *et al.*, 1999).

Although cataracts are such a common feature in DM1, congenitally affected children do not present with cataract at birth and do not develop them within the first decade of life (Harper, 2001). This would indicate that the repeat expansion does not affect the development of the lens and must therefore exert its effects postnatally. A study by Abe *et al.* (1999) showed that *DMPK* mRNA extracted from DM1 LECs had larger repeats than that extracted from peripheral blood of the same patients (Abe *et al.*, 1999). A study on cultured DM1 LECs has also shown that they contain large triplet repeats in the *DMPK* gene even in the earliest passage sampled, which are highly unstable and expand during culture (Rhodes *et al.*, 2006). This data would indicate that the lens is subject to tissue-specific somatic mosaicism which results in large repeat numbers. This could explain the sensitivity of the lens to even small repeat numbers as they could expand greatly compared to those found in other tissues, increasing their effects during the lifetime of the patient.

Cataract development results in a loss in visual acuity, but cataracts are one of the most easily treated symptoms of DM1. Despite this, the sensitivity of the lens to even the smallest of expansions in the triplet repeat makes it an ideal tissue in which to study the mechanisms behind the disease.

1.7 Aims

Despite cataract being a key feature of DM1, very little is known about the mechanisms which underlie their development in the disease. This project aims to verify the suitability of lens cell lines derived from DM1 patients as a model for the disease and to use these cell lines to investigate the mechanisms which lead to cataract development.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines and culture

A number of cell lines were obtained and employed in a variety of experimental procedures to elucidate the effects of the DM1 triplet repeat mutation on cellular signalling.

2.1.1 Cell lines

Four DM1 and four control cell lines were derived by simian virus 40 (SV40) transformation of human LECs. Capsulorhexis specimens were obtained from DM1 patients immediately following cataract surgery and from non-cataractous post mortem donor lenses. The transformations were performed by Dr A. R. Prescott (University of Dundee, UK) as follows: the cells on capsulorhexis specimens were immortalised using adenovirus 12-SV40 hybrid virus, which was added directly to each specimen in a 24-well microtitre plate. SV40 immortalises cells via the production of large T antigen which binds to and inactivates the tumour suppressor proteins retinoblastoma protein (pRb) and p53. Once the cells had migrated and covered over 50% of the well (2-3 weeks) they were trypsinised and transferred into a 6-well plate. Once the cells reached confluency they were passaged into larger flasks until a sufficient number of cells could be frozen. The four DM1 cell lines named DMCat1-DMCat4 were obtained from patients aged 67, 33, 31 and 69 respectively. The four control cell lines named CCat1-CCat4 were obtained from donors aged 28, 43, 32 and 82 years respectively.

A further human lens cell line, FHL124 (obtained from Dr J. R. Reddan, Oakland University, Michigan, USA), was also used in this study. The FHL124 cell line is a spontaneously transformed human lens cell line derived from foetal capsule-epithelial explants (Reddan *et al.*, 1999).

2.1.2 Cell culture

Cells stored under liquid nitrogen were brought up from frozen and cultured in 75 cm² flasks (Nunc, Roskilde, Denmark) in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, Poole, Dorset, UK) supplemented with 10% Foetal Calf Serum (FCS) (Gibco, Paisley, Renfrewshire, UK) (5% FCS for FHL124 cells) and 50 µg/ml gentamicin antibiotic (Sigma-Aldrich). Medium was changed twice per week and cultures were maintained in an incubator at 35°C in an atmosphere of 5% CO₂. Cells were passaged weekly by rinsing with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich), followed by treatment with trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin and 0.02% EDTA in Hank's balanced salt solution, Sigma-Aldrich) for 2-3 minutes to detach the cells from the flask. The trypsin was neutralised with EMEM supplemented with 10% FCS and the cell suspension was transferred to a universal tube. Cell number was estimated using a haemocytometer. The cell suspension was centrifuged at 1000 rpm for 5 minutes and the pellet was resuspended in EMEM supplemented with 10% FCS. New flasks were seeded with known densities of cells and growth curves showing population doublings were created from the cell counts. The cell suspension was also used to seed cells onto coverslips (VWR International, Lutterworth, Leicestershire, UK) or culture dishes (Corning Incorporated, Corning, NY, USA). Unless stated otherwise, all cells were cultured in EMEM supplemented with 10% FCS and 1 µl/ml gentamicin.

2.1.3 Conditioned medium (CM) collection

To obtain CM, cells in 75 cm² flasks were rinsed three times with 20 ml of serum-free (SF) EMEM before incubating in 10 ml of SF EMEM for 24 hours. CM was collected in universal tubes and centrifuged at 1000 rpm for 5 minutes to remove particulates. The supernatant was either used immediately or stored at -20°C until required. CM was also collected from 35 mm diameter culture dishes by the same method.

2.2 Cell treatment

A range of chemical activators and inhibitors of a variety of cell signalling molecules were employed to study the effects of particular signalling pathways on cell growth and death.

Compound name	Function	IC ₅₀ /EC ₅₀	Concentration(s) used	Company
740 Y-P	Activator of phosphatidylinositol 3-kinase (PI3K)	25 mg/ml	50 mg/ml	Tocris
AG1024	Inhibitor of the insulin-like growth factor 1 receptor (IGFR-1)	0.4 µM	1 µM	Sigma-Aldrich
Fibroblast Growth Factor-Basic (bFGF)	Fibroblast growth factor (FGF) receptor ligand	0.05-0.6 ng/ml	5 ng/ml	Sigma-Aldrich
LY294002	Inhibitor of PI3K	1.4 µM	25 µM	Tocris
PD98059	Inhibitor of mitogen-activated protein kinase kinase 1 (MEK1)	2-7 µM	10 µM	Tocris
Rapamycin	Inhibitor of mammalian target of rapamycin (mTOR)	25 nM	25 nM and 50 nM	Tocris
SP600125	Inhibitor of c-Jun N-terminal kinase (JNK)	40-90 nM	1 µM and 10 µM	Tocris
SU5402	Inhibitor of FGF receptor 1 (FGFR1)	10-20 µM	10 µM	Calbiochem

Table 2.1 – Chemical and biological activators and inhibitors of cell signalling used in cell growth experiments.

2.2.1 Cell growth experiments

To test the effects of various compounds on the levels of cell growth and death, cells were seeded into 35 mm diameter culture dishes. Cells were cultured for up to one week until they reached 60-70% confluency. The medium was replaced with SF medium without phenol red (Sigma-Aldrich) for 24 hours before the cells were exposed to experimental conditions for 48 hours. Following this, the experimental culture medium was collected and stored at 4°C and the cells were rinsed in Phosphate Buffered Saline (PBS, Oxoid, Basingstoke, Hampshire, UK) before being lysed in 1 ml of 0.1 M NaOH. The lysates were stored at 4°C.

Total protein was measured using a bicinchoninic acid (BCA) assay (Pierce, Cramlington, Northumberland, UK) and the level of lactate dehydrogenase (LDH) was measured using a Cytotoxicity Detection kit (Roche, Burgess Hill, West Sussex, UK). Additionally, some of the dishes were lysed in Mammalian Protein Extraction Reagent (MPER, Pierce) for Western blot analysis as described in section 2.8. Compounds tested in this way are summarised in table 2.1.

2.2.2 Bicinchoninic acid (BCA) protein assay

The protein concentration in cell lysates was determined using a BCA protein assay (Pierce), which is a colorimetric assay based on the reduction of Cu^{2+} to Cu^{1+} by protein. The assay was performed according to the manufacturer's instructions described as follows: a series of standards ranging from 1-1000 $\mu\text{g/ml}$ were prepared by diluting a 2 mg/ml Bovine Serum Albumin (BSA, Sigma-Aldrich) stock made up in either MPER (Pierce) or 0.1 M NaOH, depending on the method of cell lysis. Either 10 μl of cell lysate extracted in MPER or 20 μl of cell lysate extracted in 0.1 M NaOH were pipetted into a 96-well microtitre plate. The samples were made up to 50 μl with the appropriate volume of H_2O . The standards were tested in triplicate and the samples in duplicate. The working reagent was prepared by mixing Reagent A and Reagent B in a ratio of 50:1 and 200 μl was added to each well. The plate was gently shaken for 1 minute to mix the samples and working reagent together and was then incubated at 37°C for 1 hour. The plate was allowed to cool and the absorbance at 562 nm was read on a Wallac 1420 VICTOR² multilabel plate counter using WorkOut 1.5 software (Perkin Elmer, Waltham, MA, USA). A standard curve was constructed and the concentration of protein in the samples was calculated.

2.2.3 Lactate dehydrogenase (LDH) assay

Cell death was quantified using a Cytotoxicity Detection kit (Roche) which is a colorimetric assay of LDH activity released from the cytosol of damaged cells. The assay was performed according to the manufacturer's instructions described as follows: cell medium was collected in 1.5 ml microcentrifuge tubes and briefly vortexed before being centrifuged for 5 minutes at 13 000 rpm. The supernatants were collected and stored at 4°C until required. 100 μl of each sample was added to a 96-well microtitre plate. For background

correction 100 µl of fresh medium, along with any compounds used, was also added in triplicate. The reaction mixture was prepared by mixing the catalyst (diaphorase/NAD⁺) and dye solution (iodotetrazolium chloride and sodium lactate) at a ratio of 1:45 and 100 µl of this was added to each sample. The plate was incubated in the dark for up to 30 minutes at room temperature. The reaction was terminated by addition of 50 µl of 1 N hydrochloric acid (HCl) to each well. The absorbance of the reaction product was measured at 490 nm using a Wallac 1420 VICTOR² multilabel plate counter and WorkOut 1.5 software (Perkin Elmer).

2.2.4 Cell growth with conditioned medium (CM)

CM was applied to FHL124 cells which had been grown in SF medium for 24 hours. After 48 hours the cells were lysed in 1 ml of 0.1 M NaOH and protein levels were measured using a BCA assay (described in section 2.2.2).

To analyse the effects of CM addition on cell signalling in the FHL124 cells using Western blotting (described in section 2.8), cells were lysed in 400 µl of MPER (Pierce).

2.3 TaqMan[®] Quantitative real time polymerase chain reaction (QRT-PCR)

The technique of QRT-PCR was used to analyse gene expression levels in the cell lines. The TaqMan[®] QRT-PCR system is based on the use of a fluorogenic-labelled oligonucleotide probe which has a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end. When intact the reporter and quencher dyes are in close proximity and fluorescence of the reporter dye is quenched via fluorescence resonance energy transfer (FRET) by the quencher dye. Each reaction requires gene specific primers and probes. The primers anneal to the cDNA and amplify the region between the primers. The probe anneals to a sequence within the amplified region and is cleaved by the 5' nuclease activity of Taq DNA polymerase during primer extension. Cleavage of the probe results in separation of the quencher and reporter dyes, allowing fluorescence of the reporter dye. During each PCR cycle a number of probes will be cleaved and the level of fluorescence will increase in proportion to the amount of message present for the gene of interest. The fluorescent signal is recorded for a total of 40 cycles. The point at which the fluorescent signal increases above the background is referred to

as the threshold cycle (C_T) value and is used as a measure of the starting template present in each sample. The TaqMan process is summarised in figure 2.1.

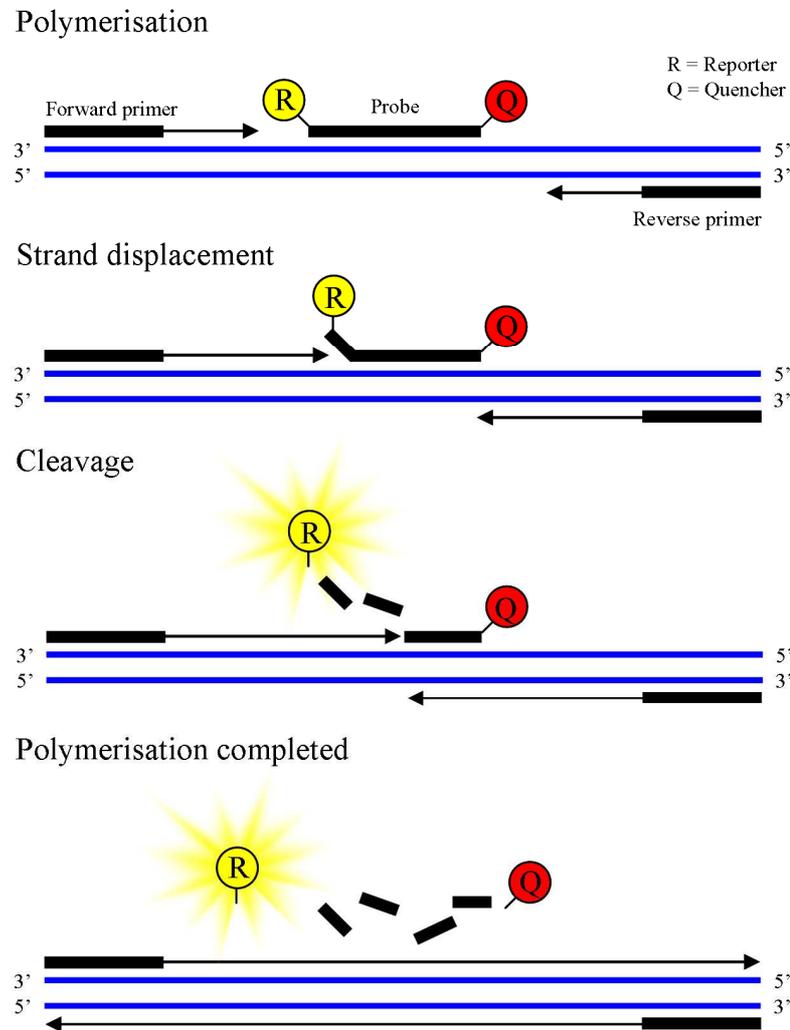


Figure 2.1 – A summary diagram of the principles behind the TaqMan RT-PCR system. The probe anneals to the cDNA but is displaced and cleaved during primer extension. The quencher and reporter dyes are separated, enabling the fluorescence of the reporter dye.

2.3.1 RNA extraction

Cells were seeded into 60 mm diameter culture dishes and grown for one week before the medium was replaced with SF medium for 24 hours. Cells were

exposed to experimental conditions and RNA was extracted using an RNeasy Mini kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's protocol described as follows: the medium was aspirated and the cell membranes were disrupted by addition of 600 μ l of buffer RLT with 10 μ l/ml β -mercaptoethanol. The dishes were scraped and the lysates were collected in 1.5 ml microcentrifuge tubes. The samples were homogenised by being vortexed and passed through a 20-gauge needle (0.9 mm diameter) at least 5 times. An equal volume of 70% ethanol was added to the homogenised sample to aid the binding of RNA to the membrane of the spin column. The lysate was passed through the columns by centrifuging for 15 seconds at 10 000 rpm. The flow through was discarded and 350 μ l of buffer RW1 was applied to the column. The column was centrifuged for 15 seconds at 10 000 rpm and the flow through discarded. On column DNase digestion was performed using an RNase-free DNase I kit (Qiagen). The DNase stock solution and buffer RDD were mixed in a ratio of 1:7 and a total of 80 μ l was then applied to each column and incubated for 15 minutes at room temperature. Following this, a further 350 μ l of buffer RW1 was added and the column was centrifuged for 15 seconds at 10 000 rpm. The flow through was discarded and 500 μ l of buffer RPE was added to each column and centrifuged for 15 seconds at 10 000 rpm. A further 500 μ l of buffer RPE was added and the column was centrifuged for 2 minutes at 10 000 rpm followed by a further 1 minute in a new collection tube to dry the membrane. The column was transferred to an RNase free 1.5 ml microcentrifuge tube and the RNA was eluted in 30-50 μ l of RNase free water. The column was spun for 1 minute at 10 000 rpm and the RNA was then stored at -80°C . The quantity of RNA was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

2.3.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was reverse transcribed into cDNA using a PTC-200 DNA engine (MJ research, Reno, NV, USA). A total of 1 μ g of RNA in 10 μ l of RNase-free water was added to a thin-walled nuclease-free microcentrifuge tube. To this, 1 μ l of Random primers (Invitrogen, Paisley, Renfrewshire, UK) and 1 μ l of 10 mM dNTP Mix (Bioline, London, UK) were added and mixed by flicking the tube. The contents were collected at the bottom of the tube by briefly centrifuging before heating the mixture to 65°C for 5 minutes and then quickly chilling on ice.

Then 4 µl of 5X First-strand buffer, 2 µl of 0.1 M Dithiothreitol (DTT) and 1 µl RNasin® Ribonuclease Inhibitor (Promega, Southampton, Hampshire, UK) were added before incubating at 25°C for 10 minutes followed by 42°C for 2 minutes. Following this, 1 µl of SuperScript™ II reverse transcriptase (Invitrogen) was added and the mixture was incubated at 42°C for 50 minutes and then at 70°C for 15 minutes. The cDNA product was stored at -20°C.

Gene Symbol	Gene Name	Assay Identification number(s)
CLCN1	Chloride channel 1, skeletal muscle	Hs00163961_m1
CUG-BP1	CUG triplet repeat, RNA binding protein 1	Hs00198069_m1
CUG-BP2	CUG triplet repeat, RNA binding protein 2	Hs00272516_m1
DMPK	Dystrophia myotonica-protein kinase	Hs01094334_g1 and Hs00189385_m1
FGFR1	Fibroblast growth factor receptor 1	Hs00241111_m1
IR	Insulin receptor	Hs00169631_m1 and Hs00961550_m1
MBNL1	Muscleblind-like 1	Hs00253287_m1
MBNL2	Muscleblind-like 2	Hs01058996_m1
RYR1	Ryanodine receptor 1 (skeletal)	Hs00166991_m1
RYR2	Ryanodine receptor 2 (cardiac)	Hs00181461_m1
RYR3	Ryanodine receptor 3	Hs00168821_m1
SIX5	SIX homeobox 5	Assay by design Forward primer: 5'-GCTGCCTTCGGCCACT-3' Reverse primer: 5'-GCCACACCCGTCACGAT-3' Probe: 5'-CCCCAGGAAACTTC-3'

Table 2.2 – The genes analysed by TaqMan QRT-PCR. The assay identification numbers for the probes purchased from Applied Biosystems are also shown.

2.3.3 TaqMan[®] Quantitative real time PCR (QRT-PCR)

Gene expression levels were measured using QRT-PCR (TaqMan[®]) performed using a standard run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, Cheshire, UK). A total of 5 ng of sample cDNA was loaded per well of a MicroAmp[®] Optical 96-Well Reaction Plate (Applied Biosystems), except for those of the standard curve which had varying amounts of cDNA and those for analysis of *I8S* which had 1 ng per well. Gene-specific primers and probes (Applied Biosystems) were used to measure expression levels (see table 2.2 for a list of genes assayed). The primers and probes were mixed with Mastermix (Applied Biosystems) containing the components necessary for the PCR reaction to give a total reaction volume of 25 μ l. The reactions were performed under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes followed by 40 cycles consisting of 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle (C_T) values were obtained and the results were normalised to the *I8S* expression values.

2.4 Northern blot

The technique of Northern blotting was used to confirm the expression levels of the *DMPK* gene previously analysed by TaqMan QRT-PCR. Gel electrophoresis separates RNA by size and following transfer to a blotting membrane the RNA of interest is detected using a radiolabelled probe complementary to the target sequence.

2.4.1 RNA extraction

RNA was extracted as previously described in section 2.3.1.

2.4.2 Agarose/formaldehyde gel electrophoresis

A 1.2% agarose gel was made in an RNase free tank containing 6.6% formaldehyde and 1x MOPS (0.02 M MOPS (3-[N-morpholino]propanesulfonic acid) (pH 7.0), 5 mM sodium acetate and 1 mM EDTA (pH 8.0) in DEPC treated H₂O). Once the gel had set it was immersed in running buffer (1x MOPS and 0.74% formaldehyde in H₂O with ethidium bromide). The samples (8.5 μ g of RNA) were concentrated to a volume of (around) 5 μ l by vacuum drying in a

Concentrator 5301 (Eppendorf, Histon, Cambridge, UK). The samples were mixed with a mastermix (1x MOPS, 7.4% formaldehyde, 50% formamide) and a denaturation reaction was performed where they were heated to 65°C for 10 minutes. The samples were chilled on ice for 5 minutes before being combined with 10% gel loading buffer (50% glycerol, 10 mM EDTA (pH 8.0) and 0.4% (w/v) bromophenol blue) and loaded onto the agarose gel. The gel was run at 100 V for approximately 2½ hours until the blue dye reached the end of the gel. The gel was removed from the tank and washed twice in sterile distilled H₂O for 45 minutes.

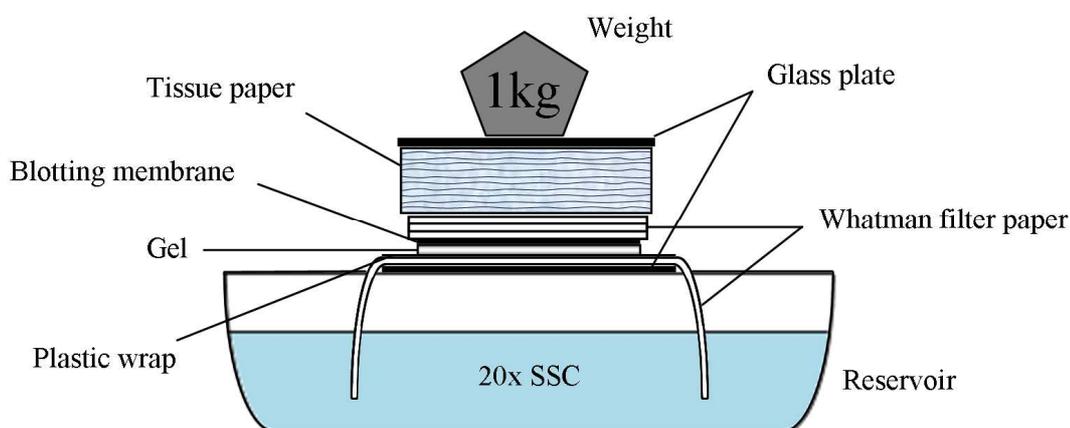


Figure 2.2 – A diagram of the capillary transfer system used to transfer RNA from the gel to the blotting membrane.

2.4.3 Capillary transfer of RNA to blotting membrane

A capillary transfer system was set up as follows (see figure 2.2): a reservoir was filled with 20x saline-sodium citrate (SSC, 175.3g of NaCl, 88.2g of sodium citrate in 800 ml distilled H₂O) and a glass plate was rested on top. A long piece of Whatman filter paper was soaked in 20x SSC and placed over the glass plate with the ends in the 20x SSC reservoir to act as a wick. The gel was turned upside down onto the filter paper and the surrounding areas were covered with clingfilm. A piece of Hybond-N membrane (GE Healthcare, Chalfont St Giles,

Buckinghamshire, UK) was placed on top after dipping in H₂O followed by 20x SSC. One layer of wet Whatman filter paper was placed on top followed by two dry layers. Air bubbles were removed and a 5 cm thick stack of tissue paper was placed on top. A glass plate was placed over this and weighed down with a 1 kg weight. The RNA was left to transfer over night. The following day the blotting system was dismantled and the membrane was removed after the position of the wells had been marked. The membrane was surrounded in filter paper and baked in an incubator at 80°C for 2 hours to cross-link the RNA to the membrane. The blot was stored at room temperature.

2.4.4 Probe synthesis

Custom made primers specific to *DMPK* and *GAPDH* transcripts were purchased from Invitrogen with the following sequences (obtained from Krol *et al.*, 2007): *DMPK* (forward, 5'-AGGCTTAAGGAGGTCCGACTG-3'; reverse, 5'-GCCAAGTGCAGCTGCGTGATC-3'), *GAPDH* (forward, 5'-GAAGGTGAAGGTCGGAGT-3'; reverse, 5'-GAAGATGGTGATGGGATTTC-3'). The primer sequences were aligned with the sequences of all known transcript variants of *DMPK* and *GAPDH* mRNA (using ClustalW software, URL - www.ebi.ac.uk/clustalw/ and the NCBI nucleotide website URL - www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide). The primer sequences were located within homologous sites of the transcript variants found.

The probes were amplified by a PCR reaction where 1 µl containing 20 ng of cDNA template was added to 28.5 µl H₂O, 10 µl HF 5x PCR buffer, 5 µl 10x dNTP (2 mM), 2.5 µl 10 µM forward primer, 2.5 µl 10 µM reverse primer and 0.5 µl PhusionTM Taq polymerase (Finnzymes Oy, Espoo, Finland). The PCR reactions were performed on a PTC-200 DNA engine (MJ research). For amplification of the *DMPK* transcript probe the PCR conditions were as follows: 98°C for 30 seconds, 34 cycles of 98°C for 10 seconds, 68°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 5 minutes and 7°C until the tubes were removed. For amplification of the *GAPDH* transcript probe the PCR conditions were as follows: 98°C for 30 seconds, followed by 34 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 5 minutes and 7°C until the tubes were removed. The PCR products were run on a 1% agarose gel and bands at the appropriate sizes were excised.

The DNA product was extracted from the gel using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions described as follows: for every 100 mg of gel 300 µl of buffer QG was added. The gel was dissolved by heating to 50°C for 10 minutes with occasional mixing before 100 µl of isopropanol was added per 100 mg of gel. The sample was applied to a QIAquick spin column in a 2 ml collection tube which was centrifuged for 1 min. The flow-through was discarded and 500 µl of Buffer QG was added to the column and centrifuged for 1 min to remove the agarose. To wash the sample, 750 µl of Buffer PE was added to the column and centrifuged for 1 minute. The flow-through was discarded and the column was centrifuged for an additional 1 minute. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl of water was added to the centre of the membrane and allowed to rest for 1 minute. The column was centrifuged for 1 minute and the eluant was stored at -20°C.

To sequence the DNA, a set of reactions were set up containing 3 µl of DNA, 2 µl of 5x buffer, 2 µl H₂O, 1 µl primer, 1 µl Big Dye v3.1 and 1 µl ½ Big Dye (Applied Biosystems). Two reactions were set up for both the DMPK and GAPDH probe amplification products, one containing the forward primer and one containing the reverse primer. The mixtures were heated to 96°C for 4 minutes followed by 24 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes; the samples were then chilled at 4°C. The products were sent to the John Innes Centre Genome Laboratory (Colney, Norwich, Norfolk) for sequencing on an ABI 3730 high throughput sequencer (Applied Biosystems). The results were viewed on Chromas Lite software (URL - www.technelysium.com.au/chromas_lite.html) and the sequences obtained were put into BLAST (URL - www.ncbi.nlm.nih.gov/blast/). The probe sequences showed specificity for the RNA they were designed to anneal to. The concentration of DNA in the samples was measured using a Qubit Fluorometer (Invitrogen).

2.4.5 Pre-hybridisation and probe preparation

The membrane was placed in a glass tube with 6 ml of ULTRAhyb[®]-Oligo hybridisation buffer (Ambion, Huntingdon, Cambridgeshire, UK) pre-hybridisation solution and incubated at 42°C for 3 hours rotating. The probe was

prepared by denaturing 50 ng of DNA in a total volume of 45 µl by heating to 100°C for 10 minutes. The DNA was immediately placed on ice for 3 minutes and then centrifuged briefly to collect the sample at the bottom of the tube. A Ready-To-Go DNA labelling bead (GE Healthcare) containing buffer, dATP, dGTP, dTTP, FPLCpure™ Klenow Fragment (7-12 units) and random oligodeoxyribonucleotides was placed in a tube and 45 µl of denatured DNA was added along with 5 µl of [α -³²P]dCTP. The tube was mixed by gently pipetting up and down and was then incubated at 37°C for 30 minutes. The reaction was stopped by adding 5 µl of 0.2 M EDTA (pH 8). Unincorporated oligonucleotides were removed by passing the mixture through a Sephadex G-50 DNA grade column by centrifuging at 2000 rpm for 2 minutes.

2.4.6 Hybridisation and detection

The DNA was denatured by heating to 97°C for 5 minutes before cooling on ice for 3 minutes. The probe was added directly to the pre-hybridisation buffer, mixed thoroughly and left to hybridise with the membrane overnight at 42°C rotating.

The following day the probe solution was removed and the blot was washed twice with 2x SSC with 0.1% sodium dodecyl sulphate (SDS, Sigma-Aldrich) for 10 minutes at 42°C, followed by two washes with 0.1x SSC with 0.1% SDS for 15 minutes at 42°C. The membrane was wrapped in clingfilm and placed in a cassette with an intensifying screen for 3 hours. The bands were visualised using a Molecular Imager FX Pro Plus with Quantity One 4.5.1 software (Bio-Rad, Hemel Hempstead, Hertfordshire, UK). The intensity of the bands was analysed using Kodak 1D 3.5 software (Kodak, Rochester, NY, USA).

2.4.7 Membrane stripping

The membrane was stripped to be re-probed for different transcripts by washing repeatedly in boiling water until no radioactivity was measured.

2.5 Fluorescent *In Situ* Hybridisation (FISH)

To detect mutant *DMPK* transcripts with large CUG repeats the technique of FISH was used. The technique involves the use of a fluorescently labelled CAG

probe complementary to the CUG repeat in the *DMPK* transcripts (protocol as described by Holt *et al.* (2007)). The probe is applied to the cells to detect the transcripts and their position within the cell.

Cells were seeded onto 12-16 mm round glass coverslips and grown for one week before the medium was replaced with SF medium for 24 hours. The cells were exposed to experimental conditions for 24 hours. The cells were washed in diethylpyrocarbonate (DEPC, Sigma-Aldrich) treated PBS (100 μ l DEPC in 100 ml PBS) before being fixed in 4% formaldehyde in DEPC treated PBS for 25 minutes at room temperature. The fixative was aspirated and the cells were washed three times for 5 minutes with DEPC treated PBS with gentle agitation. The coverslips were stored at 4°C overnight.

Cells were permeabilised in 1 ml of 0.2% Triton X-100 in DEPC treated PBS for 5 minutes at room temperature. The coverslips were then rinsed twice for 5 minutes with 2x SSC with gentle agitation. The SSC was aspirated and 500 μ l of denaturation solution (40% formamide in 2x SSC) was applied for 10 minutes. The denaturation solution was aspirated and 300 μ l of hybridisation solution (10% dextran sulphate, 40% formamide, 0.2% BSA, 0.1 mg/ml herring sperm DNA (Sigma-Aldrich), 0.1 mg/ml baker's yeast transfer RNA (Sigma-Aldrich), 4 mM ribonucleoside vanadyl complexes (Sigma-Aldrich), 200 nM (CAG)₁₀ Alexa 555 labelled probe in 2xSSC) was added. A plastic coverslip was placed over the solution to ensure even coverage and to prevent the solution drying out. The coverslips were placed in a humidified chamber and incubated overnight at 37°C protected from light. The following day the coverslips were rinsed three times with DEPC treated PBS for 5 minutes each with gentle agitation. The nuclei were labelled by addition of 100 μ l of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 1:200) in DEPC treated PBS for 30 minutes. The coverslips were washed three times for 5 minutes with DEPC treated PBS and mounted onto glass slides using Hydromount (National Diagnostics, Hesse, Yorkshire, UK). The slides were dried at room temperature for 30 minutes and imaged on a charge-coupled device (CCD) upright Zeiss fluorescence microscope with an attached digital camera and Axiovision 4.7 software (Zeiss, Oberkochen, Germany).

2.6 PCR of the insulin receptor (*IR*) mRNA

In order to analyse alternative splicing of *IR* mRNA the technique of PCR was used. The technique involves amplification of *IR* transcript cDNA. PCR products are separated by gel electrophoresis and the ratio of the two isoforms calculated (protocol as described by Savkur *et al.*, 2001).

2.6.1 RNA extraction

RNA was extracted from cells as previously described in section 2.3.1.

2.6.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was reverse transcribed into cDNA as described in section 2.3.2.

2.6.3 PCR of *IR* mRNA

PCR was carried out to amplify a region from exons 10 to 12 of the *IR* mRNA. A total of 200 ng of cDNA (in 4 μ l) was mixed with 0.5 μ l each of 50 μ M forward primer (5'-CCAAAGACAGACTCTCAGAT-3') and reverse primer (5'-AACATCGCCAAGGGACCTGC-3') (Invitrogen), 0.4 μ l of 100 mM dNTP mix, 5 μ l of MgCl₂ free PCR reaction buffer x10, 1.5 μ l of 50 mM MgCl₂ and 0.25 μ l of Taq DNA Polymerase (5 U/ μ l) (Bioline). The volume was made up to 50 μ l with H₂O before mixing and briefly centrifuging to collect the solution at the bottom of the tube. The DNA was amplified using a PTC-200 DNA engine (MJ research) by heating to 94°C for 4 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds, then 72°C for 10 minutes. The samples were stored at -20°C.

2.6.4 Agarose gel electrophoresis

The PCR products were separated by electrophoresis on a 4% agarose gel with TAE running buffer containing ethidium bromide. A total of 10 μ l of PCR product was mixed with 5 μ l of 5x loading buffer before loading onto the gel alongside a Hyperladder IV 1 kB ladder (Bioline). The gel was run at 80 V until the bands had separated. Images were taken on a UV transilluminator and the intensity was analysed using Kodak 1D 3.5 scientific imaging software (Kodak).

2.7 Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay

Apoptotic cells were detected and quantified using a TUNEL assay. The technique detects DNA fragmentation which occurs during the ordered breakdown of cells during apoptotic cell death. When the DNA is fragmented 3'-OH groups are exposed to which the terminal deoxynucleotidyl transferase (TdT) can add fluorescein-12-dUTP. The fluorescein-12-dUTP can then be detected using fluorescence microscopy.

Cells were seeded onto glass coverslips and grown for one week until the medium was replaced with SF medium for 24 hours. The cells were exposed to experimental conditions for 24 hours and then fixed in 4% formaldehyde in PBS for 25 minutes at room temperature. The fixative was aspirated and the cells were washed three times with PBS for 5 minutes each with gentle agitation. The coverslips were stored at 4°C overnight. A DeadEnd™ Fluorometric TUNEL System (Promega) was used to detect and quantify apoptotic cells within cell populations according to the manufacturer's instructions described as follows: the cells were permeabilised by addition of 1 ml of 0.2% Triton X-100 in PBS for 5 minutes at room temperature. The coverslips were washed twice with PBS for 5 minutes each with gentle agitation. The PBS was removed and 100 µl of Equilibration Buffer was added to each coverslip for 5-10 minutes. The edge of each coverslip was blotted on tissue paper to remove the majority of the Equilibration Buffer before 50 µl of rTdT reaction mix was added. The rTdT reaction mix contained 45 µl Equilibration Buffer, 5 µl Nucleotide Mix and 1 µl rTdT enzyme per reaction. A plastic coverslip was placed over the solution to ensure even distribution of the mix. The coverslips were placed in a humidified chamber and incubated for 1 hour and were protected from light from this step onwards. The reaction was terminated by immersing the coverslips in 2x SSC for 15 minutes at room temperature. The coverslips were washed three times with PBS for 5 minutes each with gentle agitation. The cell nuclei and F-actin were labelled by the addition of 100 µl of DAPI (1:200) and Texas Red-X phalloidin (1:100) diluted in PBS for 15 minutes. The coverslips were washed three times with PBS for 5 minutes each with gentle agitation and mounted onto glass slides using Hydromount. The slides were dried at room temperature for 30 minutes and

then imaged on a CCD upright Zeiss fluorescence microscope with an attached digital camera and Axiovision 4.7 software (Zeiss).

2.8 Western blot

In order to detect the presence, absence and activation of particular proteins of interest within the lysates of cell samples, the Western blot technique was used. The technique is based on the binding of a primary antibody specific to the protein of interest and its subsequent detection by a secondary antibody. The Western blot gives information about the size and the relative amount of the protein of interest in different samples.

2.8.1 Cell lysis

Cells were grown in 35 mm culture dishes and subjected to experimental conditions as previously described (see section 2.2.1). The cells were rinsed in ice-cold PBS and lysed in MPER with 10 μ l/ml of Halt protease inhibitor cocktail, 10 μ l/ml of Halt phosphatase inhibitor cocktail and 10 μ l/ml EDTA (5 mM) (Pierce). Dishes were scraped and lysates were collected in 1.5 ml microcentrifuge tubes (Eppendorf) and centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was stored at -80°C. Protein concentration was measured using a bicinchoninic acid (BCA) protein assay (Pierce) according to the manufacturer's protocol described in section 2.2.2.

2.8.2 SDS PAGE

Protein samples were heated to 100°C for 5 minutes with 20% sample buffer (4% SDS, 0.01% bromophenol blue, 30% glycerol, 12.5% β -mercaptoethanol and 160 mM tris pH 6.8) and loaded alongside DualVue Western Blotting Markers (GE Healthcare) on a 10% precast polyacrylamide protein gel (Pierce). Gels were subjected to electrophoresis in a Mini-PROTEAN 3 cell (Bio-Rad) run at a constant 100 V and a current of 100 mA per gel at room temperature until the loading dye reached the bottom of the gel.

2.8.3 Protein transfer

The gel was removed from the tank and placed in transfer buffer solution (48 mM Tris, 39 mM glycine, 4% methanol and 0.0375% SDS, pH 8.3) for 15 minutes. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer) by sandwiching the gel and the PVDF membrane (activated in 100% methanol), between thick filter papers. The sandwich was placed on a trans-blot semi-dry transfer cell (Bio-Rad) and run at a constant 15 V and a current of 0.3 A per gel for 30 minutes.

2.8.4 Blocking and antibody incubation

Following protein transfer the gels were discarded and the PVDF was rinsed three times for 5 minutes in PBS and blocked for 1 hour in PBS-T (100 ml PBS, 0.5 g Marvel milk powder and 50 μ l Tween[®] 20) at room temperature with gentle agitation. The primary antibody made up in PBS-T was left on overnight at 4°C with gentle agitation. The antibodies used in this study are summarised in table 2.3.

The following day the membrane was allowed to return to room temperature before washing three times for 15 minutes in PBS-T with gentle agitation. The secondary antibody conjugated to horseradish peroxidase was made up in PBS-T at a concentration of 1:1250. The membrane was incubated in the secondary antibody for one hour at room temperature with gentle agitation. A further two 15 minute washes with PBS-T were carried out before a final wash in PBS with 10 μ l/ml Tween[®] 20 (Sigma-Aldrich) for 10 minutes.

2.8.5 Detection

ECL plus western blotting detection reagents (GE Healthcare) were mixed at a ratio of 40:1 of solutions A and B respectively and a volume of 0.1 ml/cm² was pipetted onto the membrane and allowed to rest for 5 minutes at room temperature in the dark. The membrane was blotted onto tissue paper before being placed in a film cartridge. In a darkroom the membrane was exposed to Amersham Hyperfilm ECL photographic paper (GE Healthcare) which was then passed through Kodak GBX developer and replenisher solution (Kodak), SB 80 indicator stop bath (Phosol, Basildon, Essex, UK) and Hypam fixer (Ilford, Moberley, Cheshire, UK). The revealed bands were scanned and the intensity

was analysed densitometrically using Kodak 1D 3.5 scientific imaging software (Kodak).

Antibody	Description	M_r (kDa)	Dilution	Company
Anti-β-Actin	Rabbit, polyclonal	45	1:1000	Cell Signaling Technology
Anti-phospho-Akt1/PKBα (ser473)	Rabbit, monoclonal	60	1:1000	Upstate
Anti-Akt1/PKBα	Rabbit, monoclonal	60	1:1000	Upstate
Anti-phospho-p44/42 MAPK (Thr202/Tyr204)	Mouse, monoclonal	42, 44	1:2000	Cell Signaling Technology
Anti-p44/42 MAPK	Rabbit, polyclonal	42, 44	1:1000	Cell Signaling Technology
Anti-phospho-p38 MAP Kinase (Thr180/Tyr182)	Rabbit, polyclonal	43	1:1000	Cell Signaling Technology
Anti-p38 MAP Kinase	Rabbit, polyclonal	43	1:1000	Cell Signaling Technology
Anti-phospho-p70 S6 Kinase (Thr389)	Rabbit, polyclonal	70, 85	1:1000	Cell Signaling Technology
Anti-phospho-PKC (pan) (βII Ser660)	Rabbit, polyclonal	78, 80, 82, 85	1:1000	Cell Signaling Technology
Anti-PKC	Mouse, monoclonal	78, 80, 82, 85	1:200	Santa Cruz Biotechnology
Anti-PTEN	Rabbit, polyclonal	54	1:1000	Cell Signaling Technology
Anti-phospho-SAPK/JNK (Thr183/Tyr185)	Rabbit, monoclonal	46, 54	1:1000	Cell Signaling Technology
Anti-SAPK/JNK	Rabbit, monoclonal	46, 54	1:1000	Cell Signaling Technology
Anti-Mouse IgG HRP conjugate	Sheep, secondary	-	1:1250	GE Healthcare
Anti-Rabbit IgG HRP conjugate	Donkey, secondary	-	1:1250	GE Healthcare

Table 2.3 – A summary of the antibodies used for the detection of proteins via Western blots.

2.8.6 Membrane stripping

Membranes were stripped to be re-probed with other antibodies using Re-blot Strong solution (Millipore, Watford, Hertfordshire, UK) which was left on the membrane for 20-25 minutes. The membrane was blocked by two 5 minute washes with PBS-T before being placed in the next primary antibody.

2.9 bFGF estimation by the enzyme-linked immunosorbent assay (ELISA)

The level of bFGF in conditioned medium was measured using an ELISA. The technique involves the use of a primary antibody specific to the protein of interest which is bound to the surface of the wells of a 96-well plate. The samples are added to the wells and if the protein of interest is present it will bind to the antibody. An enzyme-linked antibody is then pipetted into the wells which also binds to the protein of interest. Addition of an enzyme substrate produces a colour change proportional to the amount of protein of interest present.

A Quantikine[®] human FGF basic (bFGF) ELISA kit (R&D Systems, Abingdon, UK) was used to quantify the levels of bFGF released into the medium by the cells. Conditioned medium (CM) was collected from cells after 24 hours and was centrifuged at 13000 rpm for 5 minutes. The supernatant was removed and frozen at -20°C until required. The bFGF ELISA kit was used according to the manufacturer's protocol described as follows: a 96-well microplate pre-coated with a monoclonal antibody specific to bFGF was supplied and 100 µl of Assay Diluent RD1-43 was added to each well. A series of standards with known concentrations of bFGF were prepared from a bFGF stock solution. Each test was performed in duplicate and 100 µl of the standards or samples were added to each well. The wells were covered with an adhesive strip and incubated for 2 hours at room temperature. Each well was aspirated and washed with 400 µl of wash buffer which was repeated three times for a total of four washes. The plate was blotted to remove as much wash buffer as possible before adding 200 µl of bFGF Conjugate to each well. The wells were covered with an adhesive strip and incubated for 2 hours at room temperature. The wash step was repeated before 200 µl of Substrate Solution was added to each well. The plate was incubated for 30 minutes at room temperature protected from light before 50 µl of Stop Solution was added. The absorbance was determined using a Wallac 1420 VICTOR²

multilabel plate counter and WorkOut 1.5 software at a wavelength of 450 nm. A standard curve was constructed and used to determine the levels of bFGF in the CM samples.

2.10 Statistical analysis

Student's T-test analysis was performed using Excel[®] software (Microsoft, Redmond, WA, USA) to determine any statistical difference between two experimental groups. Statistical analysis of multiple experimental groups was performed by one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Significance was assessed using a p value of ≤ 0.05 .

CHAPTER 3

CHARACTERISATION OF DM1 LENS

EPITHELIAL CELLS

3.1 Introduction

Diseases can be modelled in a variety of different ways in order to study the mechanisms which underlie them. This includes techniques such as the culturing of tissues and primary cells from patients, the immortalisation of cells to produce cell lines and the production of animal models to mimic disease. Although all of these techniques are valuable to research, the availability and lifespan of primary cells from patients is often limited and the use of animal models poses an ethical dilemma as well as posing problems with differences between species. Therefore, in this study, cell lines have been produced from primary human LECs (as described in section 2.1.1) to study the mechanisms behind cataract development in DM1.

The use of *in vitro* studies using cell lines in research has become commonplace due to the availability of cells, the ease with which they can be maintained, the speed with which results can be obtained and the relatively low cost of experiments. However, cell lines must be characterised in order to verify their origin and to show that they possess characteristics that are relevant to the disease they represent.

3.1.1 Molecular characterisation of DM1 lens epithelial cells

The cell lines used in this study were produced from DM1 patient samples and normal donor lenses. To confirm their origins a study by Rhodes *et al.* (2006) confirmed the presence and length of the expanded triplet repeat in the DM1 lens cell lines and showed its absence in the controls through Small Pool-PCR analysis of repeat length variation (see figure 3.1a). The controls were confirmed to have small stable normal alleles, whereas the DM1 cell lines contained large unstable expanded alleles even in the earliest passage sampled, suggesting that the primary

cells from which they were derived also contained large triplet repeats (Rhodes *et al.*, 2006).

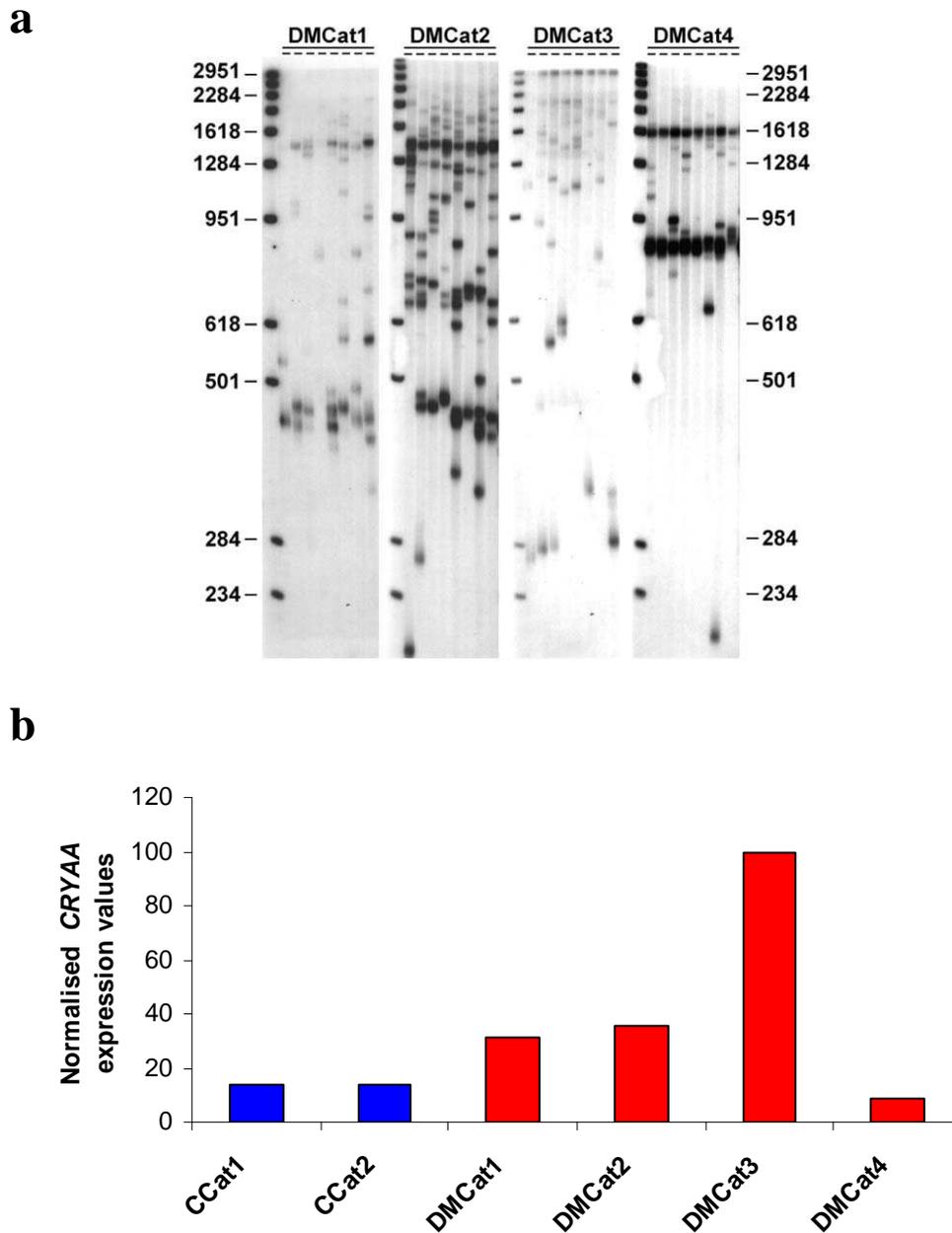


Figure 3.1 – Characterisation of DM1 lens epithelial cells. (a) Small Pool-PCR analysis of CTG repeat length at passage 10, showing 8 reactions for each cell line. No detectable expanded alleles were found in the control cell lines (data not shown), whereas the DM1 cell lines were shown to contain large triplet repeats. DMCat1 had two major alleles of 1480 and 450 repeats, DMCat2 had three major alleles of 1440, 730 and 450 repeats, DMCat3 had a single major allele of 2950 repeats and DMCat4 had two major alleles of 1590 and 880 repeats (image from Rhodes *et al.* (2006)). **(b)** QRT-PCR analysis of mRNA expression from *CRYAA* normalised to *18S* expression, showing expression in all cell lines tested (Dr. J.D. Rhodes and Dr. L.M. Hodgkinson, unpublished data).

Further characterisation performed by Rhodes *et al.* (2006) showed that the DM1 lens cells expressed mRNA from *DMPK*, *DMWD* and *SIX5*, but only *SIX5* was found to have reduced levels of both mRNA and protein. To confirm their lens cell origins, the expression of the gene *CRYAA*, which encodes the protein α A-crystallin was analysed. α A-crystallin is found in native LECs and expression of *CRYAA* was found in all of the DM1 and control cell lines tested (see figure 3.1b, Dr. J.D. Rhodes and Dr. L.M. Hodgkinson, unpublished data).

3.1.1.1 Triplet repeat-containing *DMPK* transcripts form nuclear foci in DM1 lens epithelial cells

Many reports have shown that *DMPK* transcripts accumulate in discrete foci in the nuclei of DM1 cells. Foci of *DMPK* transcripts have been found in cultured fibroblasts and myoblasts; in cells from heart, spinal cord, brain, gall bladder smooth muscle and skeletal muscle from DM1 patients; and in muscle cells and trophoblasts cultured from DM1 fetuses (Taneja *et al.*, 1995; Davis *et al.*, 1997; Hamshere *et al.*, 1997; Furling *et al.*, 2001b; Liquori *et al.*, 2001; Jiang *et al.*, 2004; Mankodi *et al.*, 2005; Bonifazi *et al.*, 2006; Wheeler *et al.*, 2007a; Cardani *et al.*, 2008). These studies indicate that all tissues which show symptoms of the disease could have foci of *DMPK* transcripts retained in the nuclei. As the lens is affected by DM1, and cataracts are a characteristic symptom of the disease, it would be interesting to confirm whether lens cells express *DMPK* and whether or not triplet repeat-containing *DMPK* transcripts accumulate in foci.

The formation of nuclear foci in DM1 is associated with sequestration of the MBNL proteins (MBNL1, MBNL2 and MBNL3) and increased levels of CUG-BP proteins (CUG-BP1 and CUG-BP2). MBNL proteins accumulate in nuclear foci as they bind to the stem of the dsRNA formed by the CUG repeats in *DMPK* transcripts (Miller *et al.*, 2000; Fardaei *et al.*, 2001; Fardaei *et al.*, 2002; Yuan *et al.*, 2007). In humans, *MBNL1* and *MBNL2* show widespread expression in all tissues tested, including pancreas, kidney, skeletal muscle, liver, lung, placenta, brain and heart, but *MBNL3* is almost exclusively expressed in the placenta (Fardaei *et al.*, 2002). *MBNL1* was shown to be expressed in the mouse eye but there is no data available for expression of any *MBNL* genes in the human lens (Miller *et al.*, 2000). Unlike MBNL proteins, CUG-BP1/2 are not recruited into nuclear foci, despite being shown to bind to (CUG)₈ repeats (Timchenko *et*

al., 1996; Lu *et al.*, 1999; Michalowski *et al.*, 1999; Fardaei *et al.*, 2001). Interestingly, CUG-BP1 levels were actually shown to be increased in both cultured cells expressing CUG repeats and in DM1 heart tissue, with greater levels being observed with increasing repeat numbers (Timchenko *et al.*, 2001a). Greater levels of CUG-BP1 have also been found in skeletal muscle tissue from DM1 patients and in cultured DM1 myoblasts (Savkur *et al.*, 2001; Dansithong *et al.*, 2005). Analysis of *CUG-BP1* expression showed that levels of mRNA were not affected and instead an increase in the half-life of CUG-BP1 was shown to be responsible for increased protein levels (Timchenko *et al.*, 2001a). The increased protein half-life was shown to result from an increase in the steady state levels of the protein due to hyperphosphorylation (Kuyumcu-Martinez *et al.*, 2007). In humans, *CUG-BP1* expression was found in all tissues tested, including liver, skeletal muscle and spleen, with high levels of expression in the brain and heart (Roberts *et al.*, 1997). Widespread expression of *CUG-BP1* was also found in mice (Ladd *et al.*, 2001). In humans, *CUG-BP2* is expressed predominantly in the heart, with low levels of expression found in the brain and skeletal muscle (Lu *et al.*, 1999). In contrast, in mouse, *CUG-BP2* was expressed in all tissues tested including heart, brain, spleen, lung, liver, skeletal muscle and testis (Lu *et al.*, 1999; Ladd *et al.*, 2001). No data is available for the expression of *CUG-BP1/2* in the eye or lens.

If *DMPK* is expressed in lens cells and foci of the transcripts are shown to form, then it is possible that the active levels of MBNL and CUG-BP proteins will be disturbed if they too are expressed in the lens.

3.1.1.2 Altered splicing events in DM1

Both MBNL and CUG-BP proteins are involved in the regulation of splicing events, many of which are disrupted in DM1. The sequestration of MBNL and increased levels of CUG-BP result in the misregulation of splicing, where the splicing patterns of certain transcripts revert back to that observed during embryonic development. Alternative splicing generates protein isoforms with different biological properties, and embryonic isoforms are likely to have different levels of sensitivity and action compared to their adult counterparts. It has been suggested that the altered splicing patterns of certain proteins in DM1 contributes to the observed phenotype of the disease. For example, the altered

splicing of CIC-1 could cause the myotonia which is a characteristic feature of DM1; altered splicing of IR could lead to insulin resistance and diabetes which is observed in the disease; and altered splicing of ryanodine receptor 1 (RYR1) could be involved in the myopathy associated with DM1 (Savkur *et al.*, 2001; Mankodi *et al.*, 2002; Kimura *et al.*, 2005). If MBNL and CUG-BP proteins are found in the lens then it is possible that splicing patterns could be altered, leading to changes in the lens which could result in the development of cataract.

Myotonia is caused by hyperexcitability in muscle fibres leading to repetitive action potentials which result in involuntary after-contractions. These effects have been linked to impaired transmembrane conductance of either chloride or sodium ions (Franke *et al.*, 1991; Charlet-B *et al.*, 2002; Mankodi, 2008). A number of non-dystrophic myotonias are caused by mutations in the *CLCN1* gene, which encodes the chloride ion channel CIC-1, and the *SCN4A* gene which encodes a skeletal muscle sodium ion channel (Mankodi, 2008). A study using human skeletal muscle tissue from DM1 patients showed that *SCN4A* was present in membrane fractions extracted from the cells, whereas CIC-1 was absent or present at greatly reduced levels compared to normal skeletal muscles (Charlet-B *et al.*, 2002). Studies in mice and humans have shown that CIC-1 is alternatively spliced in DM1. In mice expressing large CUG repeats a broad spectrum of altered splicing events were observed, with the inclusion of a novel exon, designated “7a”, being the most common (Mankodi *et al.*, 2002). In the same study Mankodi *et al.* (2002) showed that patients with both DM1 and DM2 had a high frequency of alternative isoforms of CIC-1 and that severely affected DM1 patients had aberrant splicing in 100% of the cDNAs examined. The most common alternative splicing event was the inclusion of the 7a exon, which was accompanied by the inclusion of another novel exon, designated “6b” (Mankodi *et al.*, 2002). Intron 2 (located between exons 2 and 3) was also shown to be retained in skeletal muscle tissue from DM1 patients (Charlet-B *et al.*, 2002). In the same study it was shown that coexpression of CUG-BP with a CIC-1 intron 2 minigene in a normal fibroblast cell line resulted in retention of intron 2 (Charlet-B *et al.*, 2002). A *Mbnl1* knockout in mice showed increased inclusion of exon 7a in CIC-1 and myotonia (Kanadia *et al.*, 2003). The insertion of novel exons and retention of intron 2 result in the insertion of premature stop codons and degradation of CIC-1 mRNAs by nonsense-mediated decay, leading to the reduced levels of CIC-1 seen

in DM1 skeletal muscle (Charlet-B *et al.*, 2002). The correction of CIC-1 splicing by morpholino antisense oligonucleotides in mice restored the full-length reading frame and increased CIC-1 expression, therefore eliminating myotonia (Wheeler *et al.*, 2007b). These results strongly support a role for MBNL1 and CUG-BP1 in the alternative splicing of CIC-1 transcripts leading to myotonia in DM1. As the misregulated splicing of CIC-1 transcripts in DM1 is proposed to be the cause of myotonia, which is a major symptom of the disease, it would be interesting to see whether the mis-splicing event could be the cause of other symptoms, such as the development of cataract in the lens. In the lens, the level of Cl⁻ is important for the maintenance of a constant cell volume and alterations in Cl⁻ are thought to result in cataract development through a loss of osmotic equilibrium (Zhang & Jacob, 1997). If CIC-1 channels are expressed in the lens and the splicing pattern is disturbed then this could result in disturbances in Cl⁻ levels and cataract development.

Transcripts of the α -subunit of IR are also subject to alternative splicing where exon 11 can either be excluded to produce isoform A (IR-A), or included to produce isoform B (IR-B). The A isoform has a higher affinity for insulin, however, it has a lower signalling capacity and therefore it is the B isoform which results in an increased level of insulin sensitivity and is expressed predominantly in insulin responsive tissues (Savkur *et al.*, 2001). A switch from the predominant IR-B in normal skeletal muscle, to IR-A in DM1 skeletal muscle was shown to occur and was linked to the increased steady-state levels of CUG-BP1 in the disease, although the same switch in isoforms was not observed in DM1 fibroblasts (Savkur *et al.*, 2001). A further study showed that MBNL1 and MBNL2 act antagonistically with CUG-BP1 in the regulation of IR exon 11 inclusion (Dansithong *et al.*, 2005). Down-regulation of MBNL1/2 and overexpression of CUG-BP1 were both shown to result in decreased inclusion of exon 11 in normal human myoblasts. Rescue experiments showed that loss of MBNL1 was the primary cause of the aberrant IR splicing with overexpression of CUG-BP1 playing a secondary role (Ho *et al.*, 2004; Dansithong *et al.*, 2005; Paul *et al.*, 2006). In the rat lens, insulin plays a role in the maintenance of differentiation in fibre cells and activates the transcription of crystallin genes which maintain transparency during ageing by binding damaged proteins to prevent their precipitation (Leenders *et al.*, 1997; Civil *et al.*, 2000). In bovine

lens epithelial cells, insulin has been shown to activate the phosphatidylinositol 3-kinase signalling pathway, which is involved in survival, growth and differentiation. Insulin receptors are also found in the human lens (Naeser, 1997). Insulin-like growth factor-1 (IGF-1) can also bind to IR and is present in the lens (Pavelic *et al.*, 2007). Changes in signalling levels from the insulin receptor could therefore be linked to cataract through possible alterations in levels of the crystallin proteins or through disturbed cellular signalling resulting in a reduction in survival, growth or differentiation in LECs. For this reason, it would be interesting to see whether the ratio of IR isoforms is altered in DM1 lens cells.

A suggested cause of skeletal muscle wasting in DM1 is the altered splicing of RYR1, which is a Ca^{2+} release channel situated in the sarcoplasmic reticulum (SR). RYR1 is stimulated to release Ca^{2+} from the SR in myocytes following the generation of an action potential. This stimulates muscle contraction in a process known as excitation-contraction (EC) coupling. Two regions of RYR1, known as ASI and ASII, are variably spliced and developmentally regulated (Futatsugi *et al.*, 1995). The ASI region is absent in the juvenile form and present in the adult form. In DM1 muscle the juvenile form of RYR1 is overexpressed and was shown to have a lower level of activity which was analysed by assessing the open probability of the channel (Kimura *et al.*, 2005). Interestingly, voltage-activated Ca^{2+} release during EC coupling was shown to be significantly increased in myocytes expressing the juvenile form of RYR1 and it was suggested that the ASI region forms a regulatory module which contributes to EC coupling, and therefore, its absence facilitates stronger EC coupling (Kimura *et al.*, 2009). Levels of Ca^{2+} in cultured myocytes from DM1 patients were found to be elevated when compared to normal controls (Jacobs *et al.*, 1990). Alterations in EC coupling could result in the increased levels of Ca^{2+} observed in DM1 muscle cells and could lead to muscle degeneration through increased activation of Ca^{2+} dependent proteases (Kimura *et al.*, 2009). An increase in activation of Ca^{2+} dependent proteases could cause myopathy through the breakdown of proteins in the muscle cells. RYR1 is primarily expressed in skeletal muscle, but is also expressed in the brain and at low levels in the heart (Futatsugi *et al.*, 1995). There are two further forms of ryanodine receptor, known as RYR2, which is mainly expressed in the heart, and RYR3, which is more widely expressed in tissues including the heart, brain and skeletal muscle (Dulhunty *et al.*, 2006). A

study found expression of RYR1 in a human lens cell line but found greater levels of expression of RYR3 and showed that the receptors were involved in the regulation of intracellular Ca^{2+} levels (Qu & Zhang, 2003). Excessive levels of Ca^{2+} in the lens can lead to cataract formation also through activation of Ca^{2+} dependent proteases, such as calpains, which break down fibre cell proteins (Biswas *et al.*, 2005). For this reason it would be interesting to see whether ryanodine receptors are present in our human lens cells and, if so, whether the splicing patterns are the same in DM1 and control cells.

3.1.2 Cellular characterisation of DM1 lens epithelial cells

At the cellular level, studies on cataracts in general have pointed at a loss of cells from the lens epithelium in the development of cataract due to the resulting loss of homeostasis in levels of ions and water (Konofsky *et al.*, 1987; Li *et al.*, 1995). Loss of cells from the LEC layer was shown to be particularly relevant in the development of subcapsular cataracts, as the cell densities were much lower when compared to those of nuclear cataract (Konofsky *et al.*, 1987). A study by Abe *et al.* (1999) investigated the density of the LEC layer in native human lenses from DM1 patients with cataract. They showed that DM1 lenses contained fewer cells in the LEC layer than those from age matched control lenses with senile cataract (Abe *et al.*, 1999). A significant decrease in cell density was observed with DM1 lenses having an average density of 2274 cells/mm² compared to 4627 cells/mm² in the control lenses. The decrease in cell density was also shown to be more prominent in a patient with an earlier onset of the disease and more severe symptoms. Despite the decrease in cell density, coverage of the lens was maintained by an increase in cell size (Abe *et al.*, 1999). This data would suggest that a reduced number of LECs may not be sufficient to maintain the balance of ions and water which is vital to lens transparency, therefore resulting in cataract formation in DM1. Rhodes *et al.* (2006) characterised the growth of most of the lens epithelial cell lines used in this study and showed that the DM1 cell lines had longer doubling times compared to the controls (Rhodes *et al.*, 2006). The rate of growth of the DM1 cell lines also began to decline at a much earlier stage and continued to decline until a point was reached where there were not enough cells remaining to start the next passage (Rhodes *et al.*, 2006). This data indicates that behaviour of the cell lines is similar to the situation seen

in vivo and it would be interesting to see whether the two further control cell lines employed in this study confirm these findings.

The decrease in density of DM1 LECs could be due to a decrease in cell proliferation or an increase in cell death. Cell death can occur either via apoptosis, which is the orderly breakdown of the cell into apoptotic bodies which can be phagocytosed (also known as programmed cell death), or via necrosis, which is characterised by the early breakdown of the plasma membrane resulting in the release of the cell contents. Interestingly, capsulorhexis samples from non-DM1 cataract patients were shown to have a significantly greater percentage of apoptotic cells compared to normal controls (Li *et al.*, 1995). To elucidate whether apoptosis of LECs preceded cataract development or *vice versa*, Li *et al.* (1995) performed experiments using cultured rat lenses. Hydrogen peroxide was used to induce cataract formation and was shown to trigger apoptosis in the LECs prior to cataract development (Li *et al.*, 1995). Rhodes *et al.* (2006) investigated whether reduced levels of cell proliferation or increased levels of cell death were responsible for the reduced population doubling times observed in the DM1 lens cell lines and found that fold stimulation of growth by 10% FCS was similar across the cell lines, but release of LDH was increased, indicating increased levels of cell death were the cause (Rhodes *et al.*, 2006). As cell death was implicated in the reduced population doubling times, it would be interesting to establish the mode by which it occurs, i.e. apoptosis or necrosis.

3.2 Aims

The aim of this chapter is to establish whether the DM1 and control cell lines are a valid model in which to study the disease. Expression levels of the genes at the DM1 locus will be analysed and a primary aim is to show whether *DMPK* is expressed in the lens cell lines and whether transcripts form foci as seen in other affected cell types. If foci form then splicing patterns may be altered within the lens and therefore these secondary effects of the triplet repeat will also be investigated in order to establish whether they may be the underlying cause of cataract formation. Cell growth, proliferation and death of the lens cell lines will also be investigated as these are thought to impact on cataract formation in the native human lens.

3.3 Results

3.3.1 *SIX5* and *DMPK* expression in DM1 lens epithelial cells

QRT-PCR showed that *SIX5* is expressed in human LECs, however, in contrast to previous reports, no difference in expression levels was observed between the control and DM1 cell lines (see figure 3.2, expression analysed at days 8, 50 and 78 of the culture period shown in figure 3.9) (Rhodes *et al.*, 2006). The previous study by Rhodes *et al.* (2006) used only two of the control cell lines (CCat1 and CCat2), but recently, two further control cell lines were obtained (CCat3 and CCat4) and revealed that the level of *SIX5* expression in the DM1 cell lines falls within the range of normal expression values. The mean expression value across the control cell lines was 5.10 ± 1.39 (arbitrary units) compared to 3.91 ± 0.43 (arbitrary units) across the DM1 cell lines. This data would indicate that reduced expression of *SIX5* is unlikely to be responsible for cataract formation in the lens as previously proposed (Winchester *et al.*, 1999; Klesert *et al.*, 2000).

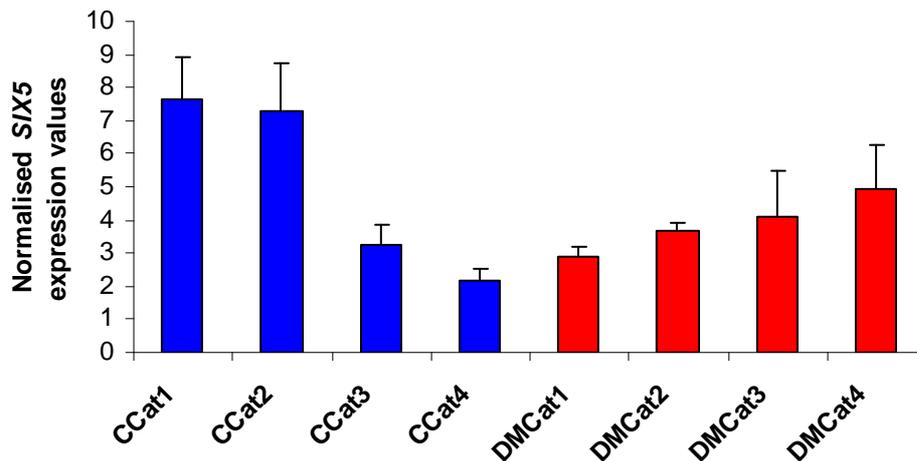
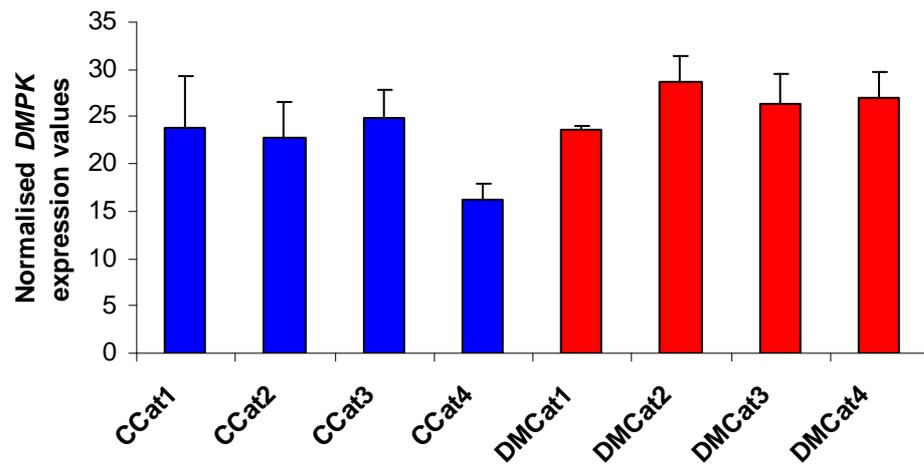


Figure 3.2 – *SIX5* is expressed in DM1 lens epithelial cells. QRT-PCR analysis of mRNA expression from *SIX5* normalised to *18S* expression. *SIX5* was expressed in both control and DM1 lens cells and no difference was observed in expression levels between the two groups (n = 3, data expressed as mean \pm SEM of three independent experiments).

a



b

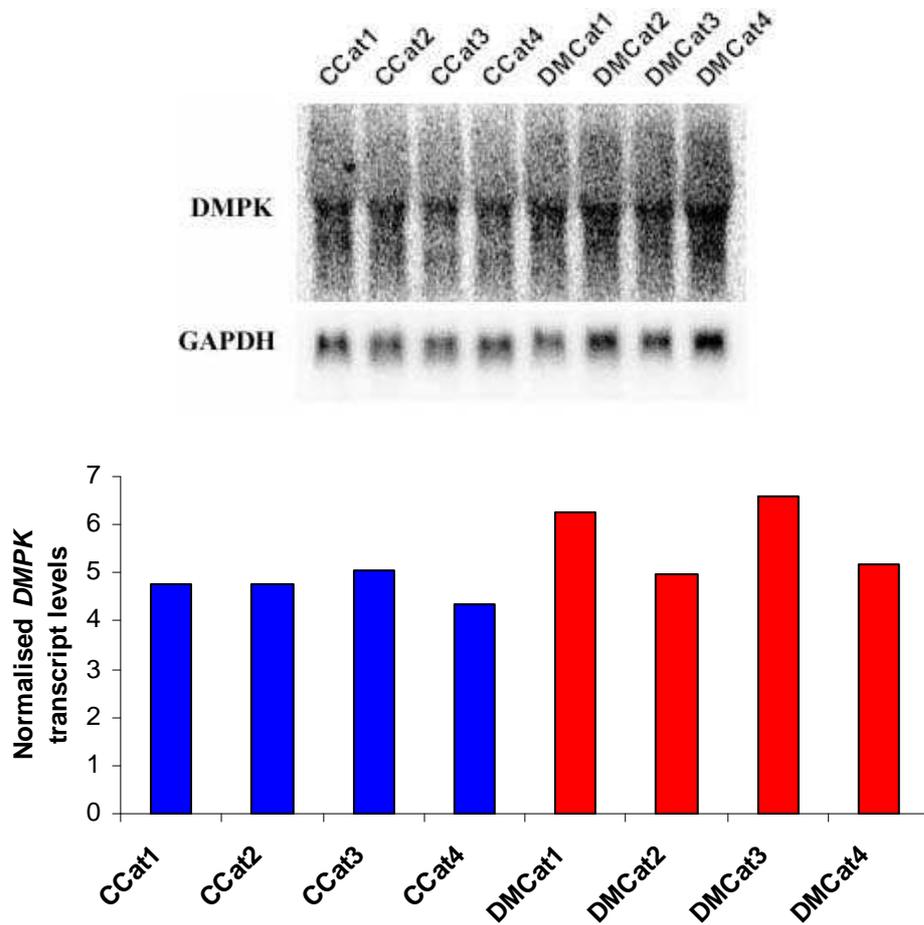


Figure 3.3 – *DMPK* is expressed in DM1 lens epithelial cells. (a) QRT-PCR analysis of mRNA expression from *DMPK* normalised to *18S* expression (n = 3, data expressed as mean \pm SEM of three independent experiments). (b) Northern blot analysis of mRNA expression from *DMPK*. Graph shows the bands analysed densitometrically and normalised to *GAPDH* mRNA levels.

Using both QRT-PCR and Northern blot analysis to detect *DMPK* mRNA, *DMPK* was shown to be expressed in human LECs (see figure 3.3). In both cases there was no significant difference in expression levels between DM1 and control LECs. Two separate probes crossing different exon boundaries were used to detect *DMPK* transcripts by QRT-PCR. Probes were selected to cross the boundaries of exons 2-3 and 8-9 (see table 2.2 for the assay identification numbers). Both probes detected similar levels of *DMPK* expression so the results are shown for only one of the probes (see figure 3.3a). The mean expression value across the control cell lines was 21.98 ± 1.96 (arbitrary units) compared to 26.46 ± 1.05 (arbitrary units) across the DM1 cell lines. Using Northern blot analysis (see figure 3.3b), one band of the same size was observed in all of the cell lines, but surprisingly no larger bands were observed in the DM1 cells. A larger band, or smear, representing transcripts of differing length depending on the size of the triplet repeat, would have been expected due to the large repeats found in the *DMPK* mutant allele. The lack of this band could indicate a problem in the RNA extraction procedure as the mutant transcripts are possibly subjected to mechanical damage or it could indicate inefficient transfer of the larger RNAs to the membrane. From the band detected, the mean values of transcript levels across the control cell lines was 4.74 ± 0.14 (arbitrary units) compared to 5.74 ± 0.40 (arbitrary units) across the DM1 cell lines. Despite not showing expression of mutant *DMPK* transcripts, both Northern blotting and QRT-PCR showed that *DMPK* was expressed in human LECs. Both techniques actually showed that expression of *DMPK* was higher in DM1 LECs, although this was not statistically significant. The presence of *DMPK* mRNA in human lens cells indicates that foci of transcripts may form in DM1 nuclei.

Figure 3.4 – *DMPK* transcripts form nuclear foci in DM1 lens epithelial cells. Triplet repeat RNA was detected using fluorescence *in situ* hybridisation with a (CAG)₁₀ alexa 555 labelled oligonucleotide probe (red) in nuclei counterstained with DAPI (blue). No foci were observed in control lens cells (a) CCat1 and (b) CCat2, however, foci (indicated by white arrows) were detected in DM1 lens cells (c) DMCat1, (d) DMCat2, (e) DMCat3 and (f) DMCat4 (bar = 20 µm). (g) Summary of mean number of foci (n = 2, data expressed as mean of two independent experiments, statistical analysis was performed by Student's T test, ** p < 0.01 relative to controls).

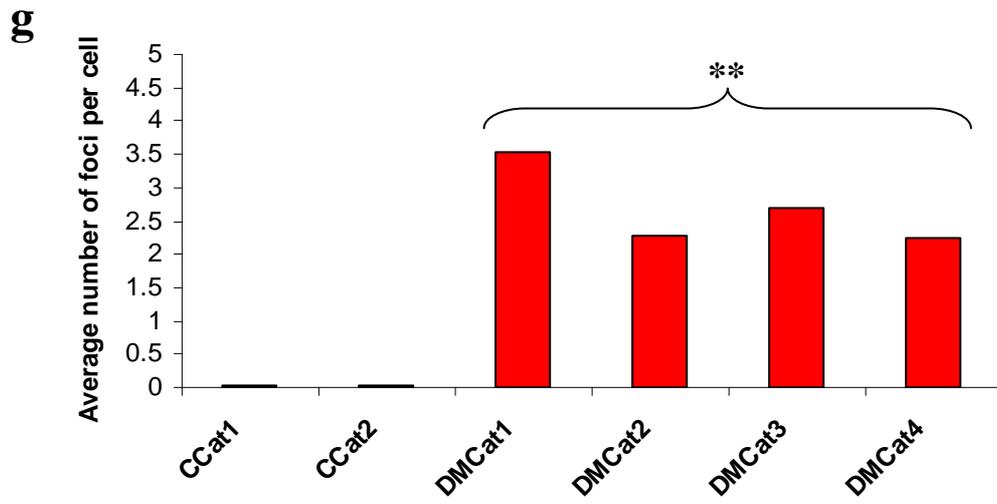
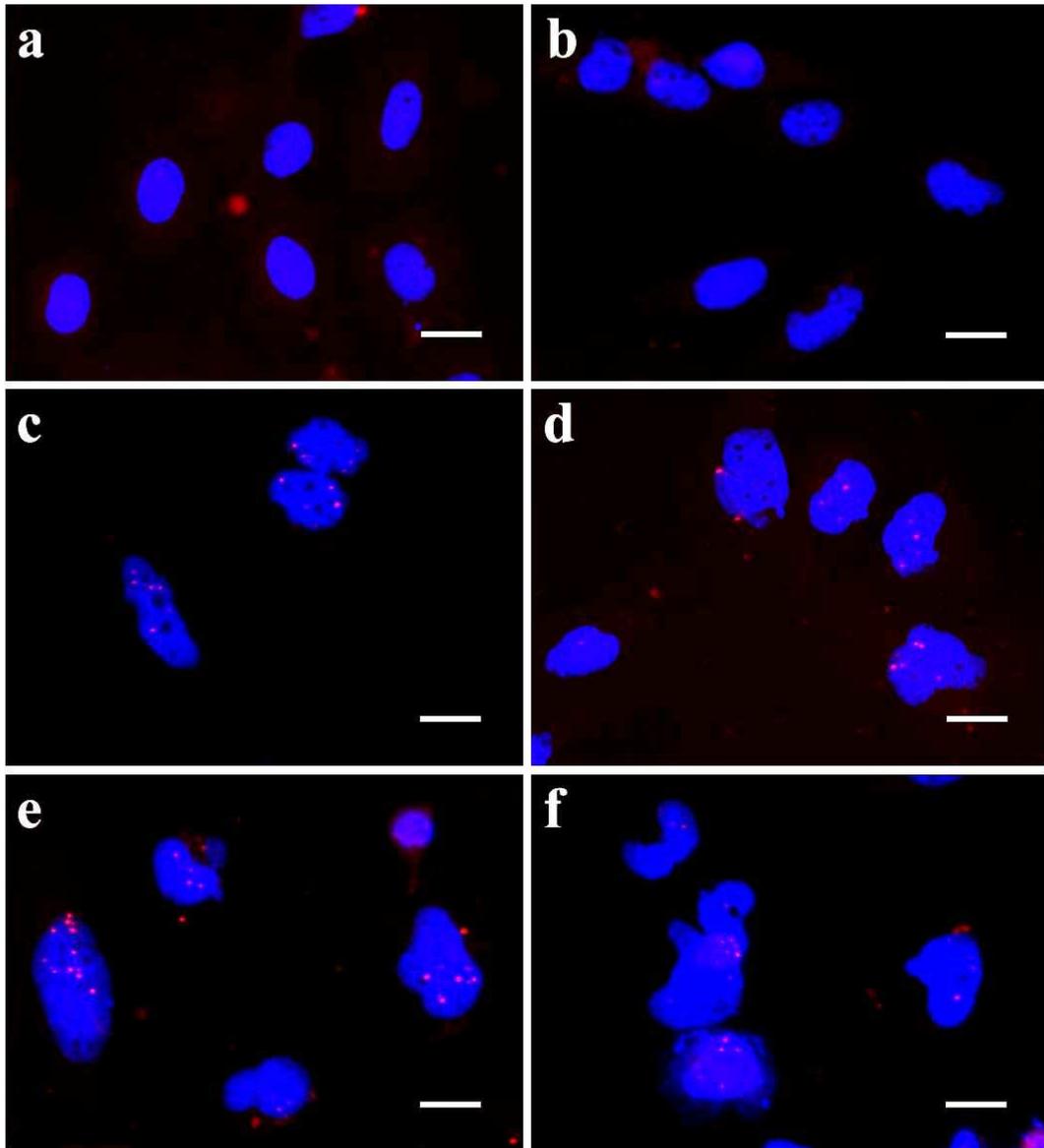


Figure 3.4 – see previous page for figure legend

3.3.2 Formation of nuclear foci of mutant *DMPK* transcripts in DM1 lens epithelial cells

Fluorescence *in situ* hybridisation (FISH) of lens cells with (CAG)₁₀ alexa 555 labelled oligonucleotide probes revealed nuclear foci of *DMPK* transcripts in all DM1 cell lines, but these were absent in the controls (see figure 3.4). This confirms that the mutant *DMPK* allele is expressed in the DM1 cells, despite not being detected during Northern blot analysis of *DMPK* expression. In all cell lines a very diffuse cytoplasmic staining could be seen, however, bright and distinct spots of staining were found only in the nuclei of DM1 cells. Many of the foci were found at the very edge of the nucleus, however, attempts to produce a 3D image of the nuclei to visualise the position of the foci in more detail using laser scanning confocal microscopy failed as the signal was of insufficient intensity. The nuclear foci ranged in size from 0.5 to 2 µm and the number of foci ranged from 0 to as many as 24 per cell, with an average of 2.7 per cell (a minimum of 153 cells were counted per cell line).

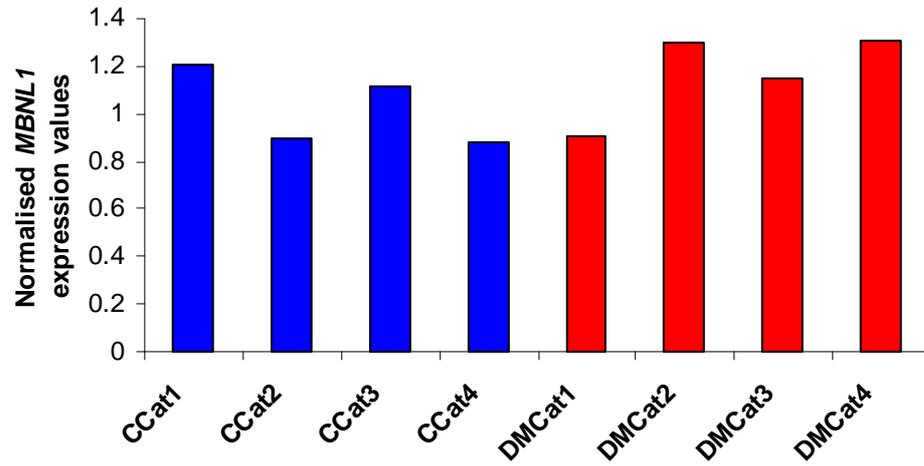
3.3.3 *MBNL* and *CUG-BP* expression in DM1 lens epithelial cells

Both *MBNL1* and *MBNL2* are expressed in control and DM1 LECs and no difference in expression levels was observed between the two groups (see figure 3.5). The expression level of *MBNL3* was not analysed in the cell lines as its expression is almost exclusively limited to the placenta in humans (Fardaei *et al.*, 2002). The mean expression value for *MBNL1* across the control cell lines was 1.03 ± 0.08 (arbitrary units) compared to 1.17 ± 0.09 (arbitrary units) across the DM1 cell lines. For *MBNL2*, the mean expression value across the control cell lines was 12.01 ± 1.86 (arbitrary units) compared to 13.12 ± 0.90 (arbitrary units) across the DM1 cell lines.

Both *CUG-BP1* and *CUG-BP2* are also expressed in control and DM1 LECs, with no difference in expression levels between the two groups (see figure 3.6). The mean expression value for *CUG-BP1* across the control cell lines was 24.46 ± 4.37 (arbitrary units) compared to 21.15 ± 3.28 (arbitrary units) across the DM1 cell lines. For *CUG-BP2*, the mean expression value across the control cell lines was 15.59 ± 6.17 (arbitrary units) compared to 10.01 ± 4.03 (arbitrary units) across the DM1 cell lines. Both MBNL and CUG-BP proteins are thought to be responsible for the altered splicing patterns of many transcripts in DM1 and their

expression in the lens makes it possible that alternative splicing may also play a role in DM1 cataract formation.

a



b

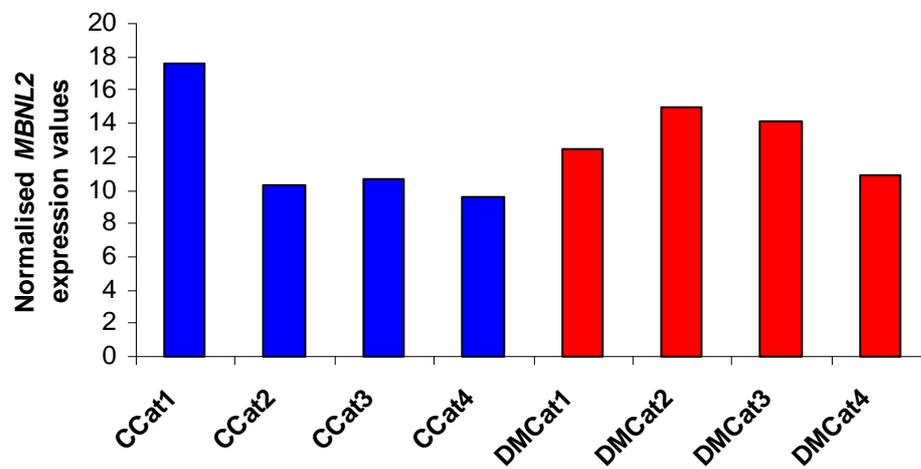
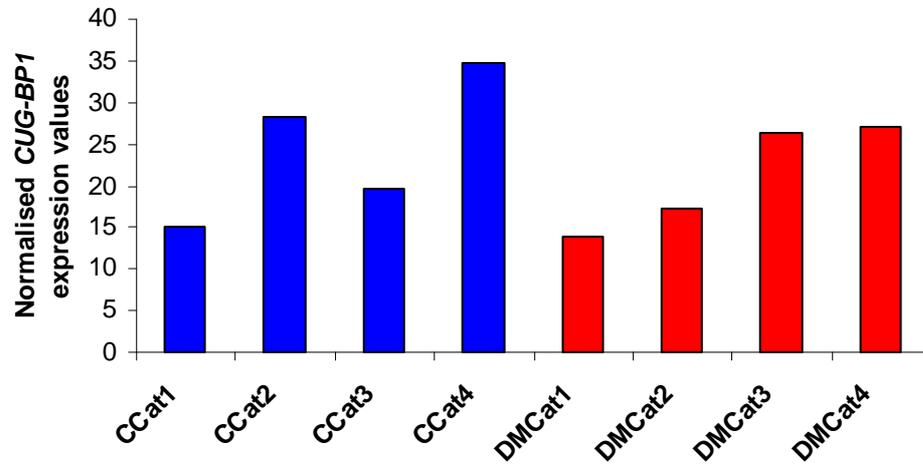


Figure 3.5 – MBNLs are expressed in DM1 lens epithelial cells. QRT-PCR showed that both (a) *MBNL1* and (b) *MBNL2* are expressed in control and DM1 lens epithelial cells. Expression is shown normalised to *18S* expression. There is no observed difference in expression levels between the two groups (n = 2, data expressed as mean, representative of two independent experiments).

a



b

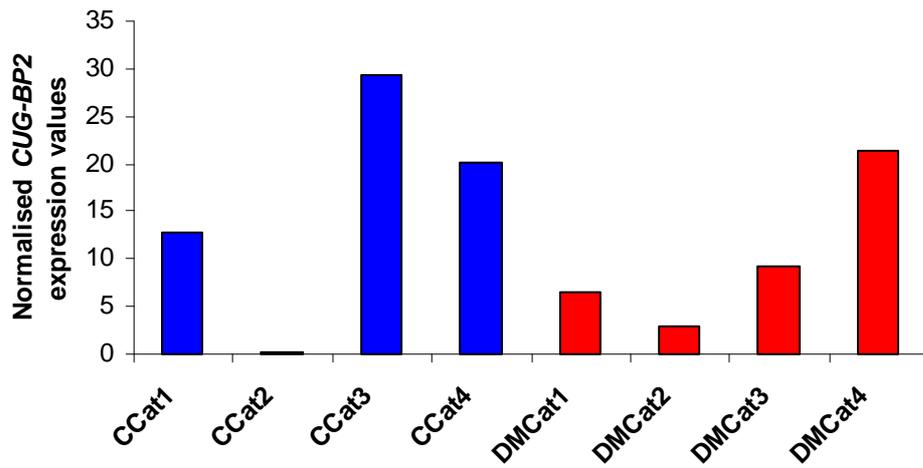


Figure 3.6 – *CUG-BPs* are expressed in DM1 lens epithelial cells. QRT-PCR showed that both (a) *CUG-BP1* and (b) *CUG-BP2* are expressed in control and DM1 lens epithelial cells. Expression is shown normalised to *18S* expression. There is no observed difference in expression levels between the two groups (n = 2, data expressed as mean).

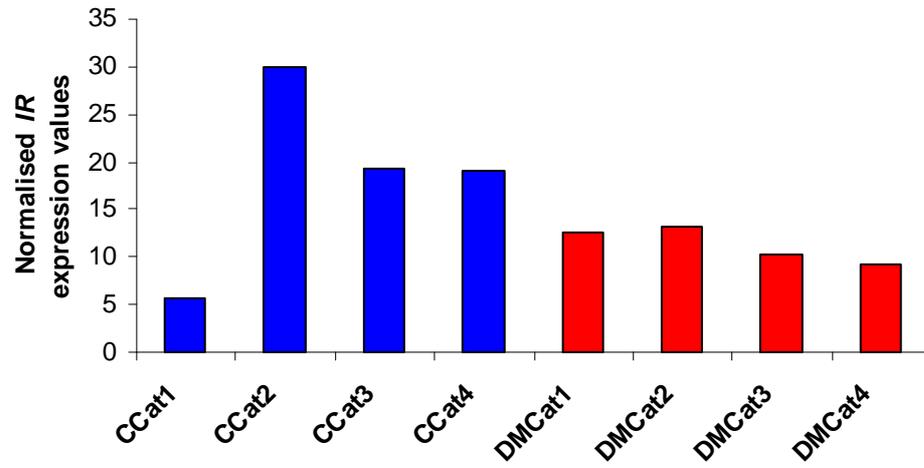
3.3.4 Alternative splicing in DM1 lens epithelial cells

A number of alternative splicing events have been characterised in DM1 and have been shown to cause a variety of the symptoms associated with the disease. In order to establish whether any of these could play a role in cataract development, the expression of a number of affected genes were analysed using QRT-PCR.

Expression of *CLCNI* was not found in any of the human lens cell lines employed in this study. Expression of *RYR1* was found in only three of the cell lines (CCat2, DMCat3 and DMCat4) at very low levels (data not shown). Expression of two further ryanodine receptors, *RYR2* and *RYR3*, was analysed and only *RYR3* was found to be expressed at low levels (data not shown). No alternatively spliced isoforms of *RYR3* have been described in DM1, however.

Expression of *IR* was found in all of the lens cell lines. QRT-PCR was used to analyse the expression levels of the two isoforms of *IR*, utilising probes which crossed different exon boundaries (see table 2.2 for assay identification numbers). As only IR-B contains exon 11, the first probe used crossed the exon 11-12 boundary and would therefore only show expression of IR-B. The second probe used crossed the exon 16-17 boundary, which is not affected by the alternative splicing event observed in DM1 and would therefore show the total expression of both *IR* isoforms. The expression of *IR* (both isoforms) appears to be slightly lower in the DM1 LECs (see figure 3.7a), with the exception of CCat1, however, this is not statistically significant. The mean expression value for *IR* across the control cell lines was 18.52 ± 5.01 (arbitrary units) compared to 11.34 ± 0.94 (arbitrary units) across the DM1 cell lines. The mean expression value for the IR-B isoform across the control cell lines was 3.20 ± 0.82 (arbitrary units) compared to 2.48 ± 0.18 (arbitrary units) across the DM1 cell lines. The expression of IR-B is more even across the cell lines (see figure 3.7b), and although it is not possible to accurately compare expression levels using different probes in QRT-PCR, the lower total *IR* expression values in DM1 cells would suggest that they actually express less of the IR-A isoform than the control cells. This is in contrast to the reports in DM1 that show that DM1 cells express more IR-A, leading to insulin resistance and diabetes (Savkur *et al.*, 2001).

a



b

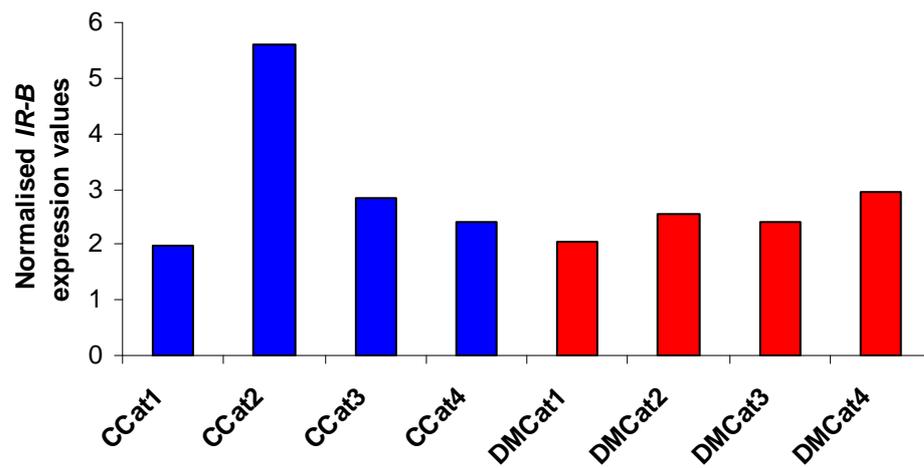


Figure 3.7 – *IR* is expressed in DM1 lens epithelial cells. QRT-PCR showed that (a) *IR* and (b) specifically the *IR-B* isoform are expressed in control and DM1 lens cells. There is no observed difference in expression levels between the two groups ($n = 2$, data expressed as mean, representative of four independent experiments).

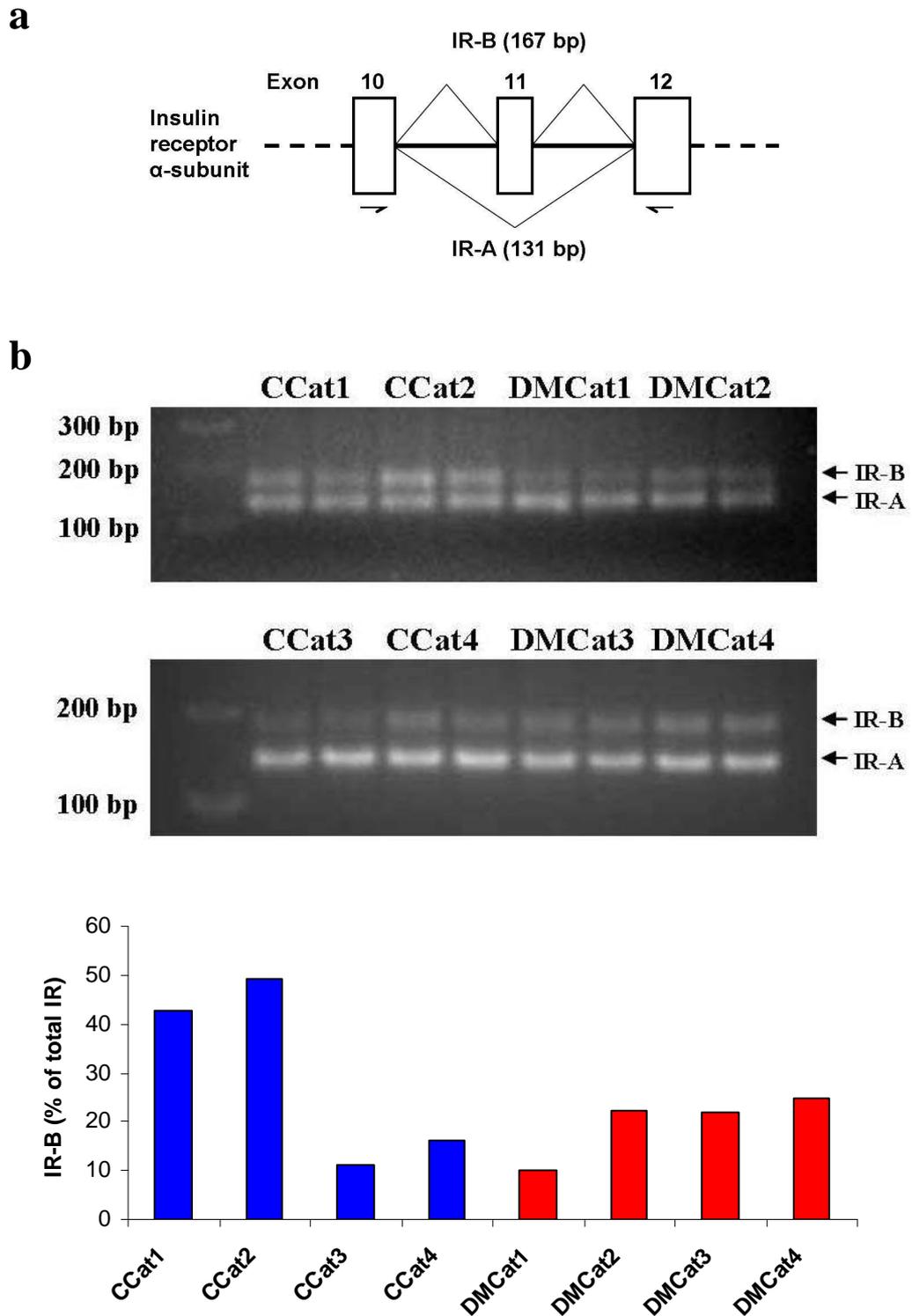


Figure 3.8 – Insulin receptor splicing is not altered in DM1 lens epithelial cells. (a) Schematic of exon splicing in IR. Primers amplify IR-B (167 bp, includes exon 11) and IR-A (131 bp, excludes exon 11). (b) RT-PCR products of IR were electrophoretically resolved on 4% agarose gels. The bands were measured densitometrically and the percentage of IR-B was calculated and is shown in the graph as an average of the two samples. The percentage of IR-B in the DM1 cells fell within the range of those of the control cells, indicating that IR splicing is not affected in the lens (n = 2, data expressed as mean).

To investigate the ratio of the expression of the IR-A and IR-B isoforms more accurately, the technique of RT-PCR was used to amplify a region of the *IR* transcripts from exon 10 to 12. Exon 11 is 36 nucleotides long and therefore the fragment produced from the IR-A isoform is only 131 bp long compared to that of IR-B which is 167 bp (see figure 3.8a). The RT-PCR products were electrophoretically resolved and the percentage of IR-B was calculated (see figure 3.8b). Two of the control cell lines, CCat1 and CCat2, showed almost equal expression of IR-A and IR-B (a total of 42.8% and 49.1% IR-B respectively), whereas CCat3 and CCat4 showed much lower levels of IR-B (11.3% and 16.0% respectively), as did all of the DM1 cell lines. The average level of IR-B in the control cell lines was $29.8\% \pm 9.4\%$ compared to $19.7\% \pm 3.3\%$ in the DM1 cell lines. Due to the large spread of the levels of IR-B in the control cell lines it appears that IR splicing is not affected in DM1 LECs, as is the case in DM1 fibroblasts (Savkur *et al.*, 2001).

3.3.5 Population doubling times of DM1 lens epithelial cells

To analyse the growth of populations of the cell lines during culture, flasks were seeded with a set number of cells and were passaged weekly and counted using a haemocytometer. Population doubling times were calculated for all of the human lens cell lines and confirmed the findings of Rhodes *et al.* (2006) (see figure 3.9). Passage numbers (P) of the cell lines at day 0 were as follows: CCat1 P = 17, CCat2 P = 14, CCat3 P = 8, CCat4 P = 6 and all DM1 cell lines P = 12. At early passages all of the cell lines were seen to be doubling at a similar rate, however, after 40 days in culture a clear separation was observed between the control and DM1 cell lines. At this point the population doubling times of the DM1 cell lines began to slow and were shown to decline at later passages as fewer cells were harvested than seeded. After 80 days in culture three out of four DM1 cell lines no longer had enough cells to start the next passage. The control cell line, CCat4, had also ceased by this point, however, this was not due to a natural decline in cell numbers as was seen in the DM1 cell lines, but rather because they had grown too fast and reached a confluent state before passaging which caused the cells to lift off the bottom of the culture flask. The remaining control cell lines continued to grow well throughout the culture period and cell counts were only stopped once the DM1 cell lines had all ceased to grow. The lifespan of the DM1

cell lines was significantly shorter than the control cell lines (CCat4 was omitted from this as cell growth was halted abruptly and did not reach a natural decline) ($p < 0.01$). The DM1 cell lines survived for an average of 79.5 ± 7.4 days in culture, whereas the controls cell lines continued to grow and were stopped after 101 days in culture. All four DM1 cell lines were shown to have significantly reduced doubling times compared to the four control cell lines ($p < 0.01$). The DM1 cell lines had an average of 1.51 ± 0.10 population doublings per week compared to 3.52 ± 0.18 in the controls.

3.3.6 Morphology of DM1 lens epithelial cells

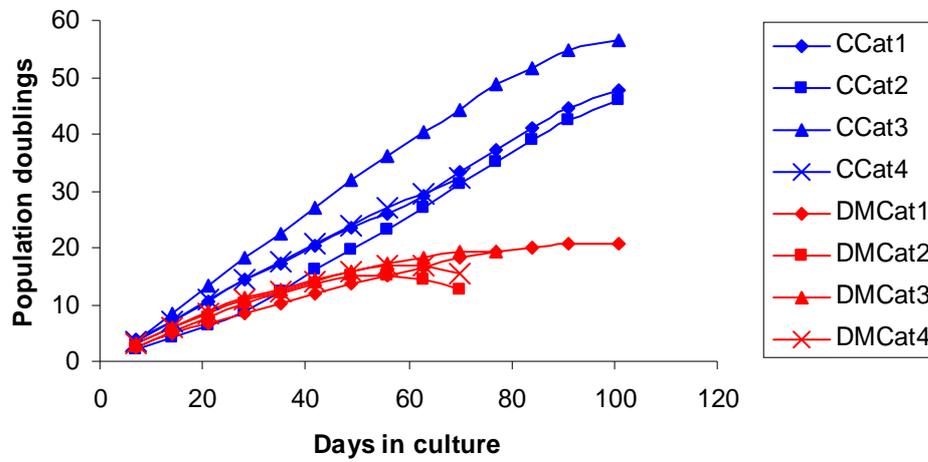
Cells were seeded in 35 mm culture dishes on day 28 of the culture period shown in figure 3.9a. Cells were grown in medium supplemented with 10% FCS for 10 days before light microscope phase images of the DM1 and control cell lines were taken (see figure 3.10). Despite the fact that the populations of all cell lines at this point were shown to be growing at similar rates by analysing the population doubling times, there are clear differences in the density of the cells seen in the images. Many more cells can be seen in the images of the control cell lines compared to the DM1 cell lines. The control cells are closely packed together, forming confluent sheets or patches of cells. In contrast, the DM1 cells are not confluent and are less densely packed.

3.3.7 Cell death in DM1 lens epithelial cells

Cell death in medium supplemented with 10% FCS and in SF medium was analysed in the DM1 and control LECs using a lactate dehydrogenase (LDH) assay. Greater levels of cell death were observed in DM1 cells in medium supplemented with 10% FCS and SF medium compared to control cells, however, neither was statistically significant (see figure 3.11). In both cell types, levels of cell death were significantly greater in SF medium compared to medium supplemented with 10% FCS ($p < 0.05$).

Further experiments were repeated using only CCat1 and DMCat1 cells and reveal that DMCat1 has significantly greater levels of cell death in both medium supplemented with 10% FCS and SF medium compared to the control CCat1 ($p < 0.01$). There was no significant difference in levels of cell death between conditions in the same cell type (see figure 3.12).

a



b

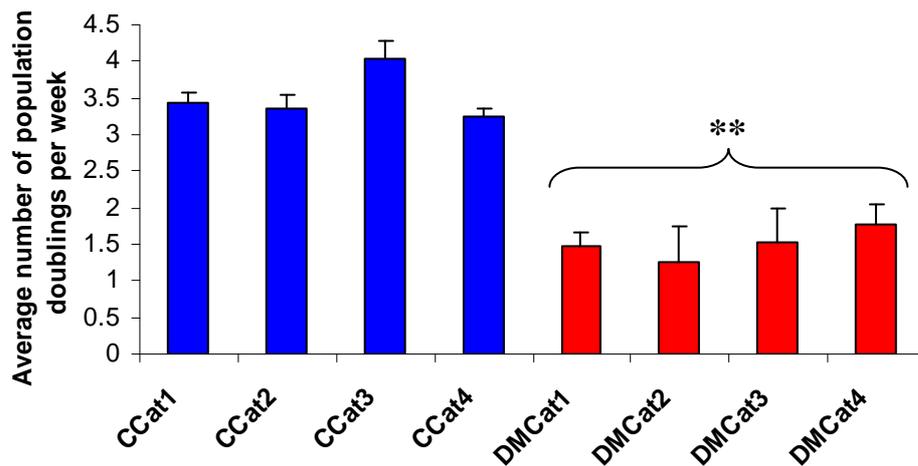


Figure 3.9 – Population doubling times of DM1 lens epithelial cells. Cells were grown in 75 cm² flasks and counted weekly using a haemocytometer. (a) Passage numbers (P) of the cell lines at day 0 were as follows: CCat1 P = 17, CCat2 P = 14, CCat3 P = 8, CCat4 P = 6 and all DM1 cell lines P = 12. At early passages all cell lines were doubling at a similar rate, however, after 40 days in culture a clear separation was seen between DM1 and control cell lines. The population doubling times of the DM1 cell lines slowed and declined at later passages as fewer cells were harvested than seeded. The lifespan of DM1 cell lines was significantly shorter than that of the controls (statistical analysis performed by Student's T test, $p < 0.05$) (b) The average number of population doublings per week, showing that DM1 lens cells have significantly reduced doubling rates compared to control lens cells (data expressed as mean \pm SEM, statistical analysis was performed by Student's T test, ** $p < 0.01$ relative to controls).

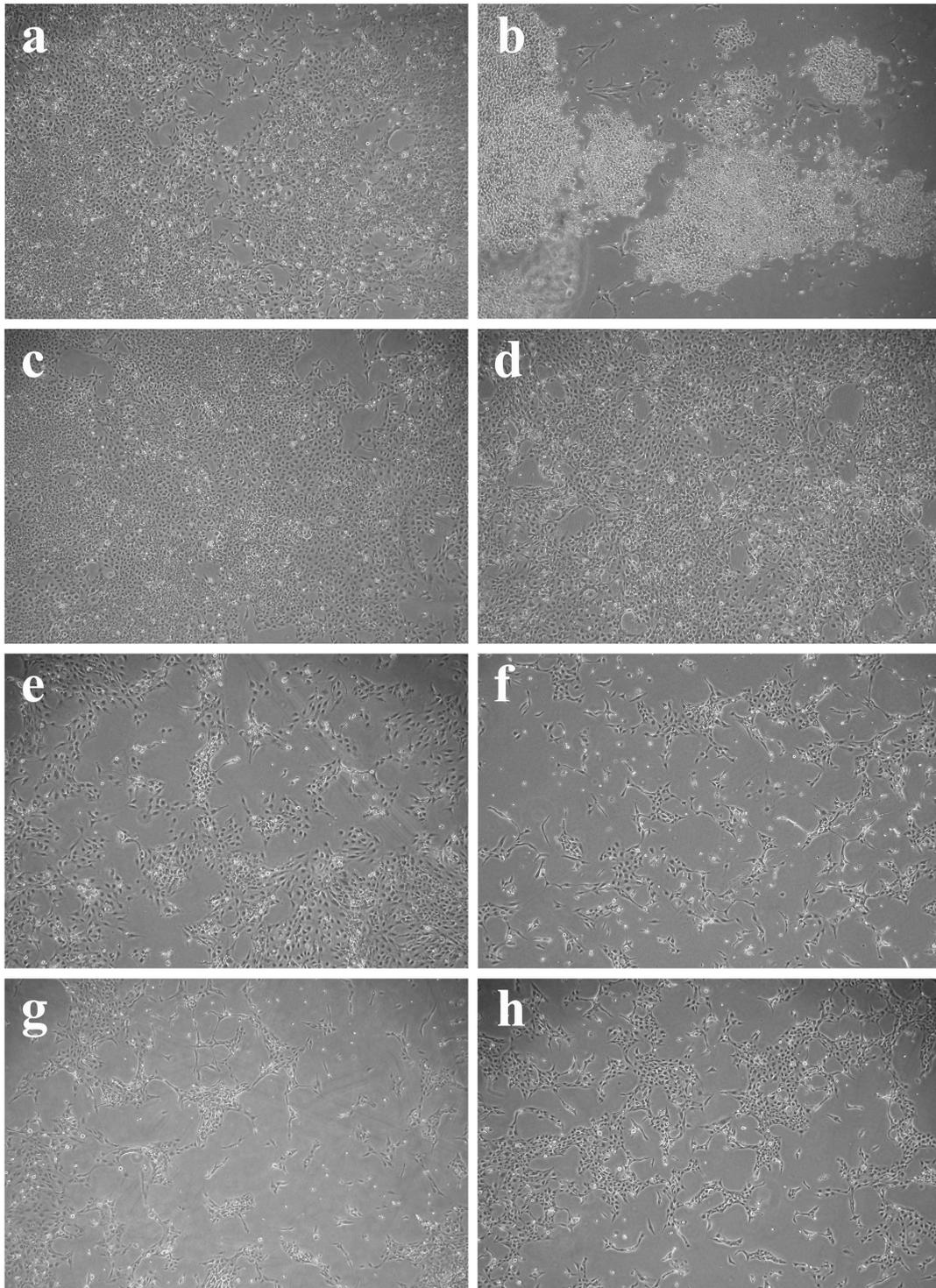


Figure 3.10 – Light microscope phase images of DM1 lens epithelial cells. Cells were grown in medium supplemented with 10% FCS and images were taken to show the density and morphology of the DM1 and control lens cells. The DM1 cell lines (e) DMCat1, (f) DMCat2, (g) DMCat3 and (h) DMCat4 never reached a confluent state, in contrast to the control cell lines (a) CCat1, (b) CCat2, (c) CCat3 and (d) CCat4.

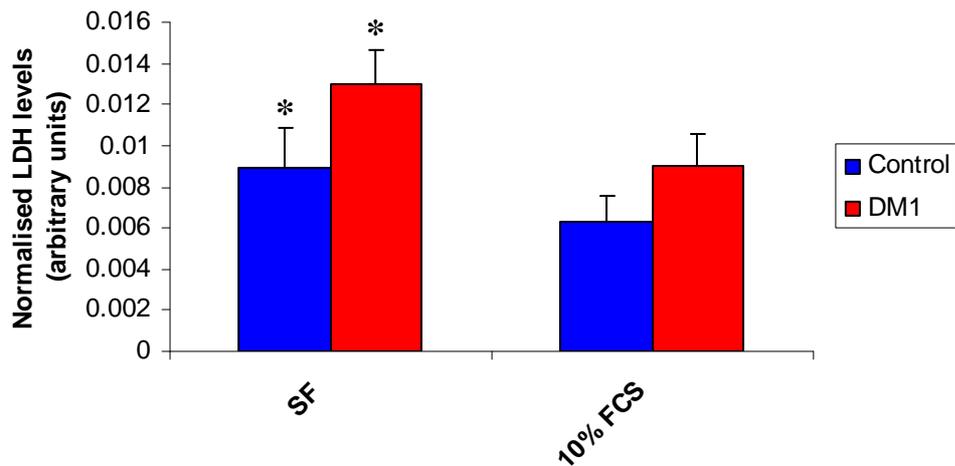


Figure 3.11 – Cell death in DM1 lens epithelial cells. All control and DM1 cells were cultured in SF medium or medium supplemented with 10% FCS for 48 hours and levels of LDH were measured and normalised to total protein at T = 0. Averages across the control and DM1 cell lines were calculated and reveal significantly increased levels of cell death in SF medium in both control and DM1 cells (n = 4, data expressed as mean ± SEM of three independent experiments, statistical analysis was performed by Student’s T test, * p < 0.05 relative to medium supplemented with 10% FCS).

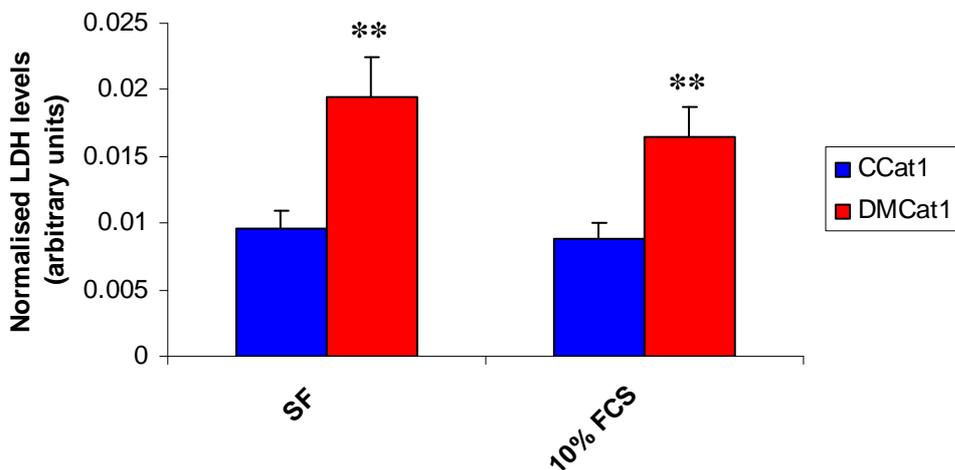


Figure 3.12 – Cell death in DM1 lens epithelial cells. CCat1 and DMCat1 cells were cultured in SF medium or medium supplemented with 10% FCS for 48 hours and levels of LDH were measured and normalised to total protein at T = 0. Cell death was shown to be significantly increased in DMCat1 cells in both SF medium and medium supplemented with 10% FCS (n = 11, data expressed as mean ± SEM of eleven independent experiments, statistical analysis was performed by Student’s T test, ** p < 0.01 relative to CCat1).

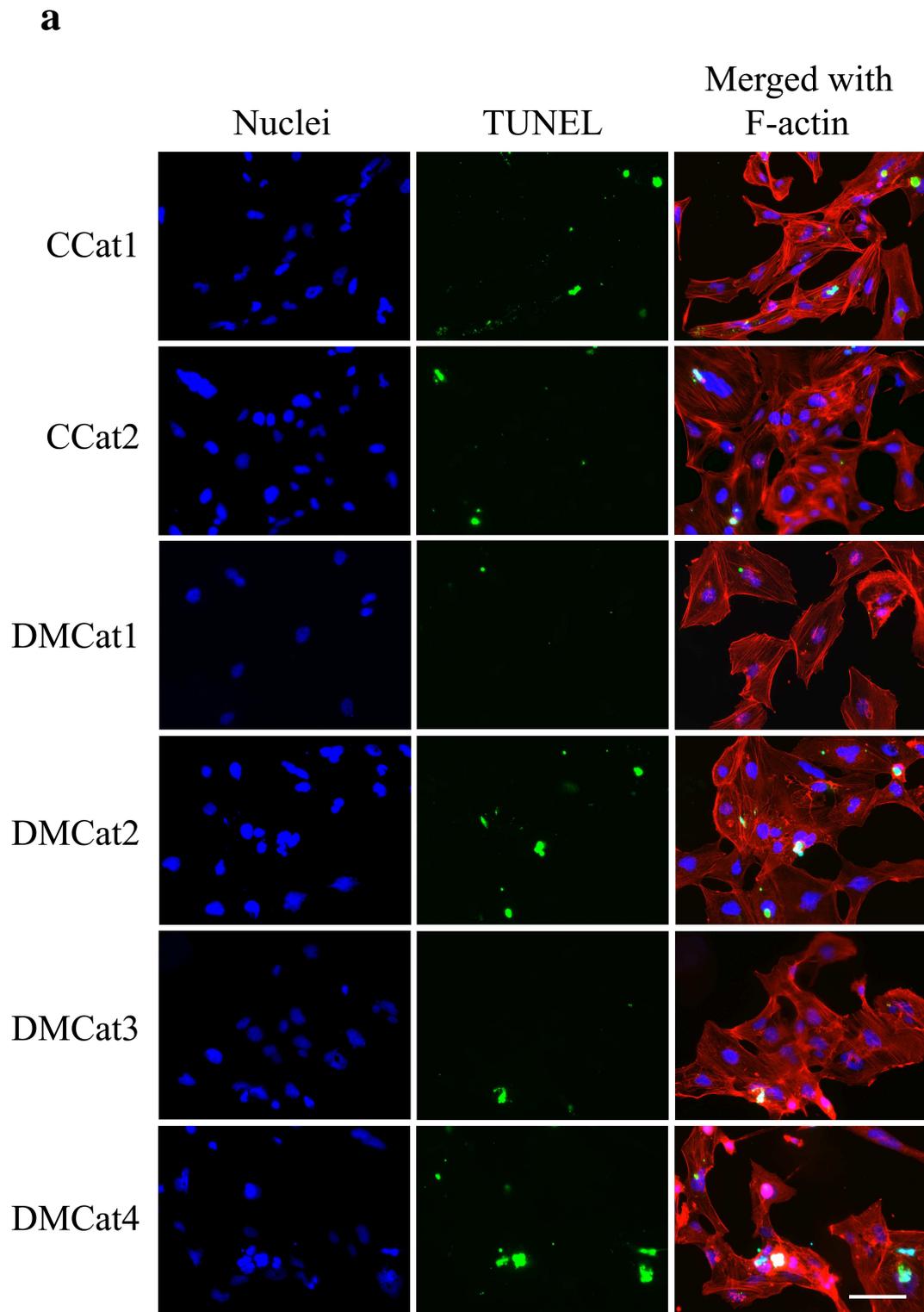


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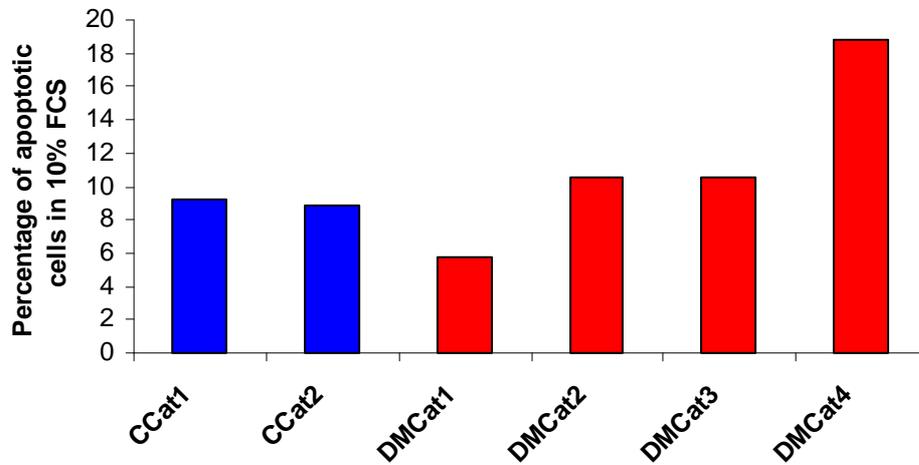


Figure 3.13 – Apoptotic cell death in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS. (a) Fluorescent micrographs of cells showing nuclei labelled with DAPI (blue), apoptotic cells labelled using a TUNEL assay (green) and F-actin labelled with Texas red-X phalloidin (red) (bar = 100 μ m). (b) Analysis of TUNEL positive apoptotic cells (n = 2, data expressed as mean of two independent experiments).

3.3.8 Apoptosis in DM1 lens epithelial cells

Cell death via apoptosis was analysed in the DM1 and control LECs using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Cells were cultured in medium supplemented with 10% FCS and in SF medium before being fixed and the assay performed. In medium supplemented with 10% FCS, similar levels of apoptosis were observed in all cell lines tested (see figure 3.13). The control cell lines had an average of $9.0\% \pm 0.2\%$ apoptotic cells, compared to $11.4\% \pm 2.7\%$ apoptotic cells in the DM1 cell lines. There was no significant difference between the two groups. In SF medium all four DM1 cell lines had greater levels of apoptotic cells than the controls (see figure 3.14). The control cell lines had an average of $8.3\% \pm 0.6\%$ apoptotic cells, compared to $17.4\% \pm 2.8\%$ apoptotic cells in the DM1 cell lines, however, this was not statistically significant. The level of apoptosis in the controls did not appear to be affected by the medium they were cultured in, with similar levels being observed

under both conditions. In contrast, the DM1 cell lines had greater levels of apoptosis in SF medium compared to medium supplemented with 10% FCS, although this was also not statistically significant.

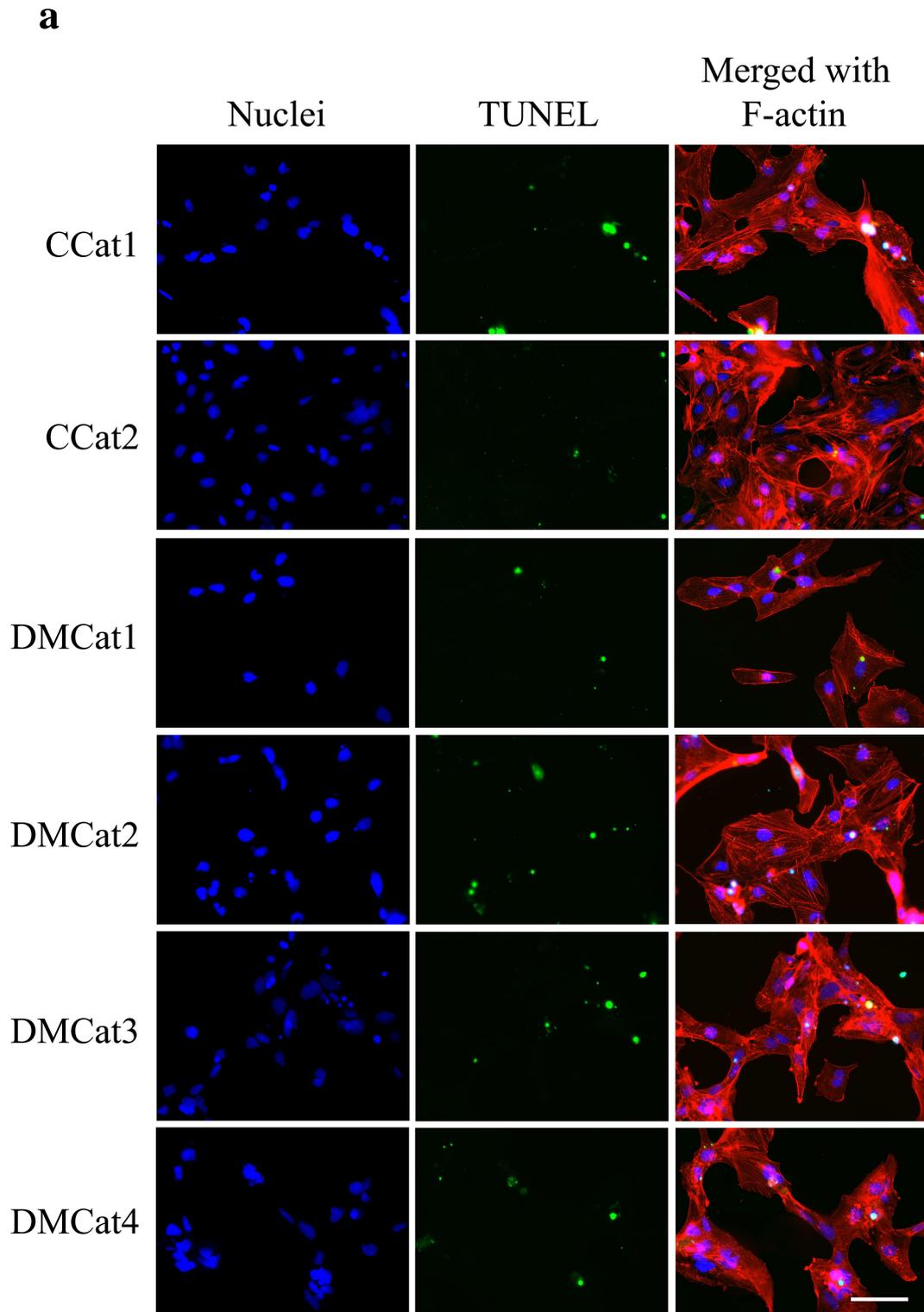


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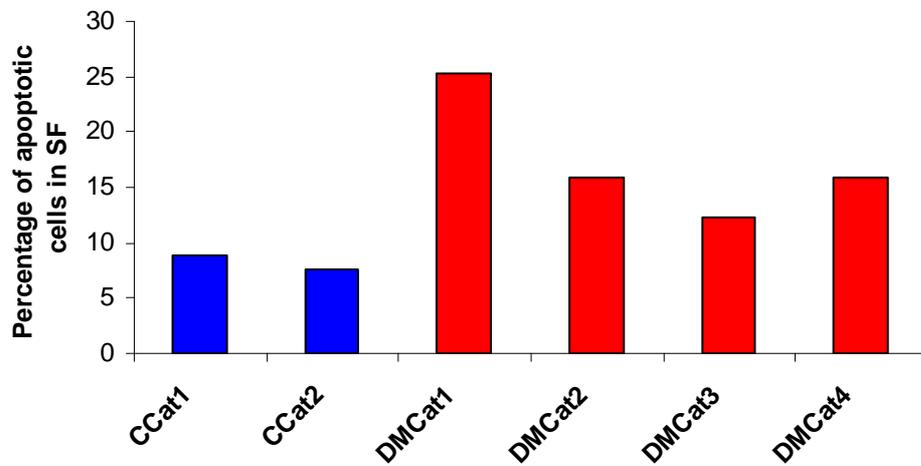


Figure 3.14 – Apoptotic cell death in DM1 lens epithelial cells cultured in SF medium. (a) Fluorescent micrographs of cells showing nuclei labelled with DAPI (blue), apoptotic cells labelled using a TUNEL assay (green) and F-actin labelled with Texas red-X phalloidin (red) (bar = 100 μ m). (b) Analysis of TUNEL positive apoptotic cells (n = 2, data expressed as mean of two independent experiments).

Although there is an increase in DM1 cell death via apoptosis in SF medium, the variability in the cell lines prevented this from being statistically significant. Therefore, further repeats were conducted using only the CCat1 and DMCat1 cell lines (see figure 3.15). CCat1 was chosen as it was typical of the control cell lines, showing even and consistent growth throughout the culture period. The DMCat1 cell line was chosen because although it had a significantly reduced growth rate compared to the control cell lines, it could be sustained over a greater number of passages than the other DM1 cell lines, enabling more experiments to be performed (see figure 3.9). In SF medium, DMCat1 had significantly greater levels of apoptotic cell death with an average of $21.1\% \pm 2.1\%$ apoptotic cells compared to only $8.8\% \pm 0.9\%$ in CCat1 ($p < 0.01$). In medium supplemented with 10% FCS, DMCat1 cells had greater levels of apoptotic cell death, as CCat1 had an average of $7.1\% \pm 2.1\%$ apoptotic cells, compared to $11.4\% \pm 1.4\%$ apoptotic cells in DMCat1, however, the difference

was not statistically significant. In CCat1 cells, no difference was observed in levels of apoptosis in either SF medium or medium supplemented with 10% FCS. However, in DMCat1 cells a significant increase in levels of apoptotic cell death was observed in cells cultured in SF medium compared to medium supplemented with 10% FCS ($p < 0.01$). Interestingly, this data suggests that the DM1 cells do not respond to stress, such as that observed during serum starvation, as well as the controls cells do, resulting in greater levels of apoptosis. A lack of serum, and therefore added growth factors, in the medium would require autocrine signalling in the cells in order to sustain growth. This data could therefore indicate an impairment in autocrine signalling in the DM1 cells.

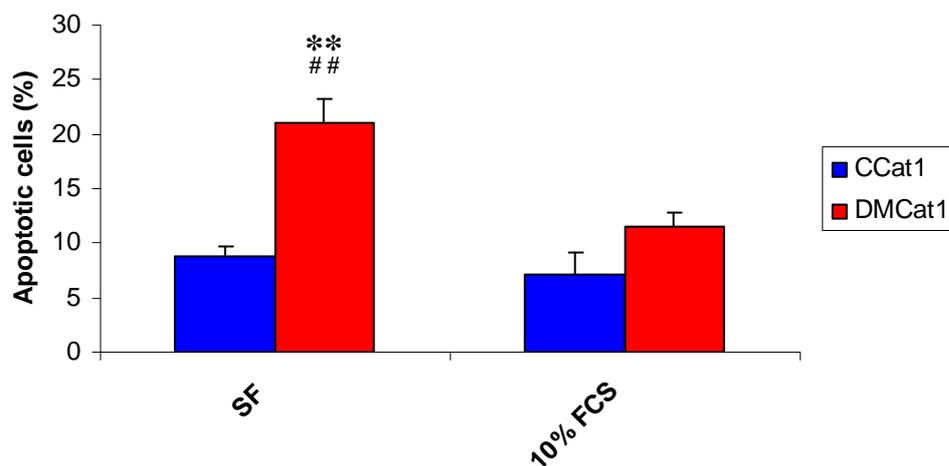


Figure 3.15 – Apoptotic cell death in DM1 lens epithelial cells. Semiquantitative analysis of TUNEL positive apoptotic CCat1 and DMCat1 cells cultured in SF medium and medium supplemented with 10% FCS. Although apoptotic cell death was greater in DMCat1 cells cultured in medium supplemented with 10% FCS than CCat1 cells, this was not significant, however in cells cultured in SF medium apoptotic cell death was significantly higher in DMCat1 cells compared to CCat1 cells. Apoptotic cell death was also significantly higher in DMCat1 cells cultured in SF medium compared to those cultured in medium supplemented with 10% FCS (n = 6, data expressed as mean ± SEM of six independent experiments, statistical analysis was performed by Student’s T test, ** p < 0.01 relative to CCat1 in SF medium, ## p < 0.01 relative to DMCat1 in medium supplemented with 10% FCS).

3.4 Discussion

Many studies have investigated the role of *SIX5* in the lens and have linked a downregulation in expression to cataract formation in DM1, adding weight to the theory that the triplet repeat causes changes to the local chromatin structure which results in downregulation of the downstream gene (Winchester *et al.*, 1999; Rhodes *et al.*, 2006). Two separate studies using *SIX5* knockout mouse models have reported cataract formation in the lens, which appeared to confirm the theory, however, the cataracts seen in the mouse models did not show the distinctive characteristics observed in human DM1 patients (Klesert *et al.*, 2000; Sarkar *et al.*, 2000). The iridescent particles were not present and the opacity was located within the nucleus of the lens as opposed to the posterior subcapsular area where it forms in humans. Cataracts with iridescent opacities have been shown to form in a mouse model for DM1, however, it was the result of a *Mbnl1* gene knockout and not *Six5*. In this study we have presented evidence that the level of *SIX5* expression in DM1 lens cells is not affected by the triplet repeat (see figure 3.2) and based on this data, we propose that although a reduction in *SIX5* expression can lead to cataract formation, it is probably not responsible for the formation of DM1 cataracts.

Previous reports investigating the expression of *DMPK* in the human lens have been contradictory (Dunne *et al.*, 1996; Winchester *et al.*, 1999; Rhodes *et al.*, 2006). Here, in support of the studies by Dunne *et al.* (1996) and Rhodes *et al.* (2006), we have shown by QRT-PCR, Northern blotting and FISH that *DMPK* mRNA is present in human LECs (see figures 3.3 and 3.4). The levels of *DMPK* mRNA analysed by QRT-PCR and Northern blotting showed that the triplet repeat had no effect on *DMPK* expression. Surprisingly, the technique of Northern blotting failed to detect the *DMPK* transcripts from the mutant allele, as no larger band or smear representing transcripts containing expanded triplet repeats was seen. The lack of this band is likely due to problems in extracting larger RNAs as the mutant transcripts are possibly subjected to increased levels of mechanical damage, resulting in shearing of the mutant RNAs, or it could indicate inefficient transfer of the larger RNAs to the membrane. However, the mutant allele was shown to be expressed in the DM1 LECs using the technique of FISH to detect the CUG repeats in the *DMPK* mRNA (see figure 3.4). We have shown that

DMPK transcripts form foci in DM1 LECs which are trapped within the nucleus. This is a significant finding because the formation of nuclear foci of *DMPK* transcripts is a common feature in cells affected by DM1 and is linked to the sequestration of MBNL proteins and the increased activation of CUG-BPs which are involved in the regulation of splicing events (Timchenko *et al.*, 2001a; Fardaei *et al.*, 2002). Changes in the levels of MBNLs and CUG-BPs results in the production of proteins with different biological properties due to disruption in the regulation of splicing events. The production of alternatively spliced proteins has been shown to be the cause of many of the symptoms associated with DM1 and could therefore also be the cause of cataract formation in the lens.

The expression of two members of the muscleblind family, *MNBL1* and *MBNL2*, were analysed in this study. We found expression of both genes in all of the cell lines and the expression levels were not found to be affected by DM1 (see figure 3.5). The expression of two members of the CELF family, *CUG-BP1* and *CUG-BP2*, were also analysed. Again, we found expression of both genes in all cell lines (albeit very low for *CUG-BP2* in CCat2) and the levels of expression were not affected by DM1 (see figure 3.6). The expression levels of the genes above are not expected to be altered by DM1, but the levels of active proteins could be; MBNL proteins are sequestered by the CUG repeats and activation of CUG-BPs is increased. However, the expression of these splicing regulators in human lens cells could indicate a role for alternative splicing events leading to cataract formation in DM1 lenses.

To investigate the role of alternative splicing in cataract development, we looked at some of the proteins known to be affected by alternative splicing events in DM1 and analysed their expression in human lens epithelial cells. We found that expression of *CLCN1* was absent in the lens cell lines and *RYR1* was only expressed in a few of the cells lines at very low levels and therefore altered splicing of the protein products CIC-1 and RYR1 is unlikely to cause cataract formation. However, the human lens cells were shown to express *IR* and both of the alternatively spliced isoforms were shown to be present (see figures 3.7 and 3.8). The ratio of *IR* isoforms in DM1 skeletal muscle was shown to shift towards the production of *IR-A*, which results in decreased insulin sensitivity (Savkur *et al.*, 2001). *IR* has been found in the epithelial layer of human lenses and is interestingly absent in lenses of diabetic cataract patients, although this was

suggested to be due to lens degeneration caused by cataract formation (Naeser, 1997). Insulin has been shown to increase the rate of crystallin production in the rat lens and therefore a reduced response to insulin signalling by production of IR-A instead of IR-B could result in cataract formation (Leenders *et al.*, 1997; Civil *et al.*, 2000). The ratio of IR isoforms in the human lens cell lines was analysed by separating PCR products of the two forms of IR on agarose gels (see figure 3.8). Although production of IR-A was shown to be favoured in the DM1 cell lines with an average of only 19.7% IR-B, it was not significantly different from the control cell line average of 29.8%. This was due to the large spread of the data from the control cell lines, with two showing an almost equal expression of IR-A compared to IR-B (CCat1 and CCat2) and the other two showing a greater bias towards IR-A expression than most of the DM1 cell lines (CCat3 and CCat4). Therefore, the splicing pattern of IR was not shown to be affected in the DM1 LECs. From our data it appears that splicing patterns of proteins in DM1 LECs are not altered, however, we did not examine the expression of these proteins in lens fibre cells where cataract forms and there are many proteins affected by mis-splicing in DM1 that we have not investigated. Therefore we cannot completely rule out a role for alternative splicing in cataract formation in DM1.

As no molecular cause underlying cataract formation has so far been elucidated, we looked at the cellular effects which could result in their development. Previous studies on cataracts in general have indicated that a loss of cells from the lens epithelium results in the development of cataract, and in particular subcapsular cataracts, due to the resulting loss of homeostasis in levels of ions and water (Konofsky *et al.*, 1987; Li *et al.*, 1995). A study by Abe *et al.* (1999) investigated the density of the LEC layer in native human lenses from DM1 patients and showed that DM1 lenses contained significantly fewer cells in the LEC layer than those from age matched control lenses with senile cataract. Despite the decrease in cell density, coverage of the lens was maintained by an increase in cell size (Abe *et al.*, 1999). This data would suggest that a reduced number of LECs may not be sufficient to maintain the balance of ions and water which is vital to lens transparency, therefore resulting in cataract formation in DM1. Here, we have shown that DM1 LECs had longer population doubling times compared to controls, which confirms the data presented by Rhodes *et al.* (2006) (see figure 3.9). We showed that the growth rate of DM1 LECs was

significantly reduced compared to control LECs and a point was reached where DM1 cell populations began to slow and decline, which was not observed in controls. DM1 LECs were therefore shown to have a significantly shorter lifespan than control LECs. Li *et al.* (1995) showed that capsulorhexis samples from non-DM cataract patients had a significantly greater percentage of apoptotic cells compared to normal controls and that apoptotic cell death occurred prior to cataract development. We therefore investigated the role of apoptotic cell death in the reduced population doubling times in DM1 LECs. We showed that under normal culture conditions there was no difference in levels of apoptotic cell death (see figures 3.13 and 3.15), however, if the cells were cultured in SF medium an increase in apoptosis was observed in the DM1 cells but not the controls (see figures 3.14 and 3.15). This is very interesting as it indicates that DM1 LECs are less able to withstand stress, such as serum deprivation, than normal LECs.

Interestingly, increased susceptibility to stress has been shown in cell lines transfected with CTG repeats. Studies on mouse myoblasts transfected with *DMPK* containing varying numbers of CTG repeats, showed CTG repeat number-dependent susceptibility to oxidative stress, resulting in apoptosis (Usuki *et al.*, 2000). Subsequently, it was shown that greater levels of reactive oxygen species (ROS) form in cells with larger repeats (160 repeats) and that these cells activate the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathways as opposed to the extracellular signal-related protein kinase (ERK) pathway which was activated in cells transfected with very short repeats (5 repeats) (Usuki *et al.*, 2008). This data is particularly relevant to lens cells as the lens is subject to oxidative stress from radiation (e.g. UV) and other sources and the production of ROS is a major contributor to age-related cataract formation (Berthoud & Beyer, 2009).

As DM1 LECs show a greatly reduced ability to maintain their own survival in the absence of added growth factors, this could indicate an impairment in autocrine signalling. As the lens is not innervated, vascularised or penetrated by lymphatic vessels, LECs in their native environment are not likely to receive many external signals in order to encourage growth or survival (Ishizaki *et al.*, 1993). Rat LECs have been shown to survive in culture, and even to divide, in the absence of signals from other cell types, but only when cultured at high densities. At low densities the cells undergo apoptosis, which suggests that they rely on

other LECs for their survival (Ishizaki *et al.*, 1993). Human LECs have also been shown to survive in protein-free medium when cultured on the lens capsule (although the lens capsule has subsequently been shown to contain growth factors which are available to the LECs (Tholozan *et al.*, 2007)) and proliferate and migrate as occurs *in vivo* (Wormstone *et al.*, 1997). Lens cells are one of the few cell types which are capable of surviving and maintaining their phenotype in the absence of external signals. Chondrocytes are also capable of this, however, they do not actively proliferate as is observed in LECs (Ishizaki *et al.*, 1994). Therefore, lens cells should be able to actively maintain their own growth and survival in SF medium, but the DM1 LECs have been shown to undergo significantly increased levels of programmed cell death. An impairment in autocrine signalling may result in increased levels of apoptosis, either due to the cells not releasing enough of their own signalling factors or an impairment in the way that they respond to the signalling factors which they produce. It is possible that, as is seen in mouse myoblasts transfected with *DMPK* containing large triplet repeats, the DM1 lens cells are activating different pathways in response to stress than the controls cells, resulting in increased levels of cell death (Usuki *et al.*, 2008).

From the data described in this chapter, it appears that the DM1 lens epithelial cell lines are a good model in which to study the disease. Expression of *SIX5* was not shown to be affected by the triplet repeat, which means the focus can be placed on *DMPK* expression and the downstream affects of mutant mRNA production. The lens cells have been shown to express *DMPK*, which is also seen in normal human lenses (Dunne *et al.*, 1996; Rhodes *et al.*, 2006). The mutant *DMPK* transcripts were also shown to form distinct nuclear foci which is a common feature in other DM1 cell lines and patient samples (Taneja *et al.*, 1995; Davis *et al.*, 1997; Hamshere *et al.*, 1997; Furling *et al.*, 2001b; Liquori *et al.*, 2001; Jiang *et al.*, 2004; Mankodi *et al.*, 2005; Bonifazi *et al.*, 2006; Wheeler *et al.*, 2007a; Cardani *et al.*, 2008). Although the characteristic splicing defects observed in other DM1 cell types have not been found in our cell lines, it is not known to what extent mis-splicing plays a role in the native human lens in DM1. In DM1 fibroblasts, *DMPK* transcripts have been shown to produce foci which sequester MBNL proteins in the nuclei. However, a further study showed that aberrant IR splicing did not occur in DM1 fibroblasts until they were transformed to a muscle phenotype, when they then showed a switch towards the IR-A

isoforms (Fardaei *et al.*, 2001; Savkur *et al.*, 2001; Fardaei *et al.*, 2002). Therefore, it is possible that foci may normally be present in some cell types without observing any impact on splicing patterns. Despite the lack of an observed splicing defect to describe the mechanism behind cataract development in the lens, the presence of mutant *DMPK* mRNA in LECs indicates that it is likely to play a role in DM1 cataract formation. DM1 LECs were shown to have significantly reduced population doubling times and serum deprivation was shown to result in significantly increased levels of apoptotic cell death. This could indicate an impairment in autocrine signalling which requires further investigation.

CHAPTER 4

AUTOCRINE SIGNALLING IN DM1 LENS

EPITHELIAL CELLS

4.1 Introduction

Cells communicate and bring about changes to their behaviour through cell signalling. Decisions made by a cell are the result of signalling pathways which are often complex and consist of signalling molecules, receptors, kinases, phosphatases, target proteins and more (Alberts *et al.*, 2002). Cells communicate with each other through the release of signalling molecules, such as growth factors and hormones, which can activate receptors on the surface of other cells. Cells can communicate to other cell types over long distances; known as endocrine signalling, or short distances; known as paracrine signalling. Cells can also signal to other cells of the same type, or themselves, over short distances; known as autocrine signalling. The activation of receptors triggers a cascade of signalling within a cell which can bring about changes in a variety of processes such as cell motility, proliferation, growth, survival and death. A lack of cellular signalling can also have serious effects on a cell as a certain level of signalling is required for survival. Cells that do not receive the level of signalling required undergo apoptotic cell death. Cell signalling is therefore tightly controlled, however, disturbances in cell signalling pathways can occur and could lead to many different diseases ranging from cancer to neurological disorders.

4.1.1 Cell signalling

In order for cells to respond to the signals they receive, they require receptors on their cell surface which can be activated by the signal molecule, which is also known as a ligand. The three major types of cell surface receptor are ion-channel-linked receptors, which are activated by neurotransmitters to alter ion permeability in electrically excitable cells; G protein-linked receptors, which regulate the activity of target enzymes or ion channels via a guanosine triphosphate (GTP) binding protein (G protein); and enzyme-linked receptors,

which when activated function as enzymes, or are linked to enzymes, commonly protein kinases (Alberts *et al.*, 2002). The enzyme-linked receptors respond to ligands known as growth factors, which are linked to signalling involved in the growth, proliferation, differentiation and survival of cells. The largest class of enzyme-linked receptors are the receptor tyrosine kinases (RTKs) (Alberts *et al.*, 2002). Binding of growth factors to their respective RTK usually results in a conformational change which leads to phosphorylation of tyrosine residues within the kinase domain of the receptor, resulting in its activation. This triggers the activation of a cascade of molecules involved in the transduction of the signal within the cell, resulting in a specific biological response.

4.1.2 Cell signalling in the lens

A number of different growth factors and their RTKs are important for cell signalling within the lens. These include epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF, or FGF-1), basic fibroblast growth factor (bFGF, or FGF-2), hepatocyte growth factor (HGF), insulin-like growth factor (IGF-1) and platelet-derived growth factor (PDGF) (Bhuyan *et al.*, 2000; Wormstone *et al.*, 2000). The growth factors which act upon the lens are involved in signalling which results in the maintenance of growth, proliferation and survival of the lens cells, as well as regulating the processes of differentiation and migration.

4.1.2.1 Paracrine signalling in the lens

Many ocular tissues secrete growth factors into the aqueous and vitreous humours of the eye. Paracrine signalling from these ocular tissues has been shown to be important in processes such as proliferation, migration and differentiation of LECs. PDGF was found to be expressed by the ciliary body and iris and its receptor PDGFR was expressed in LECs (Kok *et al.*, 2002). Studies have suggested that PDGF plays a role in proliferation, migration and differentiation in LECs (Reneker & Overbeek, 1996; Kok *et al.*, 2002; Xiong *et al.*, 2010). Growth factors found to be present within the aqueous and vitreous humours, include IGF-1 which was found in both the aqueous and vitreous humours from bovine eyes, with levels in the aqueous being twice that found in the vitreous (Arnold *et al.*, 1993). The growth factor EGF was also found to be present in the aqueous humour, but was not found in the vitreous humour (Majima, 1997). bFGF was

present in bovine aqueous and vitreous humours, with levels in the vitreous being greater than that found in the aqueous, however, aFGF was only present in the vitreous (Schulz *et al.*, 1993).

The aqueous bathes the anterior of the lens which contains the LECs and the vitreous bathes the posterior of the lens which contains the fibre cells, therefore, the distribution of growth factors is likely to affect cellular responses and the polarity and architecture of the lens. For example, bovine vitreous humour, which contains high levels of bFGF, was shown to induce fibre differentiation in rat lens epithelial explants but aqueous humour did not (Schulz *et al.*, 1993). Different levels of bFGF have been shown to have different effects on rat lens epithelial explants. Proliferation, migration and differentiation have been shown to be induced as concentrations of bFGF are increased, with half maximal responses for each at 0.15, 3 and 40 ng/ml respectively (McAvoy & Chamberlain, 1989). The distribution of bFGF in the aqueous and vitreous humours results in increasing concentrations of bFGF from the anterior to the posterior of the lens and is therefore likely to play a major role in determining the spatial patterns of proliferation, migration and differentiation (Lovicu & McAvoy, 1993).

Interestingly, lens cells have been shown to survive in culture in the absence of paracrine signalling factors, indicating that autocrine signalling plays an important role in their survival (Ishizaki *et al.*, 1993).

4.1.2.2 Autocrine signalling in the lens

Although many growth factors are present within the aqueous and vitreous humours, the lens has also been shown to synthesise many of its own growth factors which act in an autocrine manner. The lens contains just two cell types (epithelial cells and fibre cells) and is not penetrated by blood vessels, lymphatic vessels or nerves. It is therefore not surprising that lens cells are some of the only cell types that do not require signals from other cells to survive. LECs from young rats have been shown to survive in culture for weeks in the absence of added serum and proteins when cultured both with and without the lens capsule. Not only did the cells survive, but some were also shown to divide during the culture period (Ishizaki *et al.*, 1993). A subsequent study also showed that human LECs could grow in the absence of added serum and proteins, reaching confluency in 95% of cultures (Wormstone *et al.*, 1997). Human LECs cultured on the capsule

could be maintained in the absence of added protein and serum for over a year (Wormstone *et al.*, 2001). Although the lens is surrounded by the aqueous and vitreous humours which contain growth factors that are available to the lens, this data shows that these are not necessary for LEC survival or proliferation. Autocrine signalling must therefore play an important role in LEC survival during culture. Interestingly, rat LECs only survived under protein- and serum-free conditions when cultured at high densities. When cultured at low densities the LECs underwent apoptotic cell death (Ishizaki *et al.*, 1993). This data shows that although the LECs do not require signals from other cell types to survive, they do require signals from other LECs and it was also shown that this was not contact dependent, indicating that factors are being released by the cells to promote survival. Survival of low density cultures could be promoted by the addition of conditioned medium (CM) from high density cultures, which raised survival rates to those of the high density cultures (Ishizaki *et al.*, 1993). The factor, or factors, responsible for increasing survival of LECs in culture were not identified, however, addition of many of the growth factors known to act on LECs, for example, IGF-1 and bFGF, did not increase survival, indicating that these were either not responsible or were at too low a concentration to have an effect.

A number of autocrine signalling systems have been identified in the lens. Human LECs cultured on the lens capsule have been shown to synthesise bFGF and the receptor FGFR1 when cultured under protein- and serum-free conditions, establishing an autocrine signalling system. When this system was blocked, a marked attenuation of growth was observed (Wormstone *et al.*, 2001). Autocrine signalling via aFGF has also been shown to be critical to survival in bovine LECs (Renaud *et al.*, 1994). EGF and HGF autocrine signalling systems have also been revealed in both human and rabbit lenses and in cultured LECs (Majima, 1995; Weng *et al.*, 1997; Wormstone *et al.*, 2000). Signalling via EGF and its receptor, EGFR, was linked to proliferation, whilst signalling via HGF and its receptor, c-met, was shown to stimulate proliferation, protein synthesis and migration in cultured human LECs (Majima, 1995; Wormstone *et al.*, 2000). Expression of c-met was found to be upregulated following mechanical trauma, indicating a role for HGF signalling in wound healing (Wormstone *et al.*, 2000). During development, autocrine signalling via IGF-1 and its receptor, IGFR-1, in the chick lens has been shown to be important for the differentiation of fibre cells (Caldes *et*

al., 1991). A role for autocrine signalling via IGF-1 is uncertain in adult lenses as a study in the adult rat lens failed to show expression of IGF-1 (Daniais & Stylianopoulou, 1990). IGFR-1 is expressed in the adult human lens, but the expression of IGF-1 was not examined (Bhuyan *et al.*, 2000). However, cultured bovine LECs were shown to express both IGFR-1 and IGF-1 and signalling via this pathway was shown to regulate migration, proliferation and differentiation (Palmade *et al.*, 1994).

4.1.2.2.1 The EGF signalling pathway

The growth factor EGF binds with high affinity to its receptor EGFR. EGFR is an RTK which consists of an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular tyrosine kinase domain. Binding of EGF ligands results in receptor dimerisation and autophosphorylation of the tyrosine kinase domains. The phosphorylated tyrosine kinase domains recruit substrates and docking proteins which results in the activation of signal transduction pathways (see figure 4.1) (Carpenter & Cohen, 1990; Lurje & Lenz, 2009). Proteins which are recruited include: phosphatidylinositol 3-kinase (PI3K), which activates the PI3K/Akt signalling pathway; growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS) which bind directly or via the adaptor molecule Src homology 2 domain containing protein (Shc), which activate the mitogen-activated protein kinase (MAPK)/extracellular signal related kinase (ERK) cascade; phospholipase C γ (PLC γ), which results in the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP $_3$), which activate protein kinase C (PKC) and Ca $^{2+}$ release respectively; and Janus kinase (JAK), which activates signal transducers and activators of transcription (STAT) pathways (Carpenter & Cohen, 1990; Lurje & Lenz, 2009).

In serum-free conditions EGF has been shown to promote mitosis in the LECs of cultured rabbit lenses, revealing a role in cell proliferation (Reddan & Wilsonziedzic, 1983). EGF has also been shown to promote cell growth in a further study using rabbit LECs (Hongo *et al.*, 1993). In cultured human LECs, EGF has been shown to play a role in cell migration via the MAPK/ERK and PI3K/Akt signalling pathways (Jiang *et al.*, 2006). Growth of human LECs cultured on the lens capsule in serum-free medium has been shown to be reduced

in the presence of an EGFR inhibitor (Maidment *et al.*, 2004). This data indicates that autocrine signalling via EGF is important for growth in LECs.

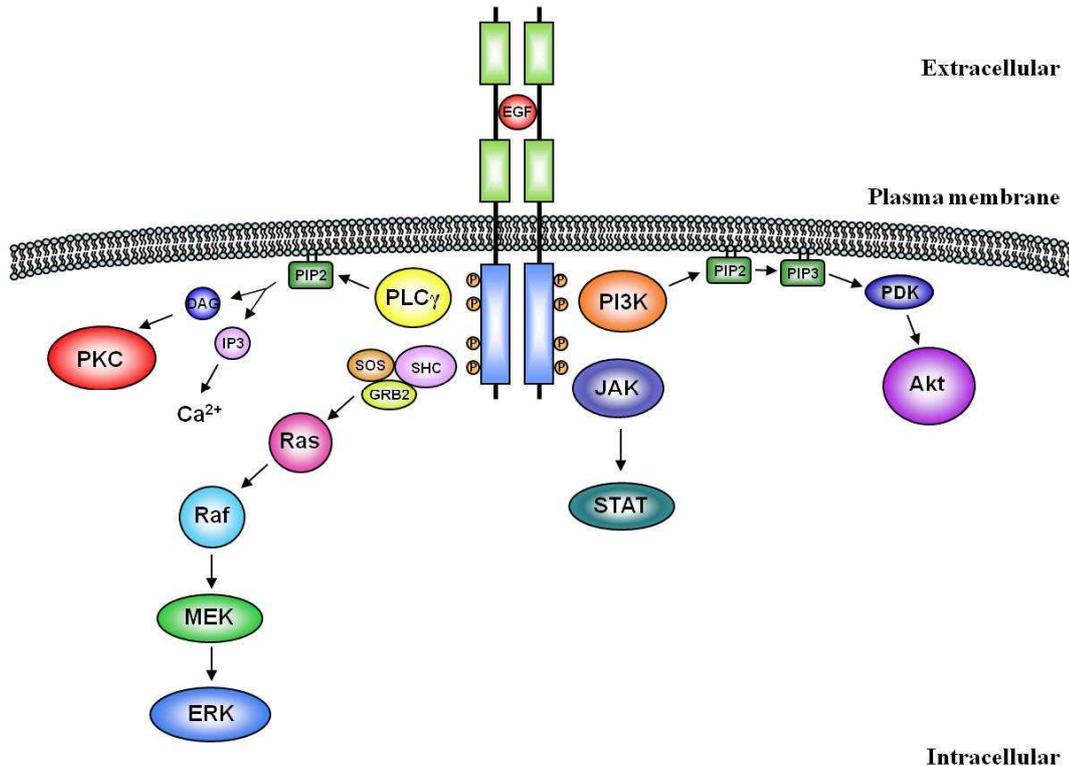


Figure 4.1 – The EGF signalling pathway. The binding of EGF ligands results in dimerisation of EGF receptors and phosphorylation of intracellular tyrosine kinase domains. Various proteins can be recruited, including PI3K which activates the PI3K/Akt pathway; Shc, GRB2 and SOS which activate the MAPK/ERK signalling pathway; PLC γ which results in the activation of PKC and the release of Ca $^{2+}$; and JAK which activates STAT pathways.

4.1.2.2.2 The FGF signalling pathway

The FGFs are a family of 22 small polypeptide growth factors, the majority of which are secreted by cells and bind to heparin or heparan sulphate proteoglycans (HSPG) in the extracellular matrix (ECM) or on the cell surface. Heparin and HSPG regulate FGF binding to their RTKs, known as FGFR1-FGFR4. The receptors contain an extracellular ligand-binding domain composed of three immunoglobulin-like domains, a stretch of acidic residues, known as the

acid box, and a positively charged region which binds to heparin. The receptors also contain a transmembrane domain and an intracellular domain containing a protein tyrosine kinase. Following ligand binding, the receptors dimerise and become activated through phosphorylation of the tyrosine residues within the tyrosine kinase domain (Powers *et al.*, 2000; Eswarakumar *et al.*, 2005). The phosphorylated tyrosine kinase domains recruit various signalling molecules, activating different signalling cascades (see figure 4.2). These include: PI3K, which activates the PI3K/Akt signalling pathway; the docking protein fibroblast growth factor receptor substrate 2 (FRS2), which recruits and activates GRB2 and SOS which activate the MAPK/ERK cascade; and PLC γ , which results in the production of DAG and IP $_3$, which activate PKC and Ca $^{2+}$ release respectively.

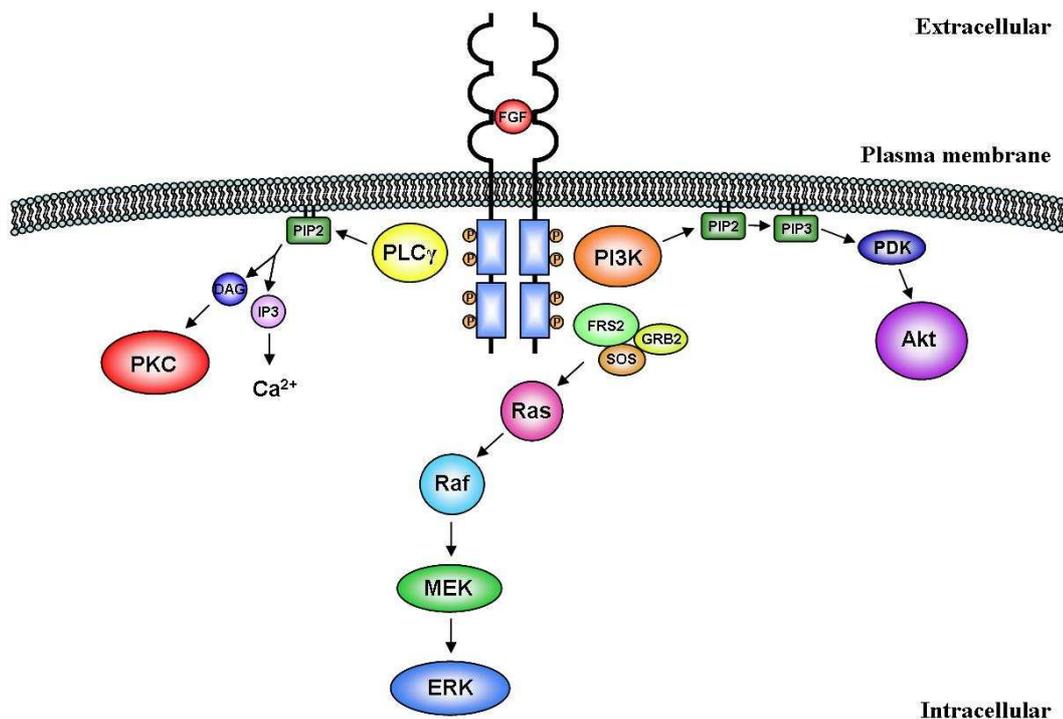


Figure 4.2 – The FGF signalling pathway. The binding of FGF ligands results in dimerisation of FGF receptors and phosphorylation of intracellular tyrosine kinase domains. Various proteins can be recruited, including PI3K which activates the PI3K/Akt pathway, FRS2 which activates the MAPK/ERK signalling pathway and PLC γ which results in the activation of PKC and the release of Ca $^{2+}$.

Neither aFGF or bFGF, which have been shown to be important in lens cell signalling, contain a signal peptide to enable them to be secreted by cells, however, receptors for these growth factors are found in the plasma membrane, indicating that they act extracellularly. Mignatti *et al.* (1991) showed that fibroblast cells produced bFGF and their motility was inhibited by the addition of bFGF antibodies to the culture medium, revealing that bFGF acted extracellularly and in an autocrine manner to promote motility. From this data it was suggested that rather than being secreted by the ER-Golgi system, the growth factors are instead released via an exocytotic pathway (Mignatti *et al.*, 1991). FGF has also been shown to be released from damaged or dead cells (D'Amore, 1990).

Both aFGF and bFGF were found to be expressed and active in the bovine lens and in the surrounding aqueous and vitreous humours (Schulz *et al.*, 1993). In bovine LECs, aFGF is thought to act as a survival factor, being produced at greater levels by serum deprived cells compared to those cultured in medium supplemented with serum. When antisense primers specific to aFGF were added to the serum deprived bovine LECs, cell viability decreased, indicating that autocrine signalling involving aFGF is required for survival (Renaud *et al.*, 1994). The lens capsule has been proposed as a reservoir of growth factors, including bFGF, and release of bFGF from the ECM of the capsule by matrix metalloproteinase 2 (MMP-2) has also been shown to be critical for survival during serum deprivation in a human lens cell line cultured on bovine lens capsules (Tholozan *et al.*, 2007). During serum deprivation, human LECs cultured on their own capsule were shown to synthesise bFGF and the receptor, FGFR1, enabling autocrine signalling via the FGF signalling pathway. When FGFR1 was blocked using a synthetic inhibitor (SU5402) growth rates and the release of bFGF were reduced (Wormstone *et al.*, 2001). In a cultured human lens cell line, bFGF was shown to suppress apoptosis induced by serum deprivation via upregulation of bcl-2 expression (Wang *et al.*, 1999). In rat LECs, bFGF has been shown to induce proliferation, migration and differentiation in a concentration-dependent manner. The half maximal concentrations of bFGF shown to induce proliferation, migration and differentiation were 0.15, 3 and 40 ng/ml respectively (McAvoy & Chamberlain, 1989). bFGF induced proliferation and differentiation were later shown to be dependent on the MAPK/ERK pathway, although a more recent study showed that both the MAPK/ERK and PI3K/Akt signalling pathways

were required for bFGF induced lens fibre differentiation in rat LECs (Lovicu & McAvoy, 2001; Wang *et al.*, 2009b). During differentiation, bFGF has also been shown to induce expression of the lens fibre protein MIP26 in rat lens epithelial explants, which is dependent on the MAPK/ERK and MAPK/JNK pathways (Golestaneh *et al.*, 2004). In porcine lenses, bFGF was also shown to strongly activate the MAPK/ERK pathway and was shown to weakly activate the PI3K/Akt pathway (Zatechka & Lou, 2002a). In human LECs, both aFGF and bFGF increase cell proliferation and differentiation, however, much higher concentrations of aFGF are required to observe effects similar to those seen with lower concentrations of bFGF (Ibaraki *et al.*, 1995). Overall, signalling via the FGF pathway, and in particular via the ligand bFGF, has been shown to be important for differentiation, proliferation, migration and survival of LECs, making it a critical signalling pathway for the function of the lens.

4.1.2.2.3 The HGF signalling pathway

HGF is a disulphide-linked heterodimeric protein which binds with high affinity to its receptor, c-Met. c-Met is an RTK which consists of an extracellular α -subunit and a β -subunit which has an extracellular region, a membrane spanning segment and an intracellular tyrosine kinase domain (Ma *et al.*, 2003). Binding of HGF to c-Met results in phosphorylation of the extracellular domain enabling homodimerisation of receptors and phosphorylation of the intracellular tyrosine kinase domains (Conway *et al.*, 2006). The activated receptor recruits signalling proteins such as: PLC γ , which results in the production of DAG and IP $_3$, which activate PKC and Ca $^{2+}$ release respectively; PI3K, which activates the PI3K/Akt pathway; GRB2 and SOS which activate the MAPK/ERK pathway; and STAT3 (see figure 4.3).

Cultured human and rabbit LECs were shown to express both HGF and c-Met using RT-PCR. Addition of HGF to rabbit LECs resulted in increased levels of proliferation and the expression of crystallin proteins (Weng *et al.*, 1997). LECs from porcine capsular bag models were shown to release HGF and addition of neutralising antibodies to the receptor, c-Met, resulted in a reduction in cell proliferation (Choi *et al.*, 2004). Addition of HGF to cultured human LECs increased cell proliferation which was also confirmed in rat lens epithelial explants (Choi *et al.*, 2004). HGF was detected in human capsular bag models

cultured in serum-free medium and the receptor, c-Met, was also detected in the epithelial cells. Following mechanical trauma the expression of the receptor increased in the cells which grew across the cell free areas, indicating a role in proliferation and/or migration. In the same study, the addition of HGF to cultured human LECs under serum-free conditions was shown to increase proliferation, protein synthesis and migration (Wormstone *et al.*, 2000). HGF was shown to activate pathways that resulted in ERK, JNK and Akt activation which were linked to cyclin D1 expression (Choi *et al.*, 2004). This data indicates that autocrine signalling via HGF plays a role in cell proliferation and also protein synthesis and migration.

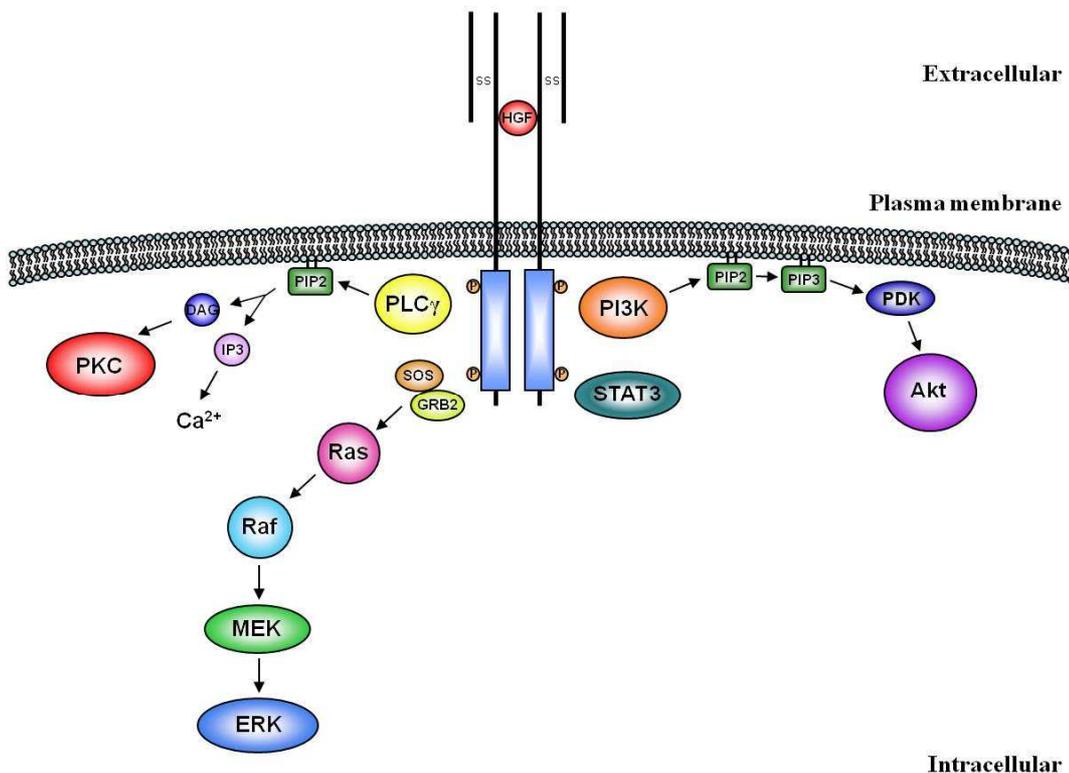


Figure 4.3 – The HGF signalling pathway. The binding of HGF ligands results in dimerisation of c-Met receptors and phosphorylation of intracellular tyrosine kinase domains. Various proteins can be recruited, including PI3K which activates the PI3K/Akt pathway; GRB2 and SOS which activate the MAPK/ERK signalling pathway; PLC γ which results in the activation of PKC and the release of Ca $^{2+}$; and STAT3.

4.1.2.2.4 The IGF-1 signalling pathway

The growth factor IGF-1 is similar to the hormone insulin in structure and function and can bind to both the IGF receptor 1 (IGFR-1) and the insulin receptor (IR), although it has lower affinity for IR. IGFR-1 is an RTK which consists of two extracellular ligand-binding α -subunits and two transmembrane tyrosine kinase domain containing β -subunits which are linked to each other by disulphide bonds. Binding of IGF-1 to IGFR-1 can be regulated by IGF binding proteins (IGFBPs), which either bind to IGF-1 or enhance its ability to bind to the receptor. Ligand binding results in autophosphorylation of the tyrosine kinase domains of the receptor, activating them and enabling the phosphorylation of downstream proteins, such as insulin receptor substrate-1 (IRS-1) and Shc (Pavelic *et al.*, 2007). IRS-1 activates the PI3K/Akt pathway, whereas Shc signals via GRB2 and SOS to activate Ras and the MAPK/ERK pathway (see figure 4.4) (Riedemann & Macaulay, 2006).

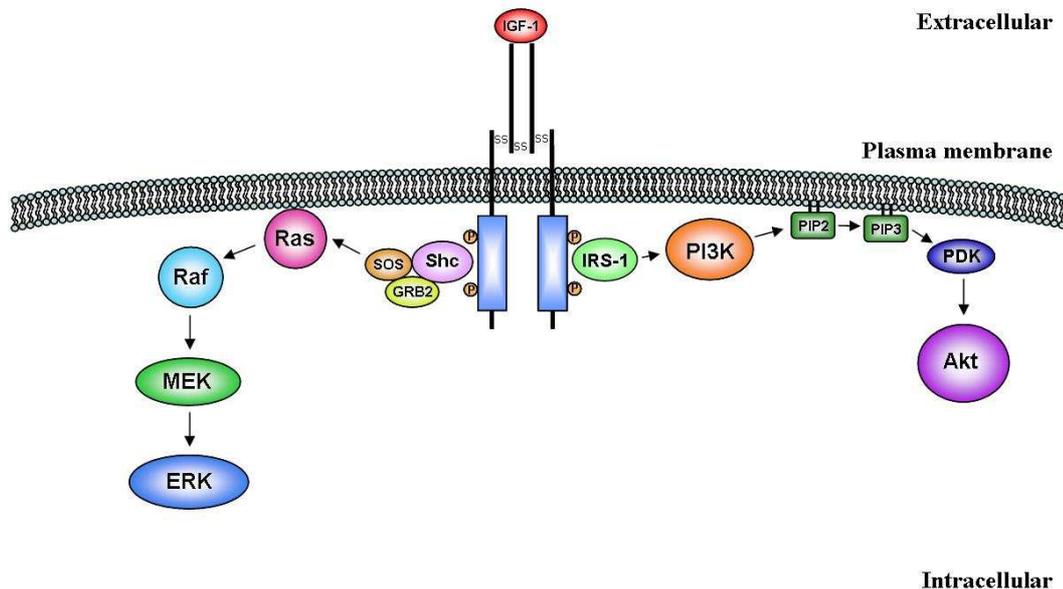


Figure 4.4 – The IGF-1 signalling pathway. The binding of IGF-1 ligands to IGFR-1 causes a conformational change resulting in autophosphorylation of intracellular tyrosine kinase domains. Proteins recruited to the activated receptor include IRS-1 which activates the PI3K/Akt pathway and Shc which recruits GRB2 and SOS which activate the MAPK/ERK signalling pathway.

Expression of *IGF-1* was not detected in the rat lens, however mRNA for *IGFR-1* was found (Burren *et al.*, 1996). IGF-1 was shown to be synthesised and released by bovine LECs and both IGF-1 and IGFBP-2 were found in the aqueous and vitreous humours which surround the lens (Arnold *et al.*, 1993; Palmade *et al.*, 1994). In human LECs, IGFR-1 was found in the cell membrane by Western blotting (Bhuyan *et al.*, 2000).

In bovine, chicken and rabbit lens cells, IGF-1 has been shown to stimulate the PI3K/Akt pathway which was linked to proliferation and differentiation of LECs (Chandrasekher & Bazan, 2000; Chandrasekher & Sailaja, 2003). In rabbit LECs, IGF-1 stimulation of the PI3K/Akt pathway was linked to survival and proliferation, with proliferation being linked to the activation of p70 S6 kinase (p70S6K) found downstream of Akt, however, the p70S6K pathway had no effect on survival (Chandrasekher & Sailaja, 2004). In the porcine lens, IGF-1 has been shown to activate both the MAPK/ERK pathway and PI3K/Akt pathway which was linked to proliferation and survival (Zatechka & Lou, 2002a). In rat LECs, IGF-1 was shown to stimulate proliferation but not differentiation, which required addition of bFGF (Civil *et al.*, 2000). By adding increasing levels of IGF-1 to the rat LECs, IGF-1 was also shown to increase the levels of β - and γ -crystallin in relation to α -crystallin, which could predispose the lens to cataract (Civil *et al.*, 2000). In human LECs, IGF-1 was shown to stimulate cell proliferation and differentiation (Ibaraki *et al.*, 1995). In serum-free conditions IGF-1 has been shown to promote mitosis in the LECs of cultured rabbit lenses, further indicating the role of IGF-1 in cell proliferation (Reddan & Wilsonsdziedzic, 1983). Signalling via the IGF-1 pathway has therefore been shown to influence lens cell proliferation, differentiation and survival which are critical to the function of the lens.

4.1.3 Autocrine signalling in DM1 lens epithelial cells

We have shown that DM1 lens epithelial cells have significantly increased levels of apoptotic cell death compared to controls when cultured in protein- and serum-free medium. We hypothesised that this was due to an impairment in autocrine signalling which promotes survival. This impairment could either be due to the cells not releasing enough autocrine signalling factors, or not responding correctly to those that they do release. As autocrine signalling systems

involving EGF, FGF, HGF and IGF-1 have been identified in the lens, it is possible that an impairment in one or more of these systems could result in the increased levels of apoptotic cell death observed in the DM1 LECs.

4.2 Aims

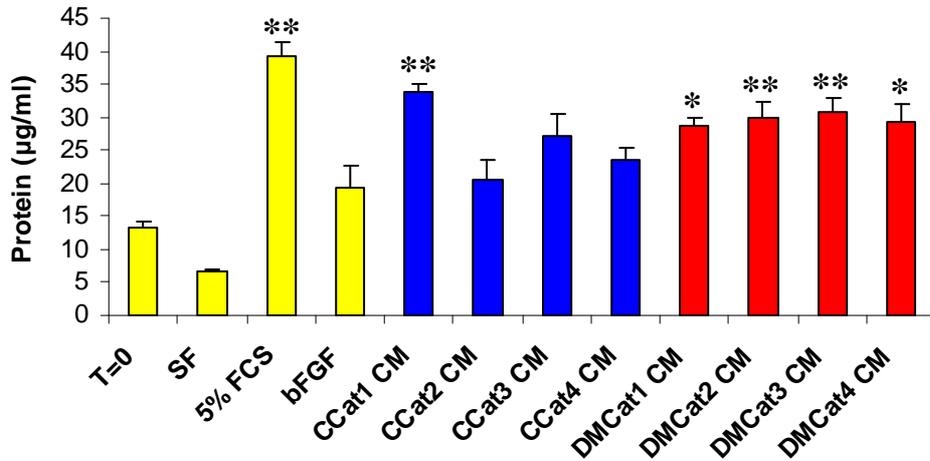
Under stress conditions, such as serum deprivation, autocrine signalling appears to be important for maintaining survival in LECs. Autocrine signalling may be impaired in DM1 resulting in increased levels of cell death. The role of autocrine signalling will be investigated further with particular emphasis on the FGF pathway which is a known autocrine signalling pathway established in the human lens that has previously been shown to increase survival.

4.3 Results

4.3.1 Autocrine signalling in DM1 lens epithelial cells

To investigate the role of autocrine signalling during conditions of serum deprivation, conditioned medium (CM) was collected from the control and DM1 cell lines after 48 hours and was applied to the foetal human lens cell line, FHL124. After 48 hours the cells were lysed and total protein was calculated as a measure of cell growth (see figure 4.5a). Culturing the FHL124 cells in SF medium resulted in a decrease in growth, whereas, a significant increase in growth was observed following addition of 5% FCS ($p < 0.01$). CM from all of the DM1 cell lines also caused a significant increase in growth ($p < 0.05$). Of the control cell lines, however, only CM from CCat1 caused a significant increase in growth ($p < 0.01$). This is interesting because the control cell lines were growing much faster than the DM1 cell lines, resulting in much higher numbers of cells in the flasks from which CM was collected (see table 4.1), therefore, more cells were available to release factors into the medium. For comparison, the growth factor bFGF was also applied to FHL124 cells as it is a known autocrine signalling factor involved in growth and survival in the human lens. Addition of bFGF (5 ng/ml) caused a slight, but non-significant increase in cell growth. The data shows that the cell lines are releasing a factor into the medium which can increase growth and/or survival in the FHL124 cells.

a



b

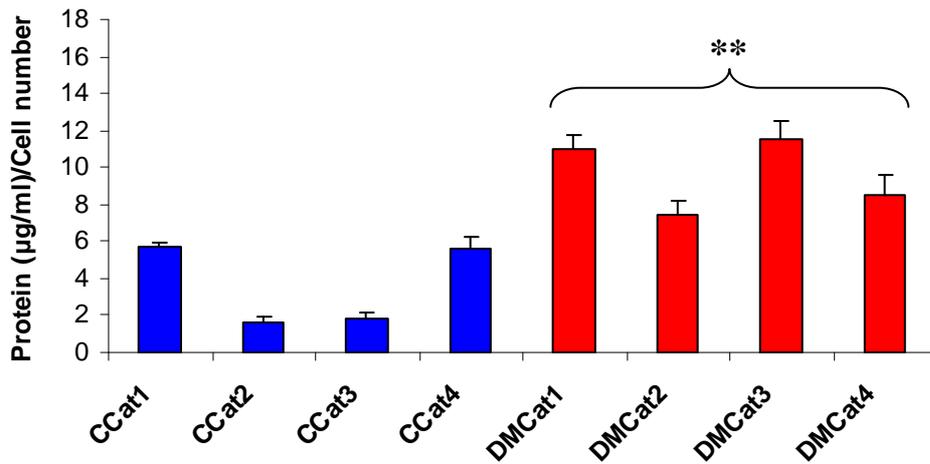


Figure 4.5 – The effect of CM from DM1 lens epithelial cells on FHL124 cell growth. (a) The growth of FHL124 cells was measured by calculating total protein ($\mu\text{g/ml}$) after 48 hours in CM. CM from all of the DM1 cell lines caused a significant increase in growth. Of the control cell lines, however, only CM from CCat1 caused a significant increase in growth ($n = 4$, data expressed as mean \pm SEM, statistical analysis was performed by one-way ANOVA with Tukey's test, * $p < 0.05$ and ** $p < 0.01$ relative to $T = 0$). (b) Growth was normalised to the number of cells from which the CM was collected and reveals that the DM1 cell lines increased growth in FHL124 by a significantly greater amount per cell than the controls ($n = 4$, data expressed as mean \pm SEM, statistical analysis was performed by Student's T test, ** $p < 0.01$ relative to controls).

FHL124 cell growth was also normalised to the number of cells from which the conditioned medium was collected (see table 4.1 and figure 4.5b) and shows that the DM1 cell lines increased growth by a significantly greater amount per cell than the controls. This data suggests that the DM1 cells are releasing greater levels of factors that are responsible for increasing growth in FHL124 cells.

Cell line	Number of cells per flask (millions)
CCat1	4.8
CCat2	8.6
CCat3	11.3
CCat4	3.0
DMCat1	2.0
DMCat2	3.2
DMCat3	2.1
DMCat4	2.7

Table 4.1 – The number of cells per flask from which CM was collected.

4.3.2 Signalling pathways activated by CM in FHL124 cells

To investigate the signalling pathways activated by CM from control and DM1 lens cell lines, CM collected over 24 hours was applied to FHL124 cells and protein was collected after 10 minutes. Levels of activated downstream signalling molecules were examined using Western blotting. The CM from both control and DM1 cell lines activated downstream signalling pathways in FHL124 cells resulting in phosphorylation of Akt and ERK1/2 (see figure 4.6). No significant difference was observed between the levels of activation of either Akt or ERK1/2 following addition of control or DM1 CM.

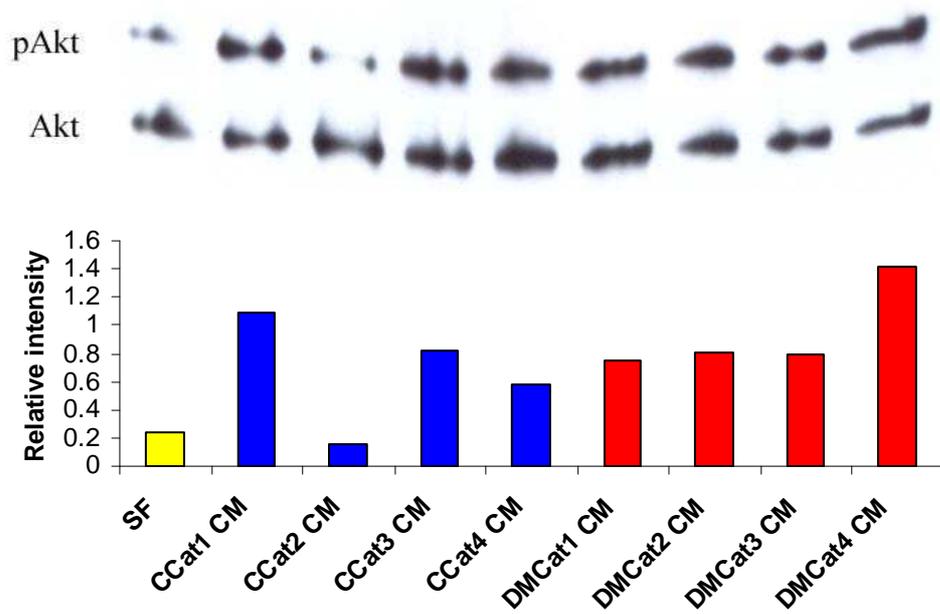
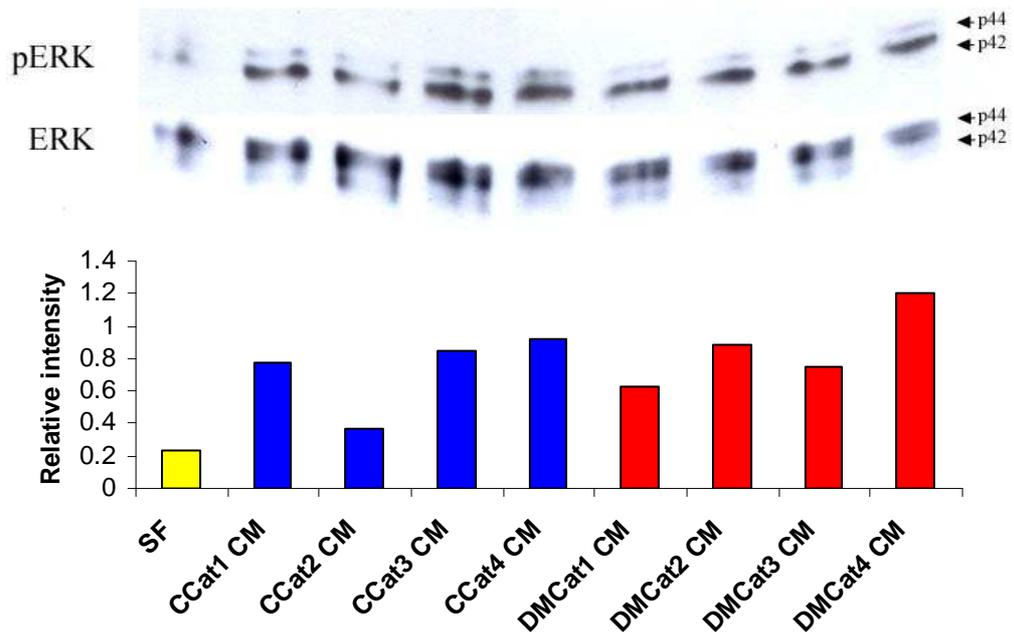
a**b**

Figure 4.6 – Signalling pathways activated in FHL124 cells by CM. Protein levels of (a) pAkt and (b) pERK were examined by Western blotting and analysed densitometrically. Graphs show the activated protein levels normalised to their total protein control. A β actin loading control was included but is not shown. Both Akt and ERK1/2 were activated in FHL124 cells by addition of CM for 10 minutes. No significant difference was observed between control and DM1 CM.

The CM from DM1 cell lines significantly increased growth in FHL124 cells, and it was therefore expected that greater levels of activation of the major pathways involved in growth and survival would have been observed. As this was not found, the CM was diluted to see whether maximal levels of activation had been reached, which were masking any differences that may have been seen. CM was diluted with SF medium to give 100%, 75%, 50%, 25% and 0% CM and was applied to FHL124 cells for 10 minutes. The levels of pAkt, pERK, pJNK and pPKC were examined by Western blotting (data from CCat1 and DMCat1 is shown in figure 4.7).

Levels of pAkt were shown to increase with increasing concentrations of CM, with higher levels seen following addition of CM from DM1 cell lines compared to control cell lines. Akt is activated to a similar level following addition of 100% CM from both CCat1 and DMCat1, however, it is possible that this represents the maximal level of Akt activation in the FHL124 cells. The dilutions of CM reveal that the factor responsible for causing Akt activation is more potent in the DMCat1 CM as the level of activation does not decrease as quickly as is seen with the control CM.

Levels of pERK were also found to increase with increasing concentrations of CM in both control and DM1 cell lines, however, levels of pERK were also very high following addition of 0% CM (SF medium), which could either be due to the stress caused by a change of medium, or a reaction to the lack of growth factors present in the medium. Although levels of pERK were higher following addition of 100% CCat1 CM compared to DMCat1 CM, neither was very different from the level observed following 0% CM addition.

Levels of both pJNK and pPKC appeared to be unaffected by the addition of different concentrations of CM, indicating that no factors capable of stimulating the pathways responsible for their activation are present in CM.

A Western blot performed using the 75% CM samples from each cell line revealed that DM1 CM results in a significant increase in levels of pAkt in FHL124 cells compared to control CM ($p < 0.05$) (see figure 4.8). No significant difference in pERK levels between DM1 and control CM were observed at this dilution (data not shown).

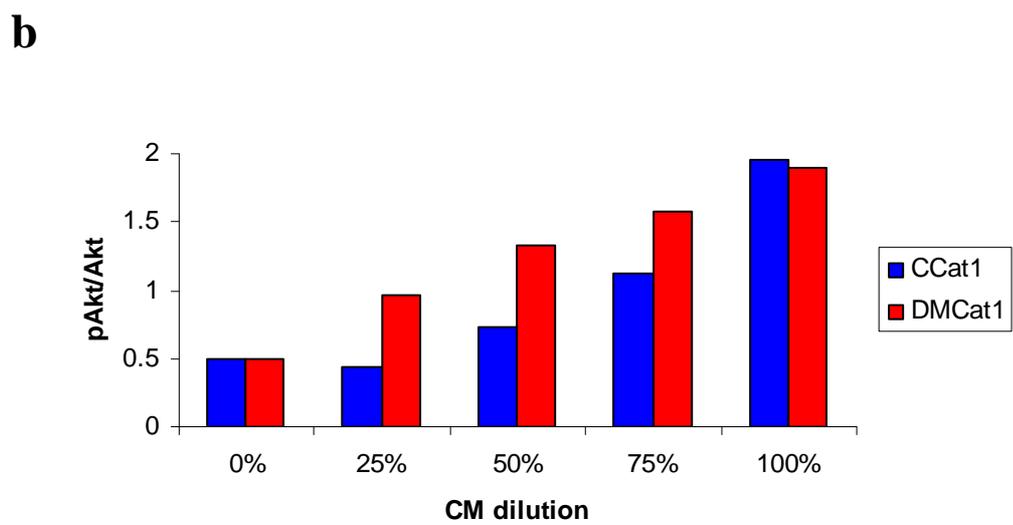
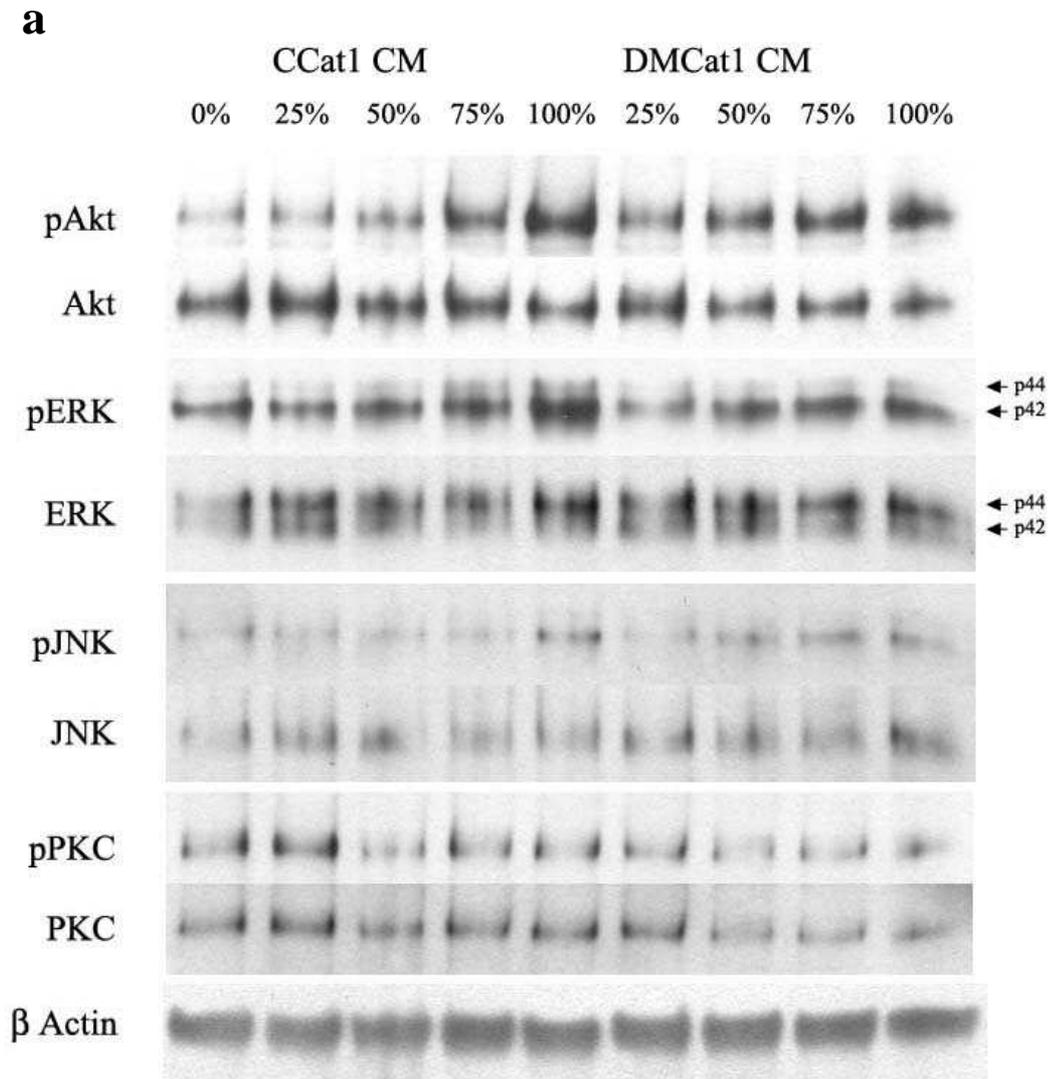
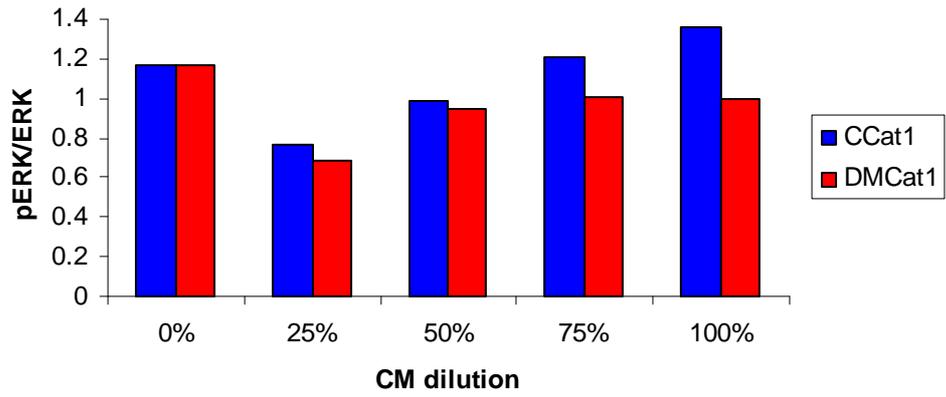
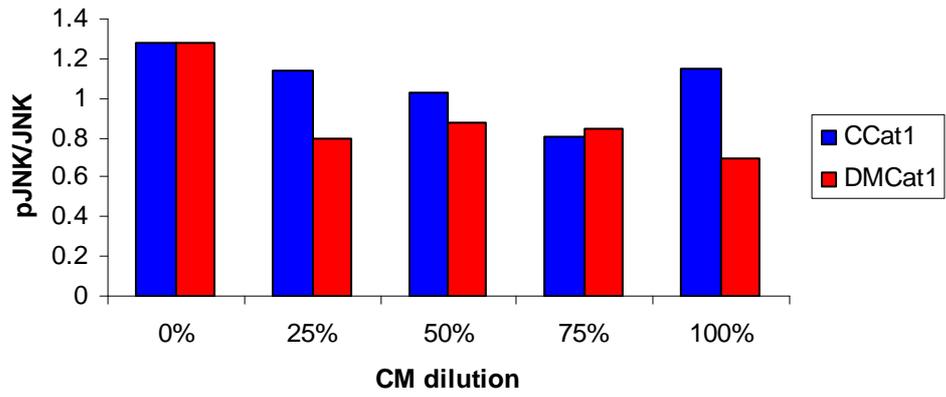


Figure 4.7 – see page 129 for figure legend

c



d



e

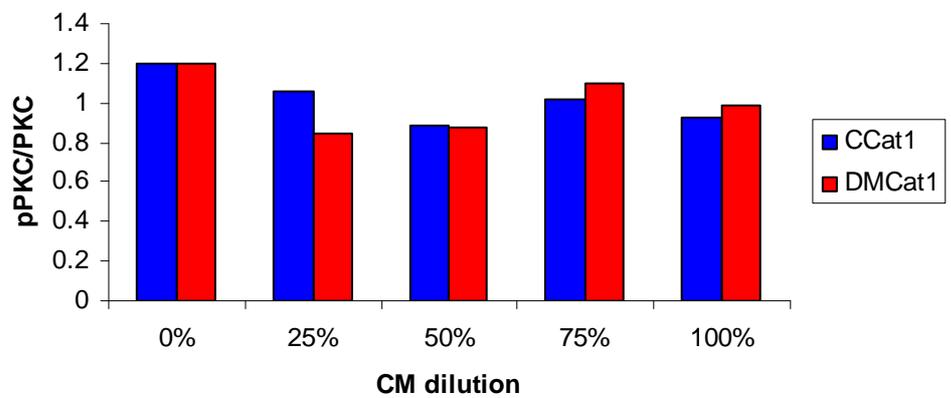


Figure 4.7 - see page 129 for figure legend

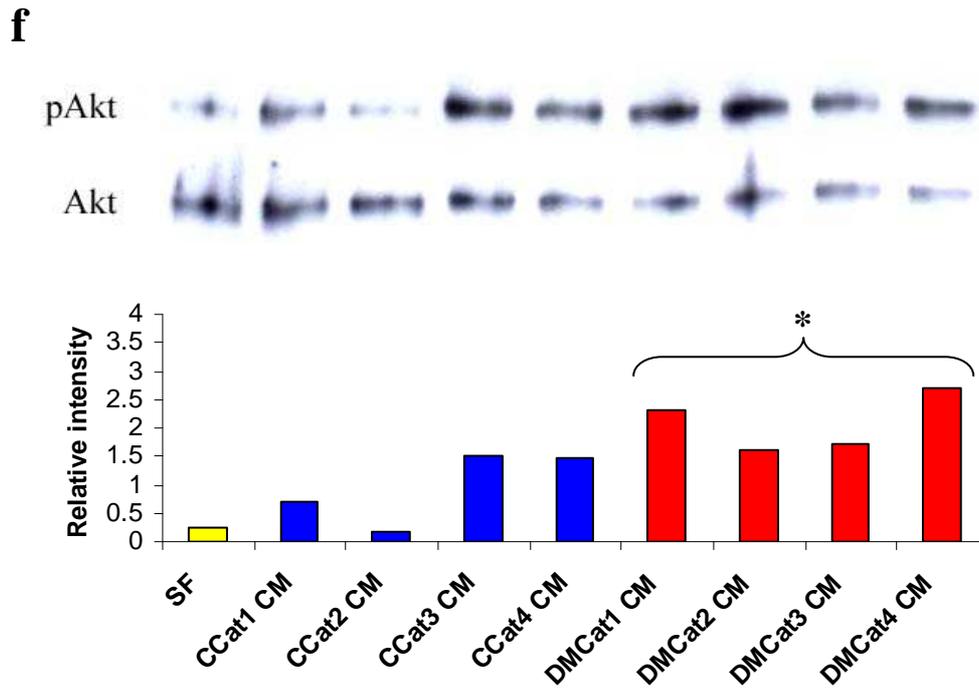


Figure 4.7 – Signalling pathways activated in FHL124 cells by CM. CM from CCat1 and DMCat1 cell lines was diluted with SF medium to give 100%, 75%, 50%, 25% and 0% CM which was applied to FHL124 cells for 10 minutes. (a) Western blotting was used to examine the activation of Akt, ERK1/2, JNK and PKC. Bands were analysed densitometrically and activated protein levels were normalised to their total protein control. Levels of both (b) pAkt and (c) pERK increased with increasing concentrations of CM, however, pERK levels also increased following addition of 0% CM. Levels of pAkt were higher with addition of DMCat1 CM compared to CCat1 CM until a maximal response was reached. Levels of (d) pJNK and (e) pPKC were unaffected by CM. (f) Levels of pAkt were significantly increased following addition of 75% dilutions of DM1 CM compared to control CM (statistical analysis was performed by Student's T test, * $p < 0.05$ relative to control CM).

4.3.3 Autocrine signalling via the FGF pathway in human lens epithelial cells

CM collected from all of the DM1 cell lines significantly increased growth in FHL124 cells, indicating that factors were released by the cells into the medium. These factors are capable of activating the PI3K/Akt pathway which likely leads to the increased levels of cell growth and/or survival. Of the autocrine signalling systems known to be active in the human lens, all can signal via the PI3K/Akt pathway. As the FGF signalling pathway is well characterised in the lens and has been shown to encourage growth and survival in LECs, we investigated its role in

CM stimulated events (Wang *et al.*, 1999; Wormstone *et al.*, 2001; Tholozan *et al.*, 2007).

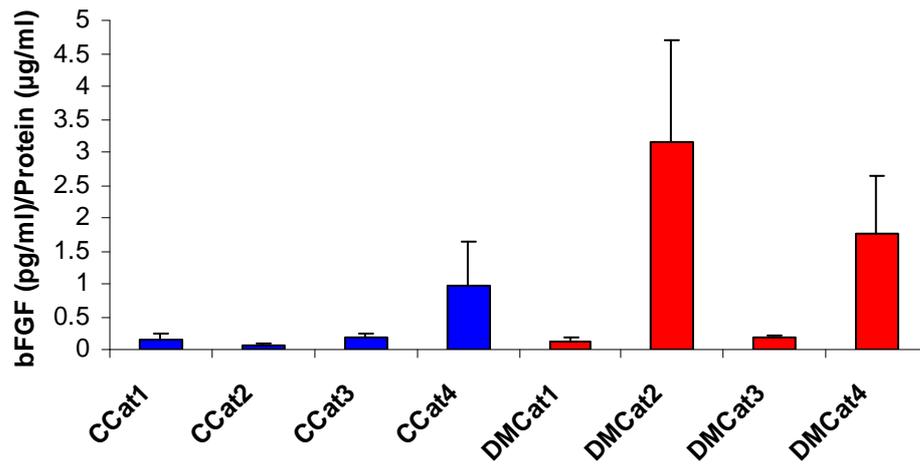
4.3.3.1 Release of bFGF by DM1 lens epithelial cells

CM was collected from cells seeded in culture dishes (after weekly passaging) every 21 days during the culture period shown in figure 3.9, beginning at day 7 and ending on day 70. CM was collected from the culture dishes after 24 hours. Levels of bFGF in the CM were measured using an ELISA. bFGF was detected in CM from each cell line, but was absent from SF medium (see figure 4.8a). The control cell lines released an average of $0.34 \text{ pg/ml} \pm 0.21 \text{ pg/ml}$ bFGF compared to an average of $1.31 \text{ pg/ml} \pm 0.73 \text{ pg/ml}$ bFGF released by the DM1 cell lines. This data confirms that the LECs release bFGF which could increase growth in FHL124 cells, however, the difference in the levels of bFGF released between the control and DM1 lens cells was not statistically significant. Interestingly, the levels of bFGF released by the cells increased over increasing passage numbers, which was especially evident in the DM1 cell lines (see figure 4.8b). Levels of bFGF increased over the culture period in all four DM1 cell lines, whereas levels only increased in two of the four control cell lines, CCat3 and CCat4. The average values for control and DM1 cell lines at each point in culture reveals a 55.3 fold increase in release of bFGF in DM1 cells from day 10 to day 73, compared to only a 10.5 fold increase in the control cells.

As CM from LECs seeded at high densities has been shown to increase survival in cultures seeded at low densities, the effect of cell number on bFGF release was analysed in the cell lines (Ishizaki *et al.*, 1993). Culture flasks were seeded with either 5 000, 10 000 or 20 000 cells per cm^2 of CCat1 or DMCat1 cells. CM was collected from the flasks after 24 hours and levels bFGF were measured using an ELISA (see figure 4.9a). The cells were lysed and total protein from each flask was calculated as a measure of cell density (see figure 4.9b). The level of bFGF in the CM was normalised to the total protein value extracted from each flask and the data shows that there is no effect of cell density on bFGF release in the densities tested (see figure 4.9c). Cells seeded at a density of 5 000 cells per cm^2 released the same level of bFGF per μg of protein as those seeded at 20 000 cells per cm^2 , however, the level of bFGF released by both cell types seeded at a density of 10 000 cells per cm^2 was slightly lower. The average level

of bFGF released per microgram of protein from CCat1 cells was $0.02 \text{ pg/ml} \pm 0.003 \text{ pg/ml}$ compared to $0.08 \text{ pg/ml} \pm 0.007 \text{ pg/ml}$ from DMCat1 cells which was significantly higher ($p < 0.01$, statistical analysis was performed by Student's T-test).

a



b

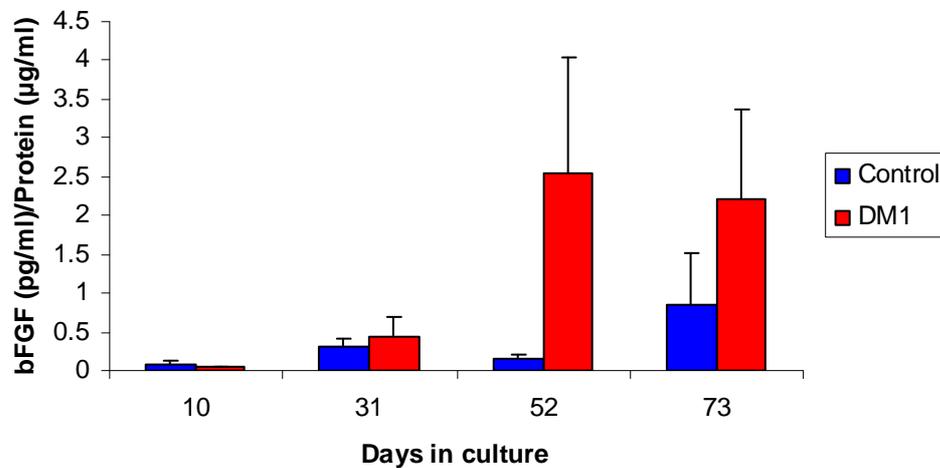


Figure 4.8 – The release of bFGF from DM1 lens epithelial cells. Levels of bFGF in the CM after 24 hours were analysed using an ELISA and normalised to the total protein values extracted from each dish. (a) Both control and DM1 lens cells released bFGF into the culture medium, however, no significant difference was found between DM1 and control lens cells ($n = 4$, data expressed as mean \pm SEM). (b) Levels of bFGF released from the cells increased during the culture period, which was especially evident in the DM1 cell lines. After 73 days in culture, control cells released an average of 10.5 times more bFGF than after 10 days in culture, compared to 55.3 times more in DM1 cells ($n = 4$, data expressed as mean \pm SEM).

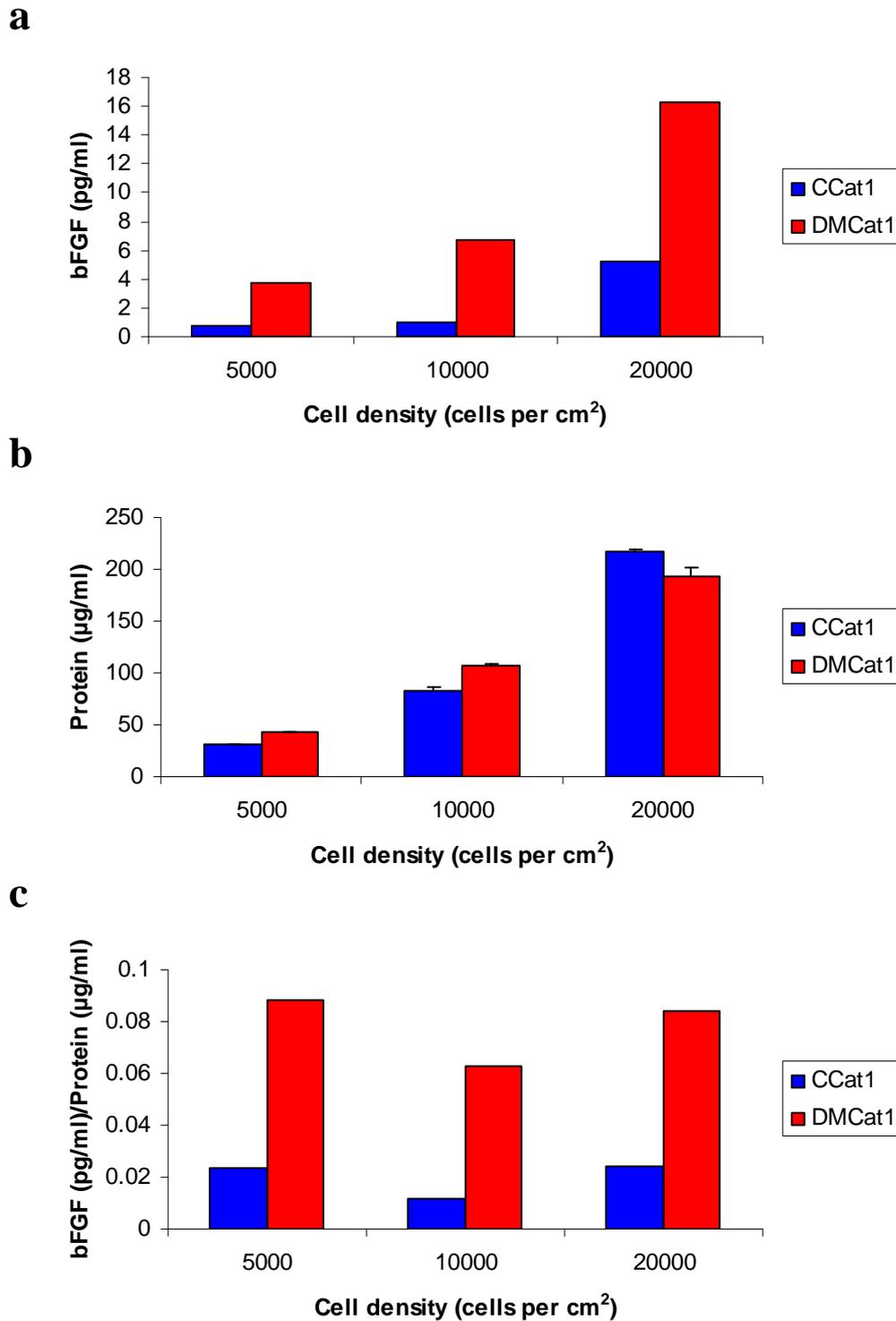


Figure 4.9 – bFGF release is directly proportional to cell density. CM was collected from CCat1 and DMCat1 cells seeded at different densities (5 000, 10 000 and 20 000 cells per cm²). (a) bFGF in CM was measured using an ELISA. (b) Total protein from each flask was calculated as a measure of cell density (n = 3, data expressed as mean ± SEM). (c) bFGF release was normalised to total protein levels revealing that bFGF release is directly proportional to cell density. DMCat1 cells release significantly greater levels of bFGF than CCat1 cells (n = 3, statistical analysis was performed by Student’s T-test, p < 0.01).

4.3.3.2 Expression of FGFR1 in DM1 lens epithelial cells

As all of the cell lines were shown to release bFGF, the presence of the receptor FGFR1 was tested by QRT-PCR analysis of *FGFR1* expression. *FGFR1* was found to be expressed in all of the cell lines, including FHL124 cells (see figure 4.10). This data reveals that autocrine signalling via bFGF is possible in the cell lines and could cause the growth effects observed by the addition of CM to FHL124 cells.

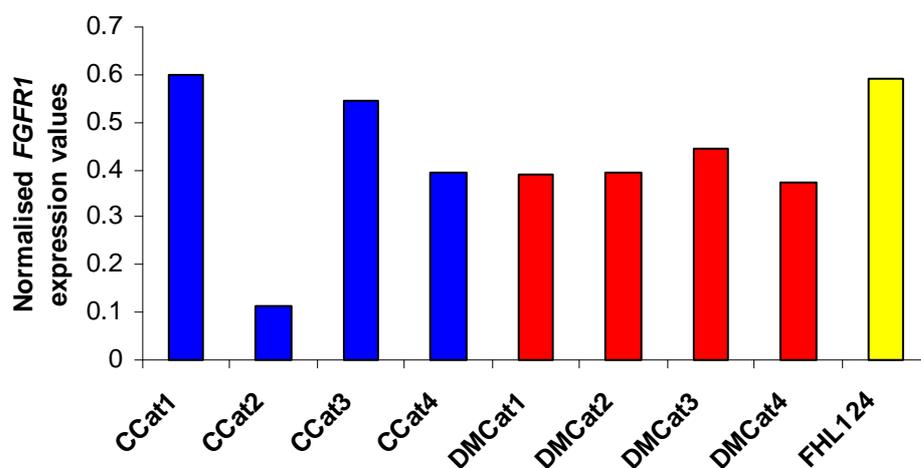


Figure 4.10 – *FGFR1* is expressed in DM1 lens epithelial cells. QRT-PCR showed that *FGFR1* was expressed in both control and DM1 lens cells and was also expressed in FHL124 cells. There was no significant difference observed in expression levels between control and DM1 lens cells (n = 2, data expressed as mean, representative of two independent experiments).

4.3.3.3 FGFR1 inhibition following CM addition in FHL124 cells

To investigate the relationship between bFGF and growth and/or survival in FHL124 cells, CM was applied to the cells in the presence of an inhibitor of FGF signalling, known as SU5402, which blocks the tyrosine kinase activity of FGFR1 (Mohammadi *et al.*, 1997). CM from CCat1 and DMCat1 was applied to FHL124 cells in the presence and absence of SU5402 (10 μ M). To rule out the effects of the solvent dimethyl sulphoxide (DMSO), a DMSO (1 μ l/ml) control

was also applied to the FHL124 cells. After 48 hours, total protein was calculated as a measure of cell growth (see figure 4.11a). CM from both CCat1 and DMCat1 cells stimulated an increase in FHL124 cell growth, however, in the presence of SU5402 (10 μ M), the stimulation from CM was significantly decreased ($p < 0.05$) when compared to the relevant DMSO control. FHL124 cell growth was also reduced in SF medium and medium supplemented with bFGF (5 ng/ml) in the presence of SU5402 compared to the relevant DMSO control, however, this was not statistically significant. The effects of the CM on FHL124 cell growth was not completely blocked following FGFR1 inhibition as cell growth was still above that observed following addition of SF medium in the presence of SU5402. This data indicates that other growth factors are released by the cells which do not act via FGFR1.

The release of LDH into the medium was measured to see whether reduced FHL124 cell growth following FGFR1 inhibition was due to increased cell death or reduced proliferation. The LDH levels were similar in each condition, showing that levels of cell death were not affected by FGFR1 inhibition. This data indicates that the reduction in FHL124 cell growth following FGFR1 inhibition was due to the reduced proliferative capacity of the cells and not due to increased cell death. However, the effects of CM on cell survival with and without FGFR1 inhibition were not analysed due to the presence of LDH already in the CM.

As inhibition of FGF signalling caused a reduction in the proliferative capacity of the FHL124 cells following addition of CM, the signalling pathways activated following SU5402 addition were also examined by Western blotting (see figure 4.12). FHL124 cells were exposed to SF medium, medium supplemented with bFGF (5 ng/ml) and CM from DMCat1 cells in the presence and absence of SU5402 (10 μ M) for 10 minutes before the cells were lysed and protein extracted.

Levels of pAkt did not increase following addition of SF medium to FHL124 cells (see figure 4.12a). Addition of bFGF led to an increase in pAkt levels, however, levels were increased to a much greater extent by the addition of DMCat1 CM. In the presence of SU5402 the levels of pAkt following bFGF addition were reduced, but levels were still elevated following DMCat1 CM addition. This data indicates that stimulation of Akt following DMCat1 CM addition is partially due to signalling via FGF, however, other factors capable of activating the PI3K/Akt pathway must also be present in the CM.

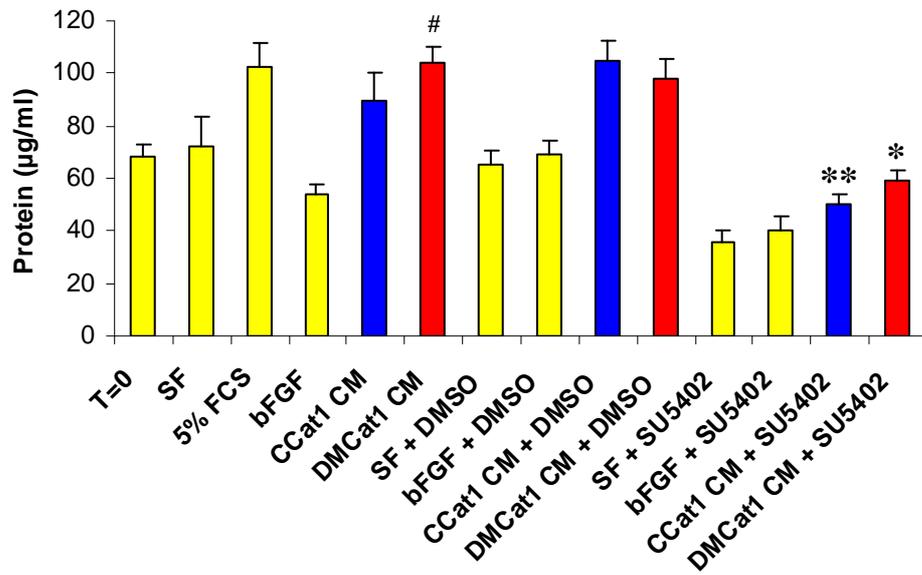
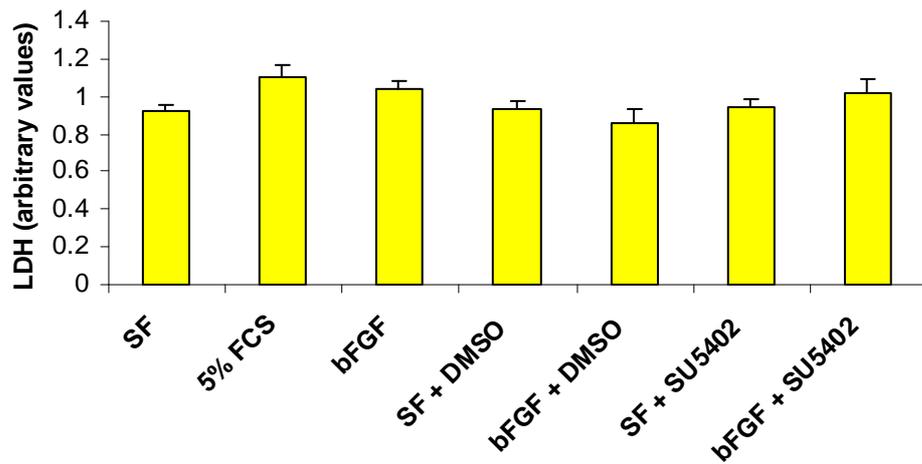
a**b**

Figure 4.11 – The effect of CM from DM1 lens epithelial cells on FHL124 cell growth following FGFR1 inhibition over 48 hours. (a) When SU5402 (10 µM) was added to CM from CCat1 and DMCat1 cell lines FHL124 cell growth was significantly decreased when compared to the relevant DMSO (1 µl/ml) control (n = 4, data expressed as mean ± SEM, statistical analysis was performed by one-way ANOVA with Tukey’s test, * p < 0.05 and ** p < 0.01 relative to the relevant DMSO control, # p < 0.05 relative to T = 0). (b) LDH levels were not affected by SU5402 (10 µM) (n = 4, data expressed as mean ± SEM).

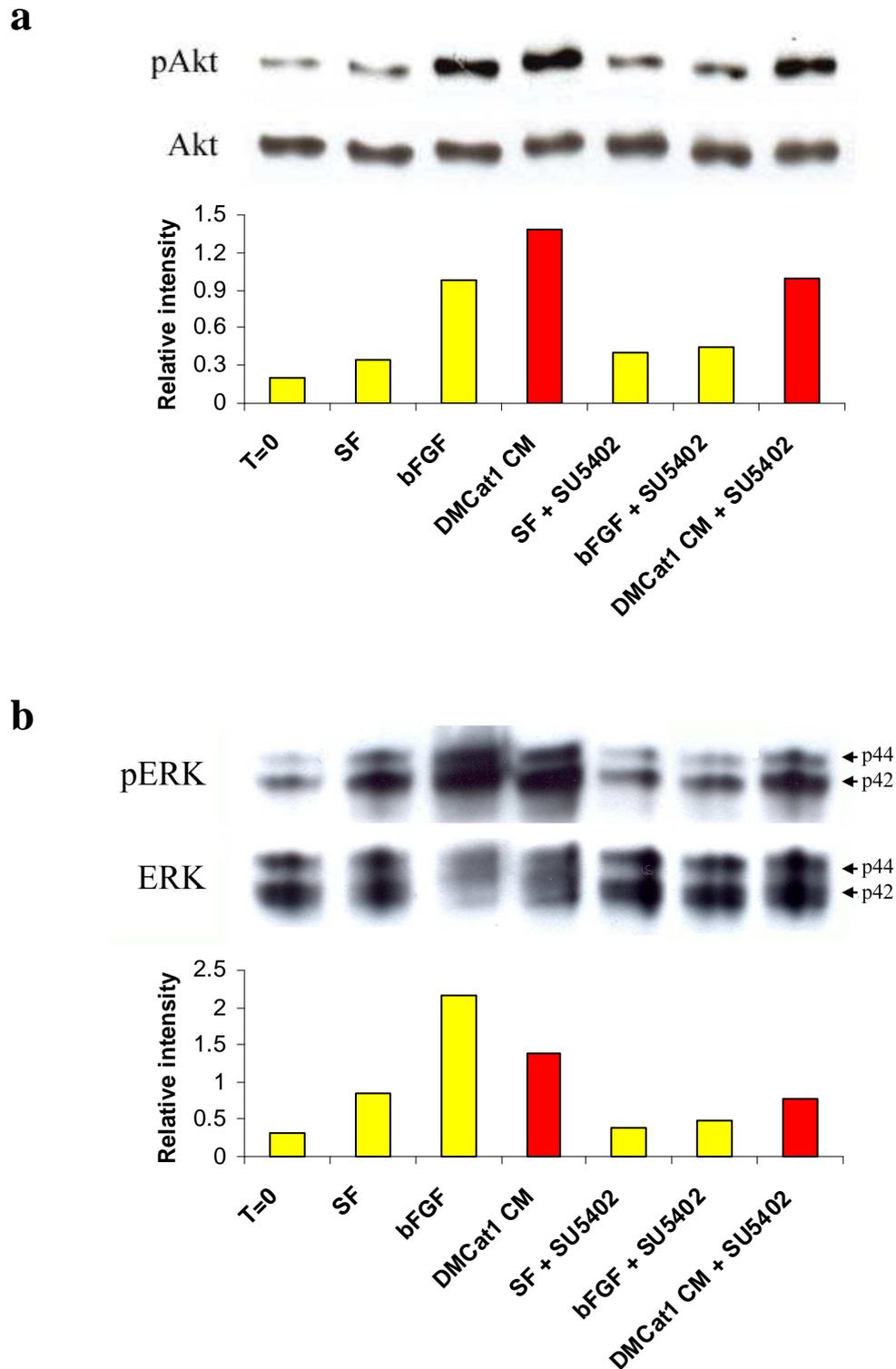


Figure 4.12 – Signalling pathways activated in FHL124 cells by CM following FGFR1 inhibition. Protein levels of (a) pAkt and (b) pERK were examined by Western blotting and analysed densitometrically. Graphs show the activated protein levels normalised to their total protein control. A β actin loading control was included but is not shown. Addition of DMCat1 CM for 10 minutes stimulated activation of Akt and ERK1/2 and in the presence of SU5402 (10 μ M) neither pAkt or pERK levels were returned to baseline levels, however, greater levels of pAkt remained compared to pERK.

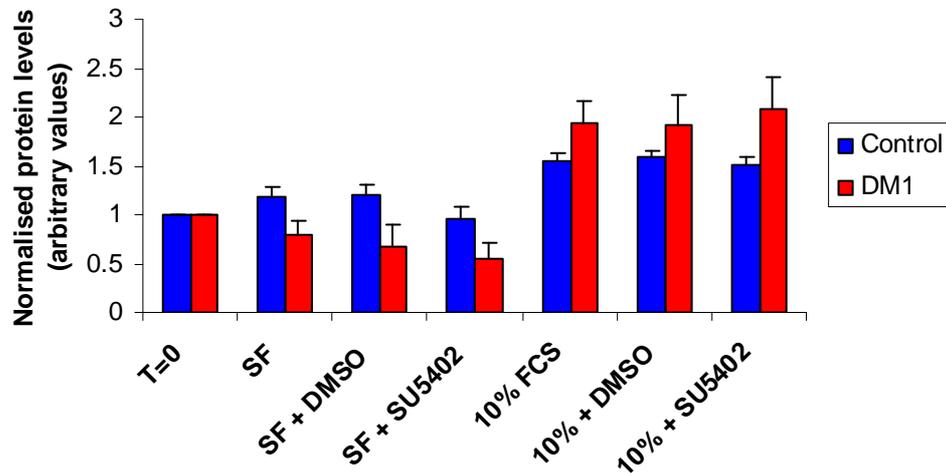
Addition of SF medium to FHL124 cells resulted in an increase in levels of pERK when compared to baseline levels ($T = 0$). The addition of bFGF resulted in a considerable increase in pERK levels when compared to SF medium, whereas levels of pERK following addition of DMCat1 CM fell between those of bFGF and SF medium. In the presence of SU5402 the levels of pERK were returned to baseline levels ($T = 0$) following both SF medium and bFGF addition. Interestingly, the levels of pERK were still slightly elevated after DMCat1 CM addition in the presence of SU5402. This data indicates that the stimulation of ERK1/2 following DMCat1 CM addition was only partially due to signalling via the FGF signalling pathway (see figure 4.12b).

4.3.4 FGF signalling in DM1 lens epithelial cells

In order to investigate the role of FGF signalling in the control and DM1 lens cells in both SF medium and medium supplemented with 10% FCS, the cells were grown in the presence and absence of SU5402 (10 μM) for 24 hours. Cells were lysed and total protein was extracted as a measure of cell growth. Protein values for each cell line were normalised to the $T = 0$ value and average values across the control and the DM1 cell lines were calculated. A reduction in cell growth was observed in both cell types with SU5402 in SF medium, however, this was not statistically significant when compared to the DMSO (1 $\mu\text{l/ml}$) controls (see figure 4.13a). In medium supplemented with 10% FCS, no effect was observed from the addition of SU5402. Levels of LDH in the medium were also calculated as a measure of cell death. LDH values were normalised to the $T = 0$ protein value for each cell line in order to observe the LDH released per $\mu\text{g/ml}$ of protein (see figure 4.13b). The $T = 0$ values were chosen to normalise to as a bias would be introduced by normalising to the protein values from the same dish due to the reduction in cell number (and therefore total protein) that occurs during cell death. A slight increase in cell death was observed in both control and DM1 cells in SF medium with SU5402, but not with SU5402 in medium supplemented with 10% FCS, however, this was also not significant. Although neither is statistically significant, the slight decrease in cell growth and increase in cell death during FGFR1 inhibition may indicate that signalling via the FGF pathway is more important in both control and DM1 cells in the absence of added growth factors,

indicating that autocrine signalling via the FGF pathway occurs in the control and DM1 LECs.

a



b

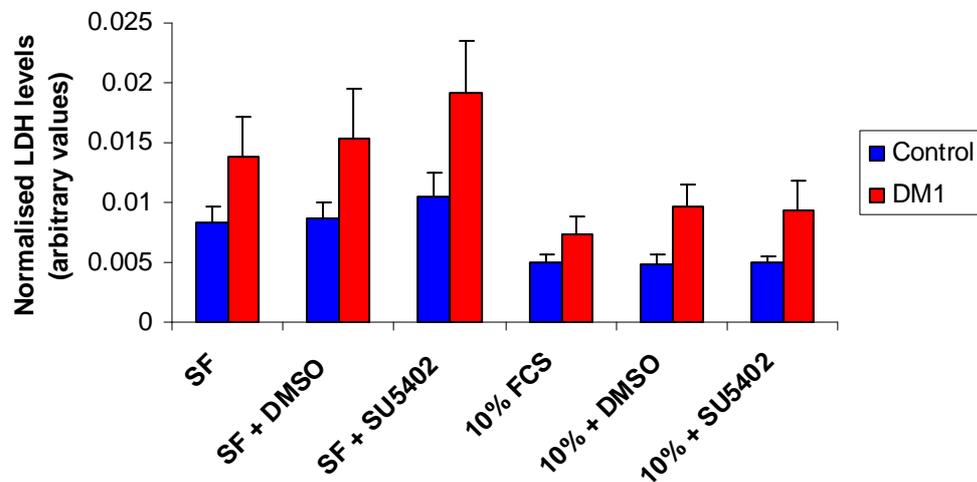


Figure 4.13 – The effect of FGFR1 inhibition on DM1 lens epithelial cells over 24 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 24 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of SU5402 (10 μM) had no significant effect on cell growth. **(b)** LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. There was no significant difference in levels of cell death following addition of SU5402 (n = 4, data expressed as mean \pm SEM, statistical analysis was performed by one-way ANOVA with Tukey’s test).

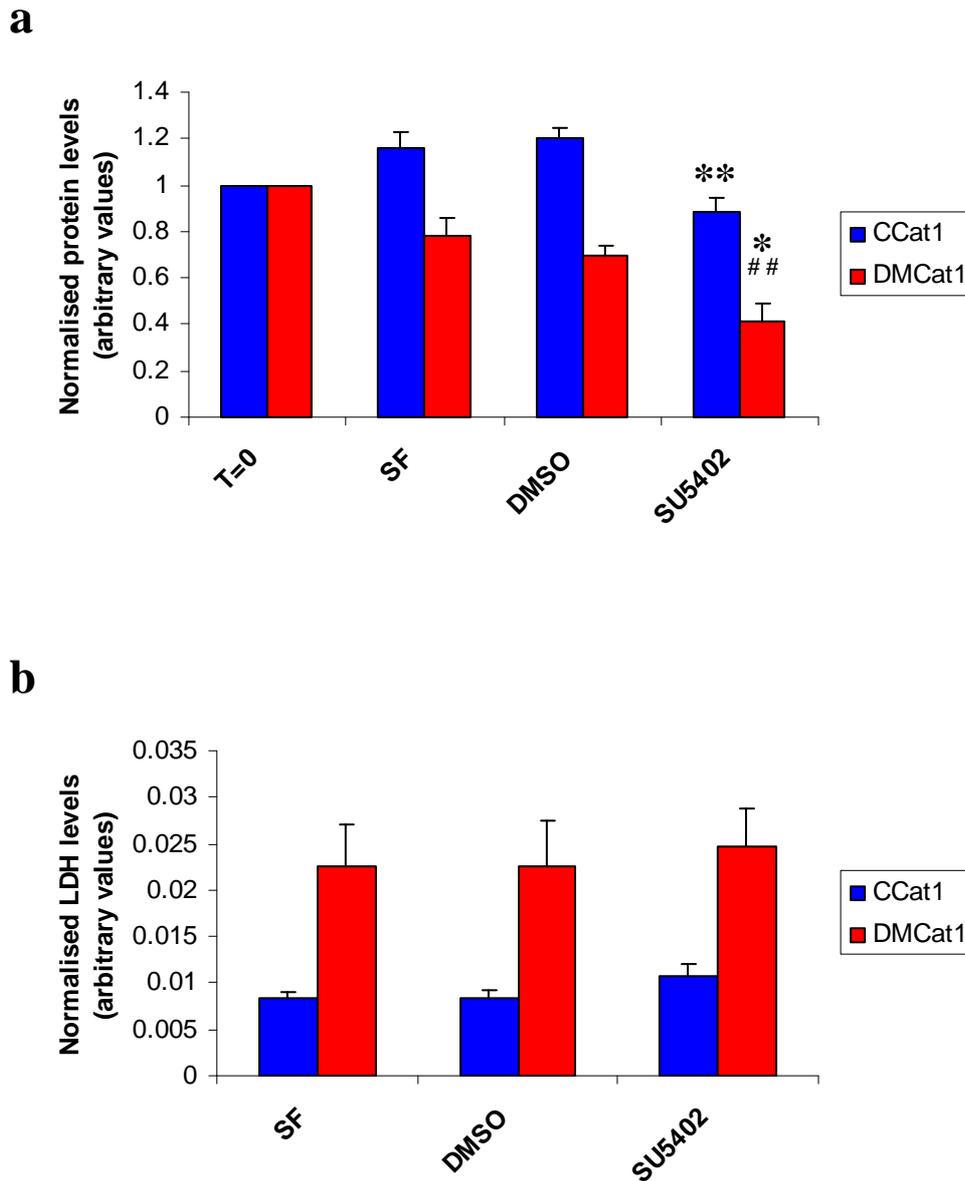


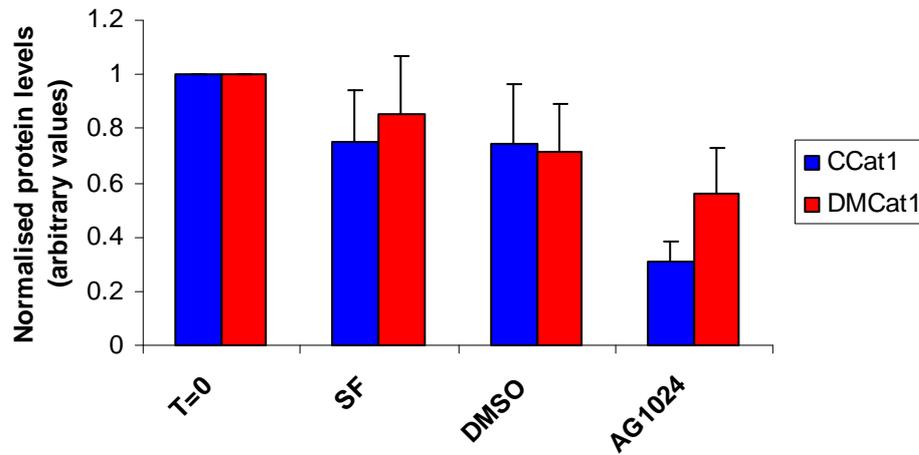
Figure 4.14 - The effect of FGFR1 inhibition on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of SU5402 (10 μM) resulted in a significant decrease in cell growth of both CCat1 and DMCat1 cells, however, DMCat1 had significantly lower levels of cell growth following SU5402 addition compared to CCat1 ($n = 4$, data expressed as mean \pm SEM of four independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test, * $p < 0.05$ and ** $p < 0.01$ relative to the relevant DMSO control, ## $p < 0.01$ relative to CCat1 treated with SU5402 (10 μM)). **(b)** LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of SU5402 resulted in a slight increase in levels of cell death but this was not statistically significant ($n = 4$, data expressed as mean \pm SEM of four independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test).

As the small effect seen after FGFR1 inhibition for 24 hours was not statistically significant, further experiments were performed on CCat1 and DMCat1 cells to analyse the effect of FGFR1 inhibition in SF medium for 48 hours (see figure 4.14). The data shows that growth is significantly reduced in both CCat1 ($p < 0.01$) and DMCat1 ($p < 0.05$) cells in SF medium with SU5402 (10 μM) compared to the relevant DMSO controls. Interestingly, cell growth following FGFR1 inhibition was also shown to be significantly lower in DMCat1 cells than CCat1 cells ($p < 0.05$). A slight increase in LDH levels was also observed following addition of SU5402, however, this was not statistically significant. The data implies that an autocrine FGF signalling loop is engaged when the cells are cultured in SF medium which is involved in cell growth, but that it is not critical to their survival.

4.3.5 IGF-1 signalling in DM1 lens epithelial cells

As signalling via FGF did not appear to be critical for survival in SF medium, the role of another growth factor, IGF-1, which is also important for survival in lens cells was analysed. As with the FGF pathway, the IGF-1 pathway can also signal via the PI3K/Akt pathway, which is activated by DM1 CM. CCat1 and DMCat1 cells were grown in SF medium in the presence and absence of the IGFR-1 inhibitor AG1024 (1 μM) for 48 hours. Normalised protein values show that cell growth was decreased in the presence of AG1024, especially in CCat1 cells, however, this was not found to be statistically significant (see figure 4.15a). There was no difference in LDH levels, indicating that cell death was not affected by IGFR-1 inhibition (see figure 4.15b).

a



b

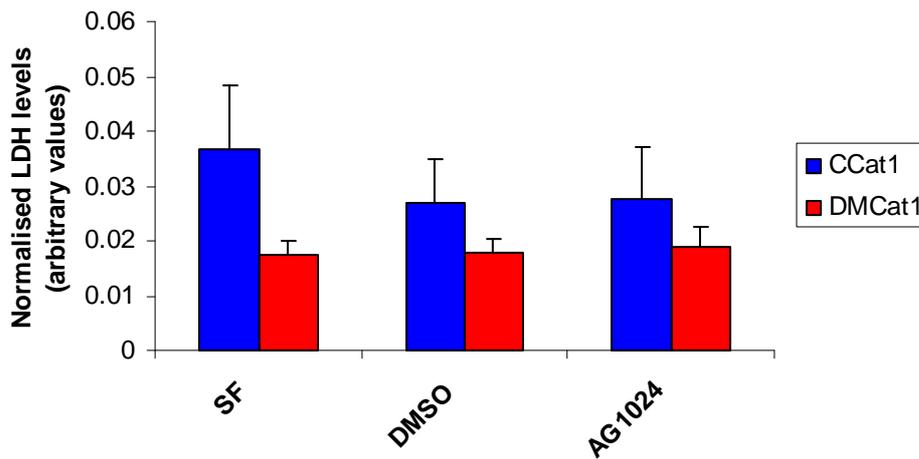


Figure 4.15 - The effect of IGFR-1 inhibition on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Although addition of AG1024 (1 μM) resulted in a decrease in cell growth of both CCat1 and DMCat1 cells, the difference was not statistically significant. **(b)** LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of AG1024 had no significant effect on cell death ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test).

4.4 Discussion

DM1 lens epithelial cells have significantly increased levels of apoptotic cell death when cultured in SF medium compared to controls (see figure 3.15). No significant difference in levels of apoptosis was seen between control and DM1 cell lines when cultured in medium supplemented with 10% FCS. It can be hypothesised that this was due to an impairment in autocrine signalling which is required for survival in SF medium. This could either be due to the cells not releasing enough of their own signalling factors or an impairment in the way that they respond to the signalling factors which they produce. Here we have shown that DM1 lens epithelial cells are releasing factors into their environment which are capable of increasing levels of cell growth and/or survival in the non-virally transformed lens cell line FHL124 (see figure 4.5). This data indicates that it is not an impaired ability to release autocrine signalling factors that results in increased levels of cell death, but rather a reduced ability to respond to these factors.

As well as increasing cell growth and/or survival in FHL124 cells, the factors released by DM1 LECs were shown to activate signalling pathways in FHL124 cells resulting in significantly greater levels of Akt activation compared to controls (see figure 4.7f). Akt is downstream of PI3K and this data indicates that the PI3K/Akt pathway is involved in growth and/or survival in FHL124 cells. It has been shown previously that signalling via the PI3K/Akt pathway is important for both growth and survival in rabbit and rat LECs (Chandrasekher & Sailaja, 2003, 2004; Iyengar *et al.*, 2006).

To elucidate the mechanisms behind the increase in apoptotic cell death in SF medium, we investigated the role of a known autocrine signalling system in the lens: the FGF pathway. Autocrine signalling via bFGF is likely to occur in the cell lines as all were shown to release bFGF and express the receptor FGFR1 (see figures 4.8a and 4.10). The DM1 cell line, DMCat1, was shown to release significantly more bFGF than the control, CCat1, and the DM1 cell lines were shown to release more bFGF at later passages, however, no significant difference was seen when all of the cell lines were analysed together (see figures 4.9 and 4.8b). The cell lines released a relatively low level of bFGF (0.17-3.17 pg/ml), however, very few cell types have been shown to release bFGF and this is

therefore an interesting finding (Rogelj *et al.*, 1988). The release of bFGF may be a feature of lens cells as human LECs cultured on their capsule have also been shown to release bFGF (Wormstone *et al.*, 2001). As bFGF has also been shown to be released from dead or damaged cells, this cannot be ruled out from our experiments (D'Amore, 1990). The observed levels of bFGF in the CM could therefore be due to release from dead cells and not a true autocrine signalling system. However, evidence that an FGF autocrine signalling system does exist in the LECs comes from the inhibition of FGFR1 in the cell lines themselves. FGFR1 inhibition had no effect on cell growth in cells cultured in medium supplemented with 10% FCS, but a decrease in cell growth was observed in cells cultured in SF medium with the inhibitor and this was found to be significant when repeated in CCat1 and DMCat1 cells (see figures 4.13 and 4.14). This data shows that in the presence of serum which contains other growth factors, signalling via FGFR1 is not required for cell growth, most likely due to other activated signalling pathways which can compensate for its loss. However, in SF medium the only growth factors available are those that the cells can synthesise themselves, revealing that signalling must occur via FGFR1 under these conditions in order for a reduction in cell growth to be observed. This data indicates that autocrine signalling via FGFR1 is present in the cells and is important for cell growth but is not critical for their survival.

Extensive investigations indicate that although signalling via the FGF pathway plays a role in growth in human LECs, it is unlikely that the factors released by DM1 LECs signal via this pathway. Addition of medium supplemented with bFGF (5 ng/ml) at much greater levels than those released from the cells themselves had no significant effect on FHL124 cell growth or survival (see figures 4.5 and 4.11). This is in contrast to the addition of bFGF (10 ng/ml) to rabbit LECs, which increased proliferation by 70-80% and was significantly blocked by the PI3K inhibitors, wortmannin (200 nM) and LY294002 (10 μ M) (Chandrasekher & Sailaja, 2003). Addition of bFGF to rat LECs, has been shown to induce proliferation, migration and differentiation in a concentration-dependent manner. The half maximal concentrations of bFGF shown to induce proliferation, migration and differentiation were 0.15, 3 and 40 ng/ml respectively (McAvoy & Chamberlain, 1989). If the same is true in human LECs, this may account for the absence in growth stimulation by addition of 5

ng/ml of bFGF as bFGF at this concentration would result in a response of migration and differentiation which were not examined in the FHL124 cells. The FHL124 cells may also be producing and releasing bFGF themselves and addition of further bFGF may therefore have no added effect. Although the addition of bFGF had no significant effect on the FHL124 cells, the addition of DM1 CM did, and as it contained much lower levels of bFGF, this data would indicate that it is not the factor responsible for increasing growth and/or survival in the FHL124 cells. Further to this data, inhibition of FGFR1 in FHL124 cells treated with CM resulted in a decrease in cell growth and/or survival and also resulted in a reduction in active levels of the downstream signalling molecules Akt and ERK (see figures 4.11 and 4.12). However, levels of growth were still greater than those observed with the FGFR1 inhibitor in SF medium alone and levels of pAkt and pERK were also not completely returned to baseline levels. This data indicates that other factors must be present in DM1 CM which activate Akt and ERK independently of FGFR1 to increase growth.

As FGF does not appear to be the factor released by the DM1 LECs which increases growth and/or survival, we investigated the role of the IGF-1 pathway in the DM1 cells as it has also been shown to signal via the PI3K/Akt pathway to mediate growth and survival in LECs (Chandrasekher & Bazan, 2000; Chandrasekher & Sailaja, 2004). Inhibition of IGFR-1 resulted in reduced cell growth in the cell lines, which was more notable in the control cell line CCat1 compared to DMCat1, however, this was not significant (see figure 4.15). No difference was observed in levels of cell death, indicating that signalling via IGF-1 may play a role in growth but not in survival in LECs cultured in SF medium. This is in contrast to a study conducted on rabbit LECs which showed that signalling via IGF-1 stimulated the PI3K/Akt pathway and suppressed apoptosis to increase survival (Chandrasekher & Sailaja, 2004). Despite the fact that the presence of IGF-1 in the CM or the effect of inhibiting signalling via IGFR-1 in FHL124 cells treated with CM was not investigated, the fact that no significant effect was seen in levels of cell growth or death in the DM1 cells lines indicates that IGF-1 is unlikely to be the factor responsible for increased growth and/or survival in FHL124 cells following addition of DM1 CM.

The factor, or factors, released by DM1 lens epithelial cells which are responsible for increasing growth and/or survival and activating Akt in the

FHL124 cells were not identified. Of the four autocrine signalling systems which have previously been identified in the lens, FGF and IGF-1 do not appear to be the factors released by DM1 LECs which are responsible for promoting growth and/or survival. This leaves the autocrine signalling systems involving EGF and HGF to still be investigated (Palmade *et al.*, 1994; Majima, 1995; Weng *et al.*, 1997; Wormstone *et al.*, 2000; Wormstone *et al.*, 2001). Both EGF and HGF have previously been shown to signal via PI3K and both have been shown to play roles in proliferation in LECs, however, neither has been shown to influence levels of survival in LECs (Reddan & Wilsonziedzic, 1983; Wormstone *et al.*, 2000; Choi *et al.*, 2004; Jiang *et al.*, 2006). As the presence of EGF and HGF autocrine signalling systems in our cell lines have not been tested, we cannot confirm or rule out a role for either growth factor in the effects observed following CM addition to FHL124 cells. Further, although the autocrine systems involving EGF, FGF, HGF and IGF-1 are known to promote growth and/or survival in LECs, other currently unidentified or less common autocrine signalling systems could also be responsible for the increase in growth and/or survival in the FHL124 cells in response to CM. Other growth factors are present within the lens, such as vascular endothelial growth factor (VEGF), transforming growth factor β 2 (TGF- β 2) and lens derived growth factor (LEDGF), however, whether autocrine signalling systems exist and whether they signal via the PI3K/Akt pathway to promote growth and/or proliferation is questionable. VEGF and its receptor VEGFR are expressed in the lens and signal via the PI3K/Akt pathway, however, the autocrine signalling system is initiated in response to hypoxic conditions and results in activation of genes involved in, for example, glucose transportation in the lens (Shui *et al.*, 2003; Radreau *et al.*, 2009). Interestingly, hypoxia has been shown to play a role in the suppression of lens growth, however, this was shown to likely be due to the increasing thickness of the lens with age and is therefore unlikely to play a role in the growth of cultured LECs (Shui *et al.*, 2008; Shui & Beebe, 2008). LEDGF has been shown to promote LEC survival and is activated under stress conditions, however, LEDGF is not known to signal via the PI3K/Akt pathway (Ayaki *et al.*, 1999; Singh *et al.*, 2000). TGF- β 2 and its receptors are expressed in the lens and addition of TGF- β 2 has been shown to increase proliferation in LECs (Kampmeier *et al.*, 2006). TGF- β 2 also plays a role in wound healing and its expression is upregulated by LECs in response to addition

of TGF- β 2 which occurs in response to injury (Wormstone *et al.*, 2006; Dawes *et al.*, 2007). However, as with LEDGF, TGF- β 2 is not known to signal via the PI3K/Akt pathway and these growth factors are therefore unlikely to be responsible for the effects of CM addition to FHL124 cells. Further investigation is required to establish the role of EGF, HGF and other possible growth factors in the effects of DM1 CM.

It is possible that no one single factor is responsible for the increase in FHL124 growth and/or survival following addition of DM1 CM. The effects observed may instead be due to a combination of factors which may be released, all activating receptors and downstream signalling pathways which increase growth and/or survival. Inhibition of FGFR1 in FHL124 cells treated with CM resulted in a reduction in growth and/or proliferation (see figure 4.11) and a reduction in the activation of Akt and ERK (see figure 4.12), however, other signalling factors must have been present as neither was returned to baseline levels. Other signalling factors may have been activating pathways which compensated for the loss of signalling via the FGF pathway. Blocking pathways in combination, such as FGF and IGF-1 together, may result in a greater reduction in the effects of CM.

Although the growth factor(s) and receptor(s) responsible for initiating the signalling which increased growth and/or survival in FHL124 cells were not identified, the downstream signalling pathway which was activated by them was revealed. Activation of the PI3K/Akt pathway was significantly increased by DM1 CM, indicating that this pathway was responsible for the subsequent increase in growth and/or survival of FHL124 cells (see figure 4.7f). This data would indicate that the PI3K/Akt pathway should also be upregulated in the DM1 cell lines themselves, unless an impairment in autocrine signalling results in aberrant activation of different pathways in response to the factors released. The aberrant activation of pathways in response to stress has been observed in mouse myoblasts transfected with *DMPK* containing small repeats (5 repeats) and large repeats (160 repeats). In response to oxidative stress the cells containing small repeats activated the MAPK/ERK pathway which led to cell survival, whereas those containing large repeats activated the MAPK/JNK pathway which led to apoptosis (Usuki *et al.*, 2000; Usuki *et al.*, 2008). This data reveals that in other cell types the expanded CTG repeats which are found in DM1 can disturb the

activation of signalling pathways. If serum deprivation in the DM1 LECs also causes stress to the cells which triggers aberrant activation of cell signalling pathways then this could cause the increased levels of cell death in SF medium. Therefore, the activation of downstream signalling pathways, and in particular the PI3K/Akt pathway, in DM1 LECs requires further investigation to elucidate the cause of increased levels of cell death when cultured in SF medium.

CHAPTER 5

CELL SIGNALLING PATHWAYS IN DM1

LENS EPITHELIAL CELLS

5.1 Introduction

Growth factor activation of receptor tyrosine kinases (RTKs) results in the downstream activation of a number of different signalling cascades in the lens, such as the MAPK/ERK and PI3K/Akt cascades. Signalling cascades transduce signals from receptors to many possible biological responses in the cell, including growth, differentiation, survival and apoptosis. Some signalling cascades which are active in the lens, such as the MAPK/JNK and MAPK/p38 cascades, are only rarely activated by growth factor activation of RTKs and are instead mostly associated with activation under stress conditions, such as exposure to UV radiation. These pathways may be of particular importance to the processes of survival and apoptosis within the lens.

5.1.1 The PI3K signalling pathway

The PI3K pathway plays roles in growth, proliferation and survival in many different cell types (see figure 5.1). PI3K is a heterodimeric protein composed of an 85 kDa regulatory subunit and a 110 kDa catalytic subunit. Src homology 2 (SH2) domains in the regulatory subunit interact with phosphorylated tyrosine residues of activated RTKs and result in stabilisation of the catalytic subunit. Through these interactions, PI3K is brought into close proximity to phosphoinositides in the plasma membrane where it catalyses the addition of phosphate groups to the 3'-OH of the inositol ring, preferentially producing phosphatidylinositol-3,4,5-trisphosphate (PIP₃) from phosphatidylinositol-4,5-bisphosphate (PIP₂) (Franke, 2008). The conversion of PIP₂ into PIP₃ is negatively regulated by phosphatase and tensin homolog (PTEN). Pleckstrin homology domains in both Akt and phosphoinositide-dependent kinase 1 (PDK1) bind to PIP₃ and are brought into close proximity where PDK1 can phosphorylate Akt at the threonine residue T308. Akt must first be phosphorylated at the serine

residue S473 by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) before it can be fully activated by PDK1 and capable of greater levels of signalling. The mechanism by which mTORC2 is activated is unknown, although it is thought to also be downstream of PI3K as Akt S473 phosphorylation is stimulated by growth factors and inhibited by LY294002 and wortmannin, which are specific PI3K inhibitors (Sarbasov *et al.*, 2005; Yang & Guan, 2007). There are three isoforms of Akt, known as Akt1-3. While Akt1 is ubiquitously expressed, Akt2 is found mostly in insulin sensitive tissues and Akt3 is most highly expressed in the brain and testis (Franke, 2008). Akt regulates cell survival in several different ways, but most notably by targeting the pro-apoptotic Bcl-2 related protein, Bcl-2-antagonist of death (BAD), and preventing its activity (Song *et al.*, 2005). Akt can also regulate protein synthesis by phosphorylating tuberous sclerosis complex 2 (TSC2) within the TSC2-TSC1 complex and inhibiting its activity, allowing the activation of mTOR complex 1 (mTORC1). mTORC1 phosphorylates p70 S6 Kinase (p70S6K) which phosphorylates the ribosomal protein S6 and induces protein synthesis (Franke, 2008). Akt can also regulate cell proliferation via phosphorylation of downstream targets such as the cell cycle regulators p21^{Cip1/Waf1} and p27^{Kip1}. Phosphorylation of these proteins prevents their localisation to the nucleus, which prevents them from inhibiting cell cycle progression and, therefore, increases cell proliferation (Manning & Cantley, 2007).

In bovine lenses, PI3K was found to be expressed and active throughout the lens, with highest activity in the LECs (Chandrasekher & Bazan, 2000). PI3K and Akt were also shown to be present in LECs from porcine lenses (Zatechka & Lou, 2002a). Inhibition of the PI3K/Akt pathway in quail LECs inhibited differentiation and was also shown to reduce survival in differentiated lens fibre cells, but not in LECs (Weber & Menko, 2006). Inhibiting the PI3K/Akt pathway has also been shown in separate studies to result in a block in proliferation and differentiation of rat LECs (Iyengar *et al.*, 2006; Wang *et al.*, 2009b). Activation of PI3K signalling was shown to play a role in both proliferation and survival in rabbit LECs and differentiation in chick LECs (Chandrasekher & Sailaja, 2003, 2004). Signalling via PI3K was also shown to play a role in cell migration in human LECs (Jiang *et al.*, 2006). These studies indicate that the PI3K/Akt pathway is involved in numerous biological responses in LECs including differentiation, migration, proliferation and survival.

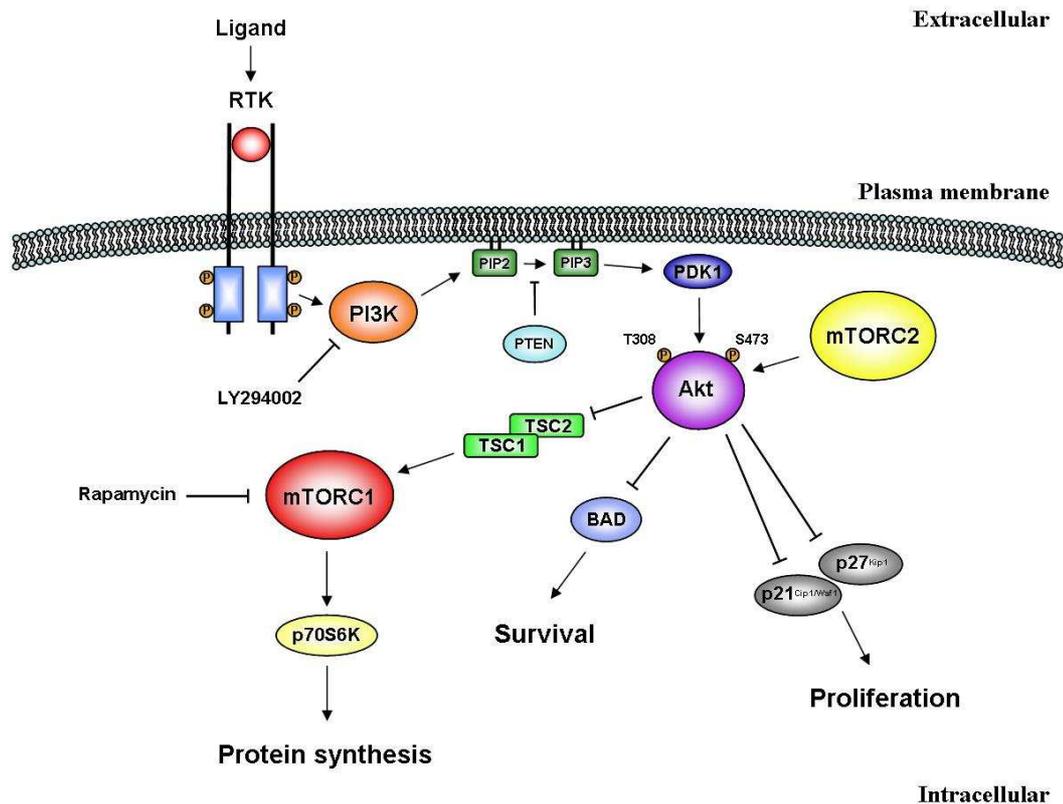


Figure 5.1 – The PI3K/Akt signalling pathway. PI3K is recruited to tyrosine kinase domains of phosphorylated RTKs and is brought into close proximity to the membrane where it converts PIP₂ to PIP₃. PIP₃ recruits Akt and PDK1 resulting in phosphorylation of Akt at T308 by PDK1. Akt must first be phosphorylated by mTORC2 at S473 before it can be fully activated by PDK1. Akt regulates many biological processes including protein synthesis via mTORC1 and p70S6K; survival via inhibition of the pro-apoptotic protein BAD; and proliferation via the cell cycle regulators p21^{Cip1/Waf1} and p27^{Kip1}. The PI3K/Akt pathway is negatively regulated by the tumour suppressor protein PTEN which negatively regulates the conversion of PIP₂ into PIP₃.

5.1.2 MAPK signalling pathways

Cell signalling via MAPK signalling pathways results in the activation of a MAPK, of which there are six distinct groups. The pathway leading to the activation of the MAPK ERK1/2 is one of the most characterised, along with c-Jun NH₂-terminal kinase (JNK) and p38. The remaining MAPKs are ERK3/4, ERK5 and ERK7/8, however, little information is known about the pathways that lead to their activation and therefore these will not be discussed. The three main MAPK signalling pathways are well characterised and the signalling cascades

consist of three sequentially activated kinases (summarised in figure 5.2). The MAPKs ERK1/2, JNK and p38 are activated by phosphorylation of both threonine and tyrosine residues within a conserved threonine-proline-tyrosine motif by a MAPK kinase (MAPKK, also known as MEK or MKK). The MAPKK itself is activated by a MAPK kinase kinase (MAPKKK), which are serine/threonine kinases. MAPKKKs are activated by phosphorylation and/or interactions with small GTP proteins. Activated MAPKs phosphorylate target substrates on serine and threonine residues, including transcription factors and other proteins or kinases. MAPK pathways are regulated by the opposing actions of phosphatases which deactivate kinases by removing phosphate groups (Krishna & Narang, 2008).

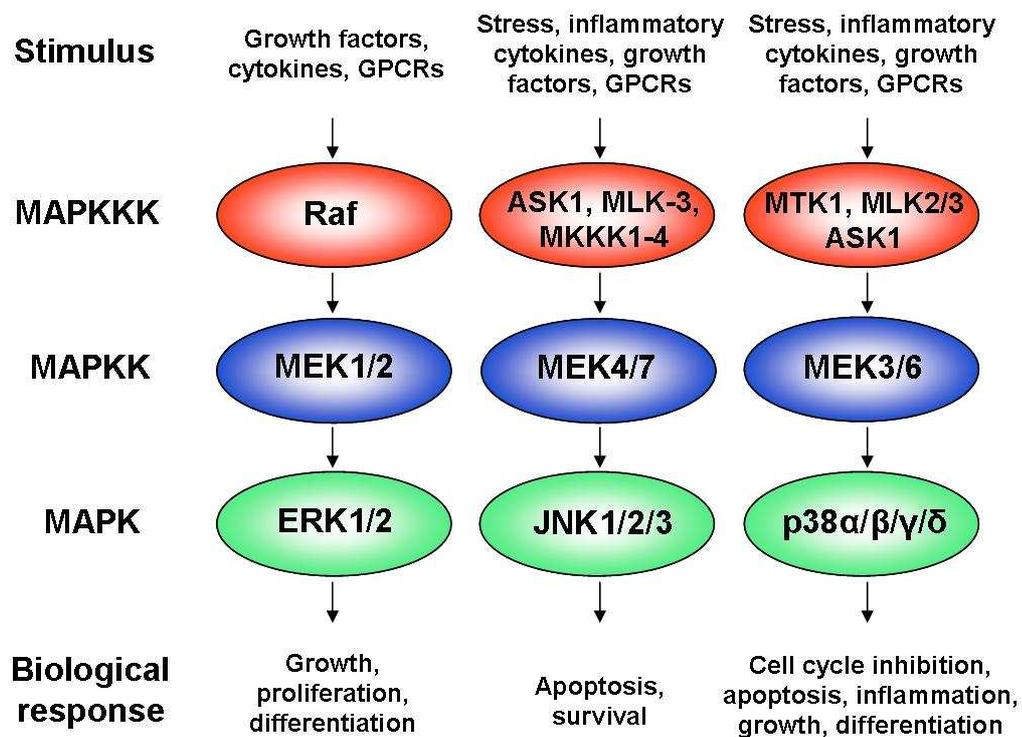


Figure 5.2 – A summary of the major MAPK signalling cascades. MAPK cascades are initiated by small GTPases and/or other protein kinases and are organised into three tiers: initially MAPKKKs are activated which lead to the phosphorylation and activation of MAPKKs, which in turn phosphorylate and activate MAPKs. The three major pathways are the ERK1/2 pathway, the JNK1/2/3 pathway and the p38α/β/γ/δ pathway, all of which are activated by various mitogens and external stimuli. The signalling pathways bring about a variety of biological responses including growth, proliferation and survival.

5.1.2.1 The MAPK/ERK signalling pathway

The MAPK/ERK pathway plays roles in proliferation, differentiation and survival in many different cell types (see figure 5.3). The MAPK/ERK pathway is most commonly initiated following recruitment of proteins to the membrane during RTK activation. Docking proteins such as GRB2 bind to the phosphorylated tyrosines of the receptor via SH2 domains. GRB2 recruits SOS via Src homology 3 (SH3) domains, enabling SOS to become activated and to subsequently activate the small membrane bound G protein Ras by the exchange of bound guanosine diphosphate (GDP) for GTP. G protein-coupled receptors (GPCRs) can also activate the MAPK/ERK pathway via Ras. Ras activates Raf and a cascade of kinase activation is initiated. Raf activates the MAPKs MEK1/2, which activate ERK1/2 (Junttila *et al.*, 2008). Activated ERK1/2 translocate to the nucleus where they activate several transcription factors, such as c-Fos, Elk-1, c-Jun and c-Myc, which regulate the transcription of many genes involved in proliferation, differentiation and survival (Krishna & Narang, 2008).

In porcine LECs, ERK1/2, Raf-1 and MEK1/2 were all shown to be present and in a separate study ERK1/2 and MEK1/2 were shown to be expressed and active in human, bovine and rat LECs (Zatechka & Lou, 2002a; Li *et al.*, 2003). Inhibiting the MAPK/ERK pathway has been shown to result in a block in proliferation and differentiation of rat LECs (Lovicu & McAvoy, 2001; Iyengar *et al.*, 2006; Wang *et al.*, 2009b). Expression of a dominant-negative form of Ras in transgenic mice resulted in the development of smaller lenses due to inhibition of cell growth. A small increase in levels of apoptosis and a delay in differentiation were also observed (Xie *et al.*, 2006). The activation of ERK in the transgenic mice was shown to be significantly reduced, whereas activation of Akt, JNK and p38 were not affected. This data reveals that signalling via Ras and the MAPK/ERK pathway is essential for cell proliferation and, to a lesser extent, for survival and differentiation (Xie *et al.*, 2006). Signalling via the MAPK/ERK pathway, as well as via PI3K, was also shown to play a role in cell migration in human LECs via an increase in the activity of the matrix metalloproteinase, MMP-2 (Jiang *et al.*, 2006). This data indicates that the MAPK/ERK pathway is involved in proliferation, differentiation, survival and migration in LECs.

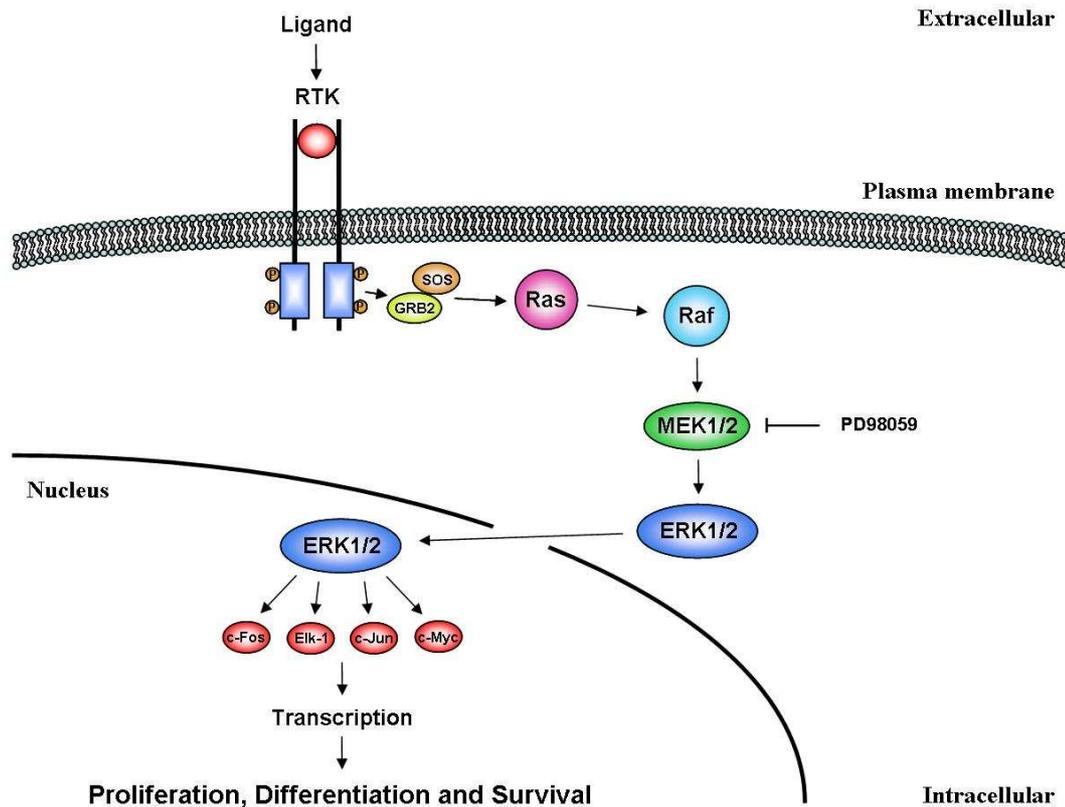


Figure 5.3 – The MAPK/ERK signalling pathway. GRB2 binds to tyrosine kinase domains of phosphorylated RTKs and recruits and activates SOS, which activates Ras by the exchange of bound GDP for GTP. Ras activates Raf and a cascade of kinase activation is initiated. Raf activates the MAPKKs MEK1/2, which activate ERK1/2. Activated ERK1/2 translocate to the nucleus where they activate several transcription factors, such as c-Fos, Elk-1, c-Jun and c-Myc, which regulate the transcription of many genes involved in proliferation, differentiation and survival.

5.1.2.2 The MAPK/JNK signalling pathway

The MAPK/JNK pathway is strongly activated by cytokines, UV radiation, growth factor deprivation, DNA damaging agents and some growth factors and GPCRs (see figure 5.4). For this reason JNKs are also known as stress-activated protein kinases (SAPKs). Three genes encode forms of JNK, *JNK1-3*, and alternative splicing leads to ten different isoforms. While *JNK1* and *JNK2* are ubiquitously expressed, *JNK3* expression is found only in the brain, heart and testis. JNK is activated by the MAPKKs, MKK4/7, which are activated by several

upstream kinases such as apoptosis signal-regulating kinase 1 (ASK1), MLK-3 and MKKK1-4 (Krishna & Narang, 2008). The activation of JNK by cytokine receptors is mediated by the tumour necrosis factor receptor associated factor (TRAF) family of adaptor proteins. Scaffolding proteins known as JNK interacting proteins (JIPs) are thought to help organise the transduction of the signal through specific kinase cascades in response to different signals (Davis, 2000). Many JNK substrates are transcription factors, such as c-Jun, ATF-2, Elk-1, c-Myc and p53, and the downstream activation of the transcription factor AP-1 appears to be regulated by JNK activation following stress. JNK signalling is mostly involved in regulating apoptosis and survival. JNK has also been shown to target both pro- and anti-apoptotic members of the Bcl-2 family of proteins. The opposing effects of JNK signalling are thought to be mediated by the duration and magnitude of the signal, with prolonged activation leading to apoptosis and transient activation promoting survival (Krishna & Narang, 2008).

JNK was shown to be present in LECs from porcine lenses and in LECs from human, bovine and rat lenses JNK1/2 were expressed and active (Zatechka & Lou, 2002a; Li *et al.*, 2003). The MAPK/JNK pathway is mostly activated under stress conditions and has been shown to be activated following exposure of human LECs to UV radiation (Bomser, 2002). A subsequent study showed that inhibition of the MAPK/JNK pathway following UV radiation resulted in increased cell death in human LECs (Long *et al.*, 2004). JNK has also been shown to be activated by oxidative stress following addition of hydrogen peroxide to human LECs. Activation of JNK, and also of ERK1/2, was linked to cell cycle arrest via expression of the cell cycle regulator p21^{Cip1/Waf1}, which is thought to help protect against DNA damage (Seomun *et al.*, 2005). This data indicates that the MAPK/JNK pathway is primarily involved in survival in LECs.

5.1.2.3 The MAPK/p38 signalling pathway

The MAPK/p38 pathway is activated by many of the same stimuli as the JNK pathway, i.e. environmental stresses, cytokines and some growth factors and GPCRs (see figure 5.4). There are four isoforms of p38, known as p38 α , p38 β , p38 γ and p38 δ , which are activated by different MAPKKs. All four isoforms are activated by MKK6 and all but p38 β are activated by MKK3. MKK4/7, which activate the JNK pathway, can also activate p38, enabling crosstalk between the two pathways.

The MAPKKs are activated by several upstream kinases, including MTK1, MLK2/3 and ASK1. Activated p38 can move to the nucleus and activate transcription factors such as ATF-1/2/6, CHOP, p53 and Elk-1 and can also activate other protein kinases in the cytoplasm such as MNK1/2 and MK2/3. The wide range of downstream substrates results in a wide range of biological responses such as cell cycle inhibition, apoptosis, inflammatory responses, growth and differentiation (Krishna & Narang, 2008).

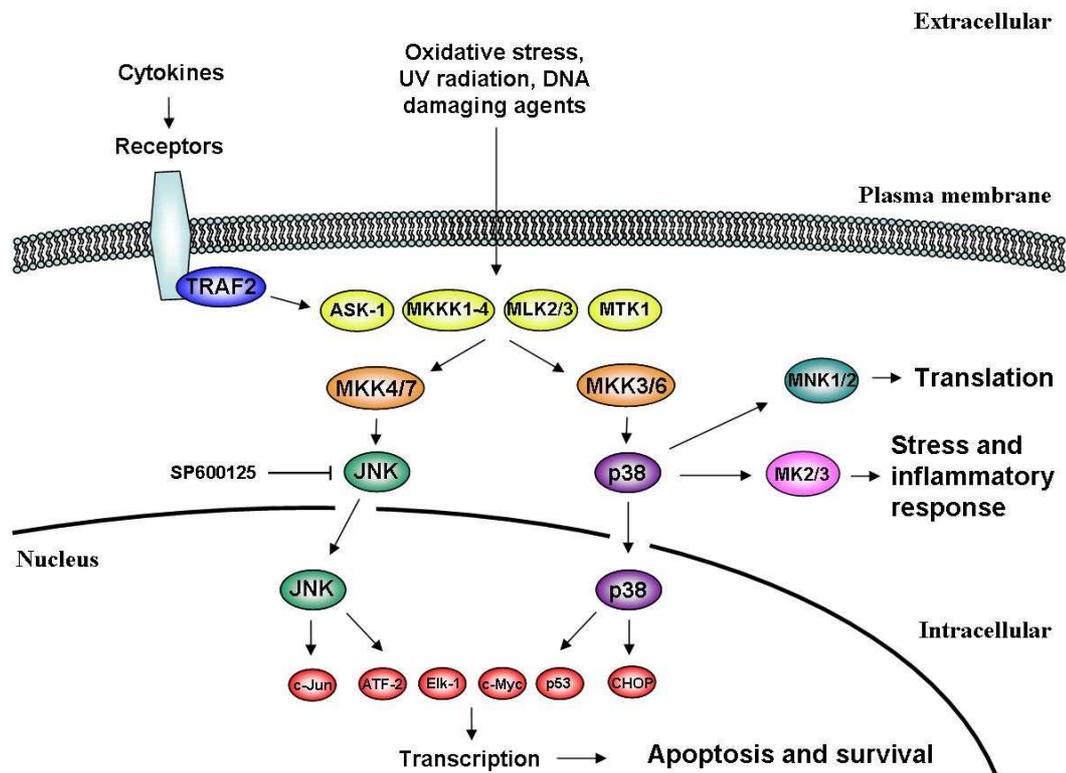


Figure 5.4 – The MAPK/JNK and MAPK/p38 signalling pathways. The MAPK/JNK and MAPK/p38 signalling pathways are mostly activated by environmental stressors and cytokines which result in activation of upstream kinases, such as ASK-1, MKKK1-4, MLK2/3 and MTK1. The MAPKKs MKK4/7 and MKK3/6 are activated and phosphorylate and activate JNK and p38 respectively. Activated JNK and p38 translocate to the nucleus where they activate several of the same transcription factors, such as ATF-2, Elk-1, c-Myc and p53 which regulate the transcription of many genes involved in apoptosis and survival. Activated p38 can also activate other kinases in the cytoplasm such as MNK1/2 and MK2/3 which have roles in translation and stress and inflammatory responses.

The MAPK/p38 pathway is mostly activated under stress conditions and has been shown to be activated following exposure of human LECs to UV radiation (Bomser, 2002). In porcine lenses, p38 was found to be present and active, with greater levels of active p38 being found in lenses subjected to osmotic stress (Zatechka & Lou, 2002a). p38 was also shown to be activated in porcine LECs treated with a Rho-associated protein kinase inhibitor and its activation was linked to an increase in α B-crystallin (Khurana *et al.*, 2002). α B-crystallin was shown to localise to the leading edge of migrating porcine LECs which was dependent on its phosphorylation by p38 (Maddala & Rao, 2005). Activation of p38 was also shown to play a role in the pathway leading to cataract formation induced by mechanical stress in embryonic chick lenses via its downstream activation of Src kinase (Zhou & Menko, 2004). In a later study it was shown that signalling via p38 and Src kinase led to cataract formation due to apoptosis in LECs and a loss in cadherin junctions, leading to fibre cell disorganisation (Zhou *et al.*, 2007). This data indicates that p38 could play a role in migration and apoptosis in LECs.

5.1.3 The PLC γ pathway

A further cell signalling pathway which could play roles downstream of the growth factors known to be important in the lens is the PLC γ pathway (see figure 5.5). PLC is a class of enzymes which cleave phospholipids. There are 13 mammalian isozymes of PLC, which are divided into six groups, known as PLC β , γ , δ , ϵ , ζ and η . PLC γ is a subclass of PLC which is activated by receptor and non-receptor tyrosine kinases. It contains two SH2 domains and an SH3 domain which enable it to be activated by the receptor and mediate interactions with other proteins (Kim *et al.*, 2000). PLC γ can be phosphorylated and activated by the EGF receptor (EGFR), FGFR and PDGF receptor (PDGFR) and once activated it hydrolyses PIP₂ to form two products: IP₃ and DAG. IP₃ binds to calcium channels on the membrane of the ER, known as IP₃ receptors (IP₃R), activating them and resulting in Ca²⁺ release and a subsequent rise in intracellular Ca²⁺ levels, whereas DAG remains in the plasma membrane. PKC can be activated by a combination of DAG, Ca²⁺ and a phospholipid such as phosphatidylserine. PKC is a family of serine/threonine kinases, which consists of 10 isozymes which are divided into three groups known as the conventional PKCs (PKC α , β I, β II and γ),

the novel PKCs (PKC δ , ϵ , η and θ) and the atypical PKCs (PKC ζ and ι), based upon the composition of their regulatory domain (Newton, 2010). The regulatory domains control the sensitivity of different PKC isozymes to DAG, Ca²⁺ and phospholipids.

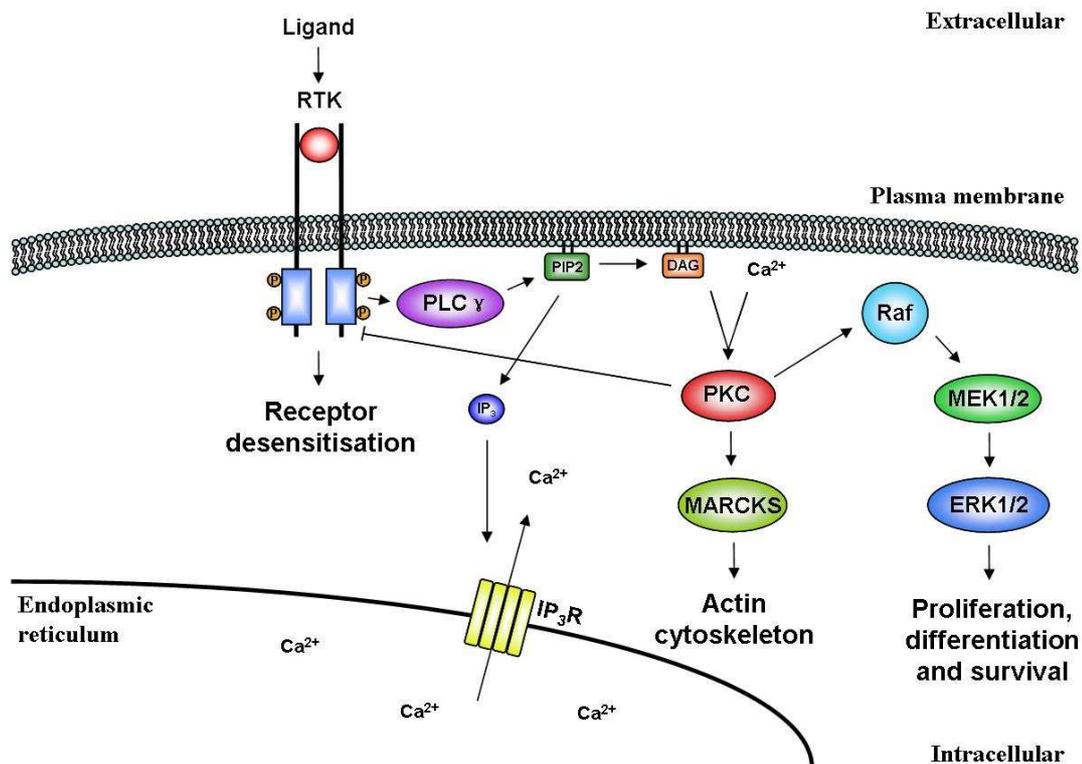


Figure 5.5 – The PLC/PKC signalling pathway. PLC γ binds to tyrosine kinase domains of phosphorylated RTKs and is activated, enabling it to hydrolyse PIP₂ to form two products: IP₃ and DAG. IP₃ binds to IP₃Rs, which results in Ca²⁺ release and a subsequent rise in intracellular Ca²⁺ levels, whereas DAG remains in the plasma membrane. DAG and Ca²⁺ activate PKC which plays roles in proliferation, differentiation and survival via interactions with the MAPK/ERK signalling pathway; the structure of the actin cytoskeleton via interactions with MARCKS; and receptor desensitisation.

PKC can bind to many different substrates, such as EGFR, which results in desensitisation of the receptor; glycogen synthase kinase 3 β (GSK-3 β), which is inactivated by PKC; growth-associated protein 43 (GAP-43), which is specific to the nervous system and plays a role in neurite formation; Myristoylated alanine-

rich protein kinase C substrate (MARCKS), which regulates the structure of the actin cytoskeleton; and Raf (Casabona, 1997). PKC activation of Raf activates the MAPK/ERK pathway and PLC γ itself can also activate the MAPK/ERK pathway via interactions between SOS and its SH3 domain. The PLC γ signalling pathway is involved in the processes of secretion, cell proliferation, growth and differentiation in many different cell types (Kim *et al.*, 2000).

A study in human lenses found expression of PLC γ in LECs with the highest levels of expression in cells near the equator of the lens (Maidment *et al.*, 2004). Proliferation and differentiation are also highest at the equator of the lens, which could indicate a role for PLC γ in these processes. PKC α and γ were found in bovine LECs, whereas PKC α , γ , ι and ϵ were found in developing chick lenses and were most highly concentrated in the LECs (Gonzalez *et al.*, 1993; Berthoud *et al.*, 2000). Overexpression of PKC α and γ in rabbit LECs revealed a role for these proteins in the initiation of differentiation as the cells began to elongate and express α A- and α B-crystallins (Wagner & Takemoto, 2001). This data indicates a role for the PLC γ pathway in differentiation of LECs.

5.1.4 Cross-talk between signalling pathways in the lens

Many of the signalling pathways described above result in activation of the same proteins and biological processes within a cell. Some signalling molecules have been shown to activate more than one pathway within a cell and activation of particular pathways can also result in opposing effects such as apoptosis and survival. This complexity is the result of cross-talk between pathways and the regulation of signal transduction that occurs within cells including mechanisms such as the duration and strength of the signal; scaffolding proteins; subcellular localisation; and the presence of different isoforms within the cascade (Krishna & Narang, 2008). A study investigating the intercommunication and cross-talk between pathways in the lens showed that inhibition of the MAPK/ERK and MAPK/p38 cascades resulted in an increase in PI3K/Akt signalling, most likely due to signalling via Ras. Inhibition of Ras suppressed PI3K activation but stimulated the MAPK cascades possibly via the action of p21-activated kinase. Inhibition of MEK downregulated signalling via ERK and JNK when stimulated by bFGF and downregulated signalling via p38 when stimulated by osmotic stress. Inhibiting p38 suppressed ERK but stimulated JNK and

downregulation of PKC suppressed PI3K and stimulated ERK (Zatechka & Lou, 2002b). This data highlights the complexity of the signalling network. Communication between the signalling systems results in particular pathways being upregulated to compensate for the loss of others. It also demonstrates that a reduction in a particular component of a given signalling pathway could result in the transduction of a signal down a different signalling cascade, which could have different effects to those which would usually occur, e.g. apoptosis instead of survival.

5.1.5 Signalling pathways in DM1

The cell signalling pathways which transduce signals from external stimuli into biological responses within the cell are highly complex. Disturbances in the regulation of cell signalling pathways are responsible for many diseases from cancer to neurological diseases. Studies have shown that the expanded CTG repeats in *DMPK* may result in altered activation of cell signalling pathways (Usuki *et al.*, 2000; Usuki *et al.*, 2008; Befly *et al.*, 2010). Mouse myoblasts transfected with *DMPK* containing small repeats (5 repeats) and large repeats (160 repeats) were shown to activate different signalling pathways in response to oxidative stress caused by the addition of 0.3 μM methylmercury. Cells transfected with *DMPK* containing small repeats activated the MAPK/ERK pathway, in comparison to the MAPK/JNK pathway activated in cells transfected with *DMPK* containing large repeats. This difference in activated signalling pathways resulted in apoptotic cell death in the cells activating the MAPK/JNK pathway and survival in those that activated the MAPK/ERK pathway (Usuki *et al.*, 2000). It was subsequently shown that the intracellular concentration of ROS accumulated faster in cells expressing larger repeats and that ASK1 was activated much earlier and to a greater extent in cells expressing large repeats compared to small repeats (Usuki *et al.*, 2008). ASK1 is found upstream of both the MAPK/JNK and MAPK/p38 pathways, which are both involved in cell survival and apoptosis. The aberrant activation of signalling pathways has also been observed in human myoblasts from congenitally affected DM1 fetuses. When the DM1 cells were treated with differentiating medium, fewer cells were shown to differentiate compared to controls. Differentiation of myoblasts is dependent on the activation of the MAPK/p38 pathway and the inactivation of the MAPK/ERK

pathway, however, in DM1 myoblasts activation of p38 in response to differentiating medium was 50% lower than controls and activation of both MEK and ERK1/2 were found to be markedly higher (Befly *et al.*, 2010). These studies indicate that the regulation of cell signalling pathways in DM1 cells may be disturbed.

Under conditions of serum deprivation, DM1 LECs have been shown to have significantly increased levels of apoptosis, despite also being shown to release signalling factors capable of significantly increasing growth and/or survival in the non-virally transformed cell line, FHL124 (see figures 3.15 and 4.5). This data would indicate that their responses to these signalling factors are somehow impaired. The DM1 LECs may be activating different pathways in response to the factors released which could result in the alternative outcome of apoptosis. Alternatively, the DM1 LECs may be activating pathways which initiate apoptosis in response to the stress caused by serum deprivation and may be releasing factors in an attempt to counteract this. Investigating the activation of pathways involved in growth, proliferation, survival and apoptosis in DM1 LECs may help to elucidate the cause of increased levels of cell death during serum deprivation and also of reduced population doubling times when compared to control LECs.

5.2 Aims

The aim of this chapter is to elucidate the signalling pathways which are active in the human DM1 lens epithelial cell lines. These will be analysed during serum deprivation in order to establish pathways activated by autocrine signalling and during normal culture conditions (medium supplemented with 10% FCS). The roles that these pathways play in cell growth, proliferation, survival and apoptosis will also be investigated. Differences in the activation of particular pathways between DM1 and control lens cells could help to identify the cause of reduced population doubling times and the increased levels of cell death following serum deprivation in DM1 LECs.

5.3 Results

5.3.1 Signalling pathways activated in DM1 lens epithelial cells cultured in SF medium

To establish which downstream signalling pathways are active during autocrine signalling, levels of the activated signalling molecules pAkt, pERK, pJNK, pp38 and pPKC were measured in CCat1 and DMCat1 cells in response to serum deprivation. Activated protein levels were measured throughout the culture period shown in figure 3.9 using Western blotting.

Levels of pAkt in both cell lines decreased during the culture period (see figure 5.6). At all but one time point (day 57) the DMCat1 cells had greater levels of pAkt compared to CCat1. Average levels revealed significantly greater levels of pAkt in DMCat1 cells compared to CCat1 ($p < 0.01$).

Levels of pERK were detected throughout the culture period in DMCat1 cells, however, after 43 days in culture pERK was undetectable in CCat1 cells, despite a relatively stable expression of total ERK (see figure 5.7). Until that point the two cell types had similar levels of pERK and average levels throughout the culture period did not reveal a significant difference despite DMCat1 having greater levels.

Levels of pJNK in both cell lines decreased during the culture period (see figure 5.8). At each time point the DMCat1 cells had much greater levels of pJNK compared to CCat1. Average levels revealed that DMCat1 had significantly greater levels of pJNK ($p < 0.01$).

Levels of pp38 decreased in both cell types during the culture period and could not be detected at later passages (see figure 5.9). There was no significant difference in the levels of pp38 between CCat1 and DMCat1 cells.

Levels of pPKC and PKC varied during the culture period in both CCat1 and DMCat1 cells (see figure 5.10). After 36 days in culture, the levels of both pPKC and PKC were barely detectable in CCat1 cells, whereas both were still detected in DMCat1 cells. Average levels throughout the culture period reveal greater levels of pPKC in DMCat1 cells, however, this was not significant.

Differences in the levels of activated signalling molecules in DMCat1 cells compared to CCat1 cells are summarised in table 5.1.

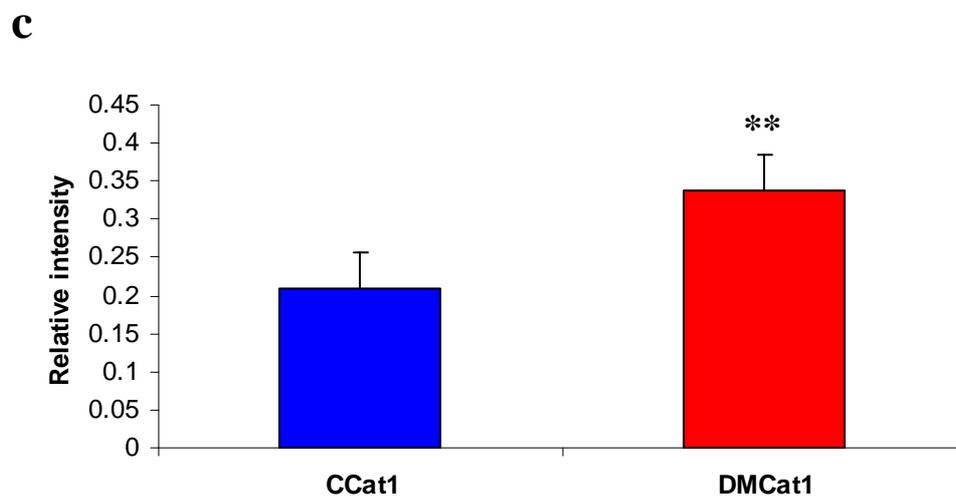
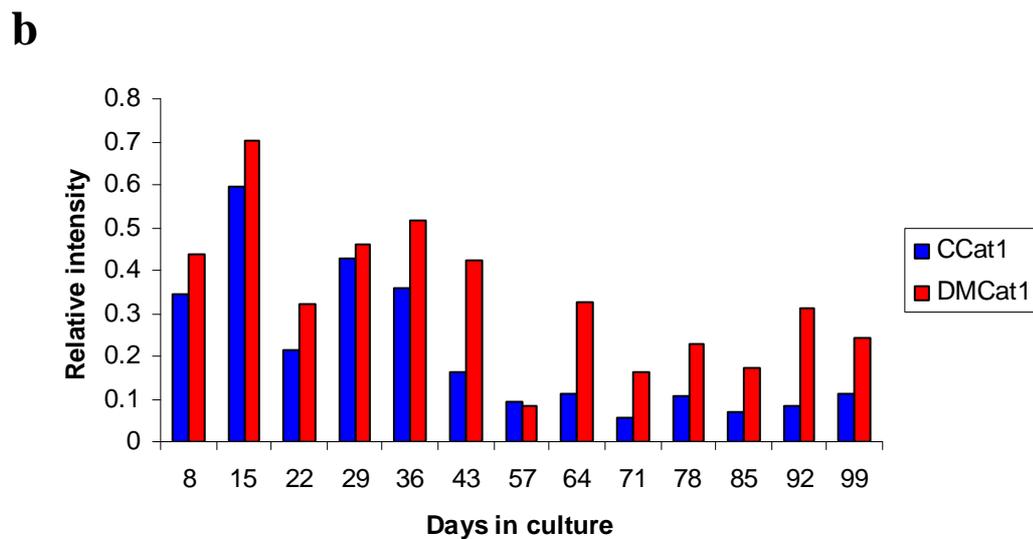
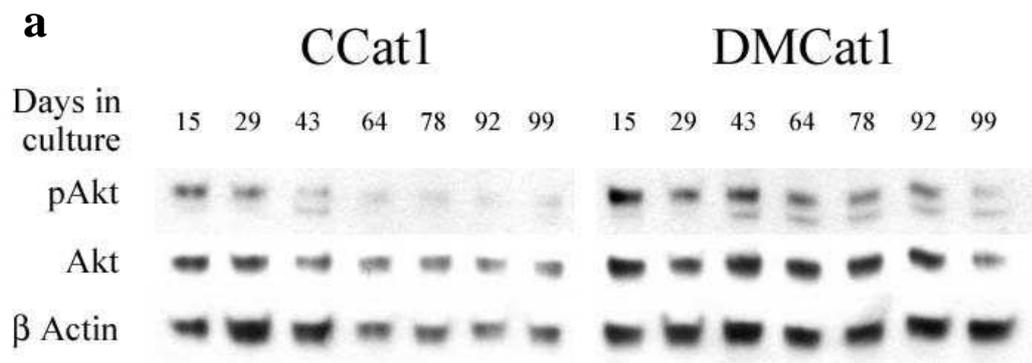


Figure 5.6 – Activation of Akt in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of pAkt and total Akt from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pAkt were normalised to total Akt. (c) Average pAkt levels show that DMCat1 has significantly greater levels of pAkt compared to CCat1 ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test, ** $p < 0.01$ relative to CCat1).

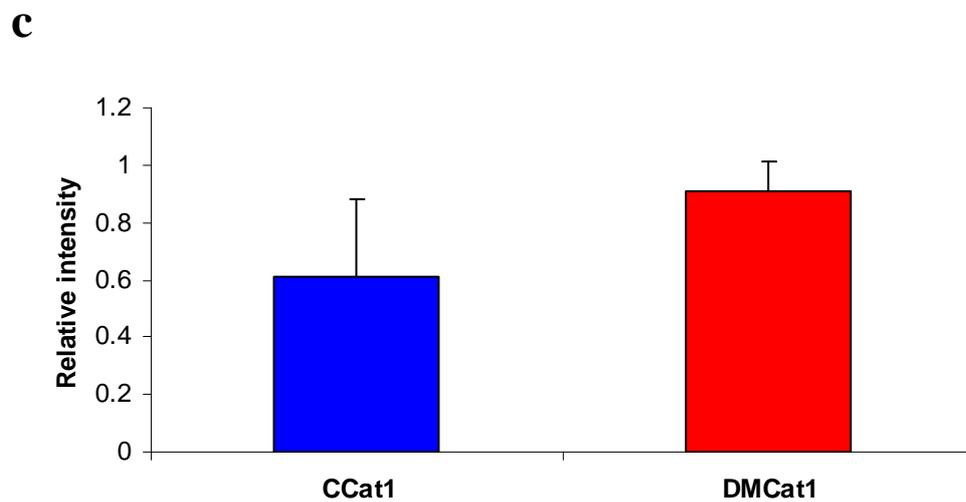
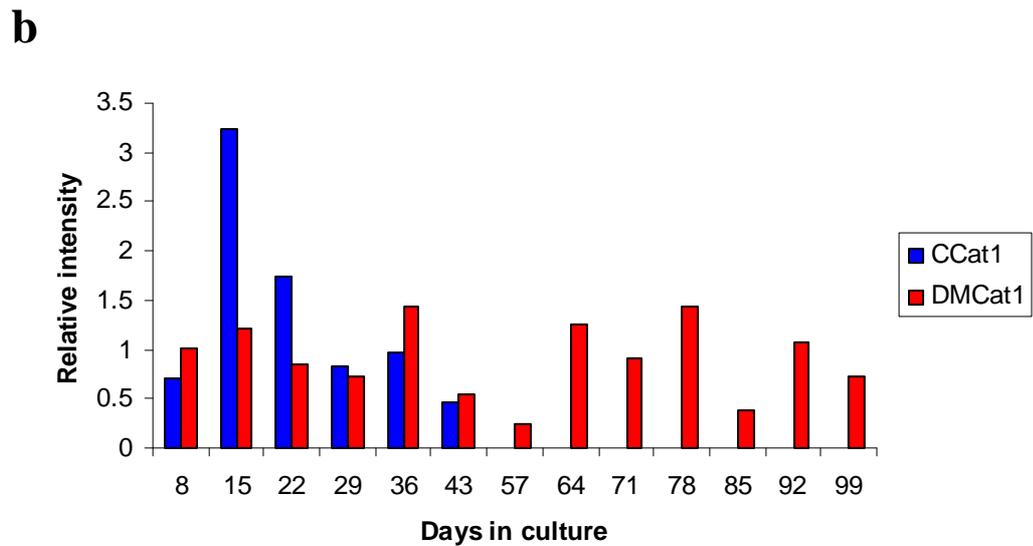
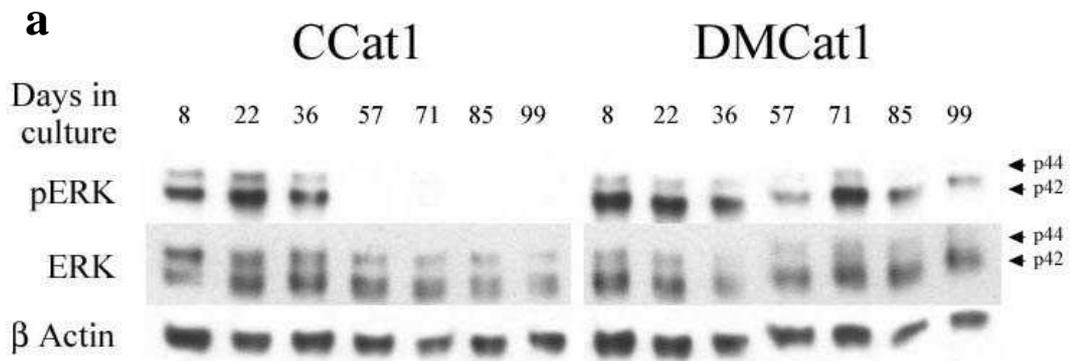


Figure 5.7 – Activation of ERK in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of pERK and total ERK from a selection of samples. β actin is included as a loading control. **(b)** Bands were analysed densitometrically and levels of pERK were normalised to total ERK. **(c)** Average pERK levels show that DMCat1 has greater levels of pERK, but this is not significant ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test).

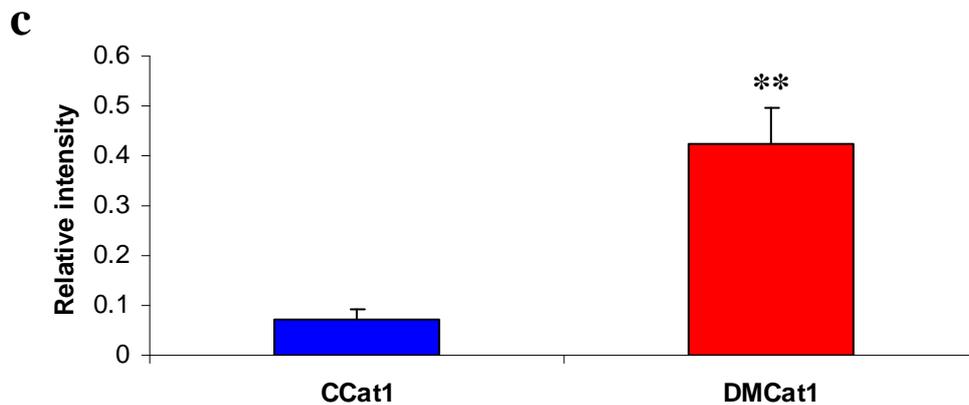
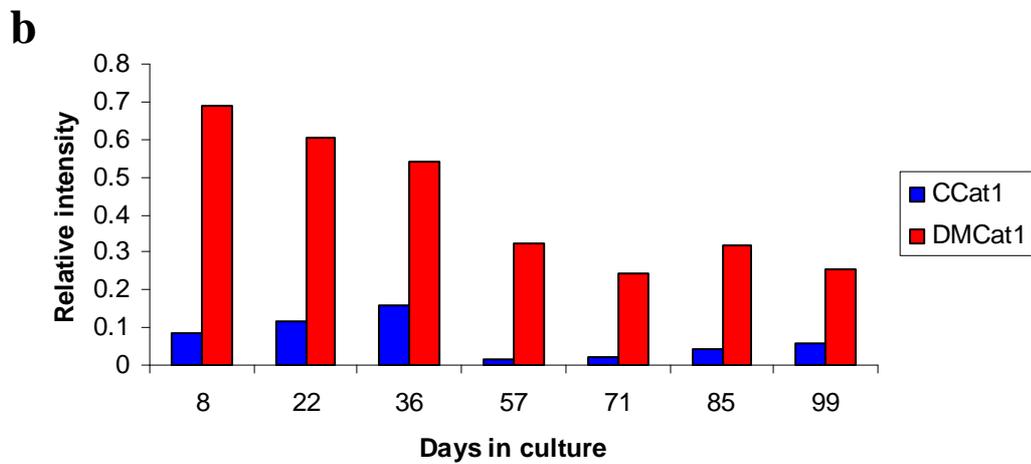
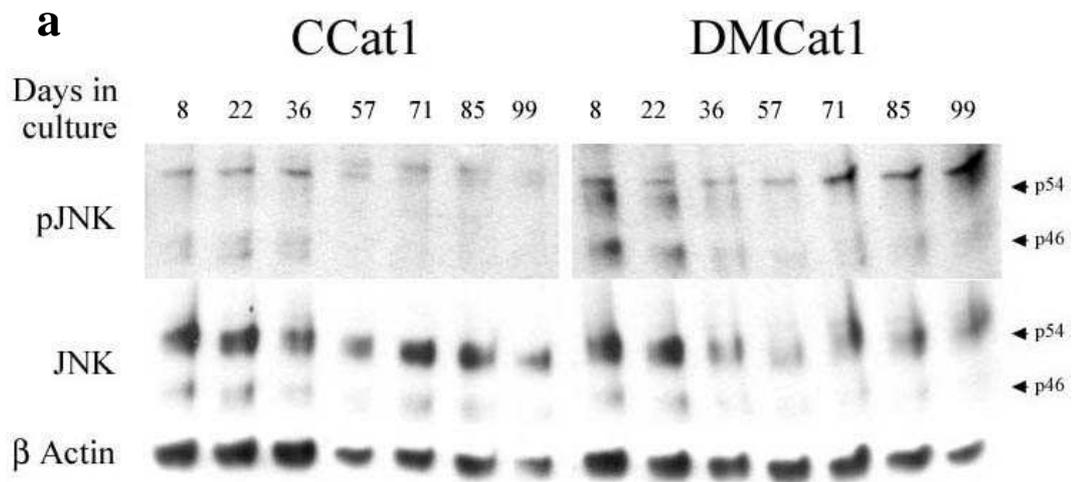


Figure 5.8 – Activation of JNK in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of pJNK and total JNK from a selection of samples. β actin is included as a loading control. **(b)** Bands were analysed densitometrically and levels of pJNK were normalised to total JNK. **(c)** Average pJNK levels show that DMCat1 has significantly greater levels of pJNK compared to CCat1 ($n = 7$, data expressed as mean \pm SEM of seven independent experiments, statistical analysis was performed by Student's T test, ** $p < 0.01$ relative to CCat1).

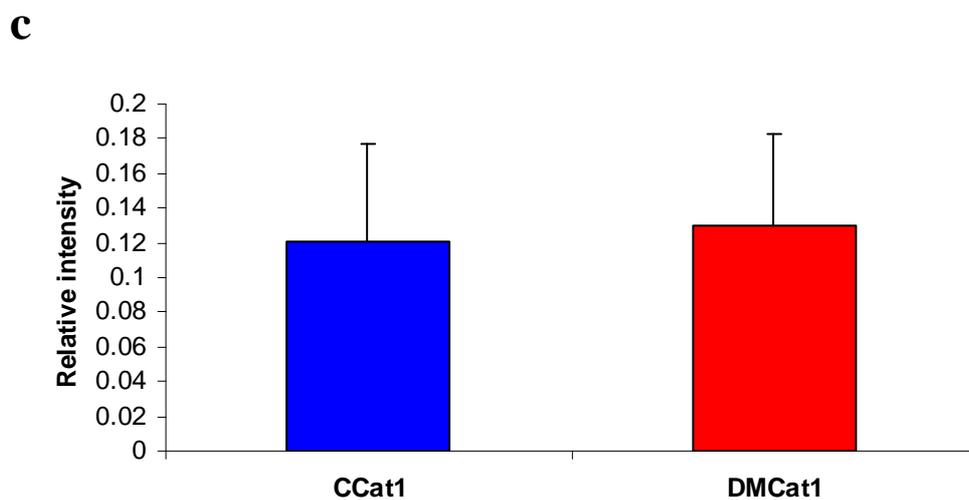
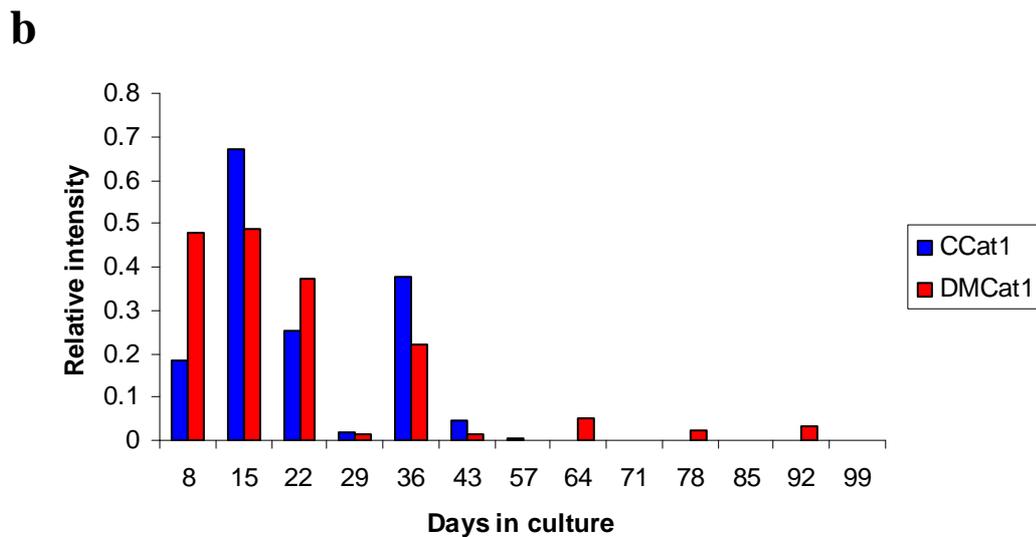
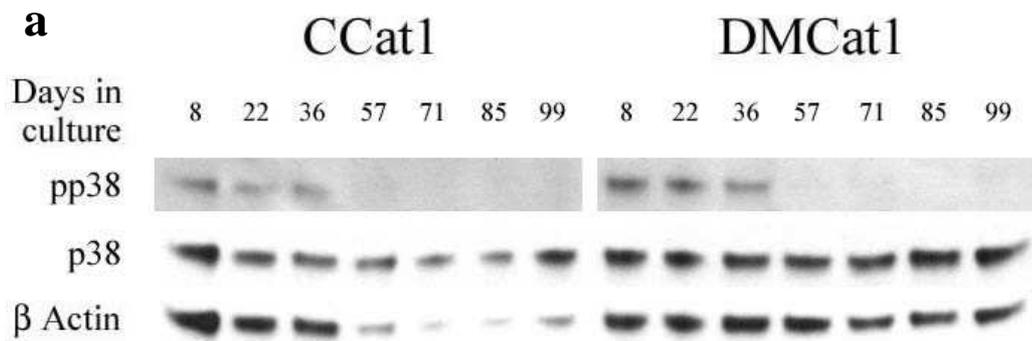


Figure 5.9 – Activation of p38 in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of pp38 and total p38 from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pp38 were normalised to total p38. (c) Average pp38 levels reveal no significant difference between CCat1 and DMCat1 cells ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test).

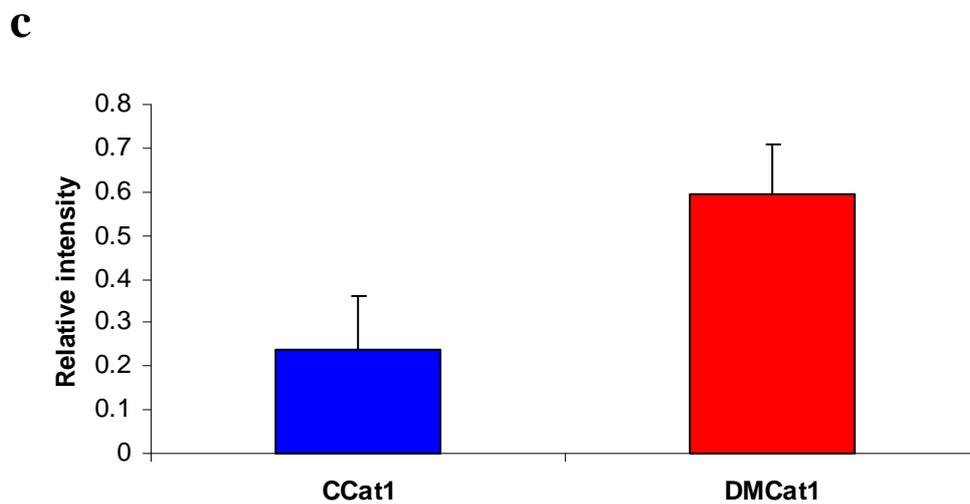
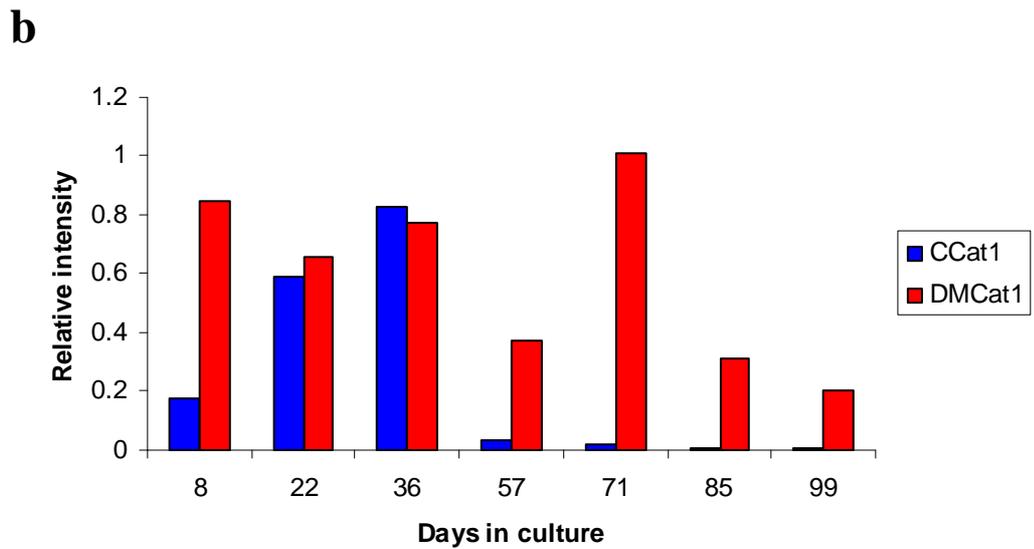
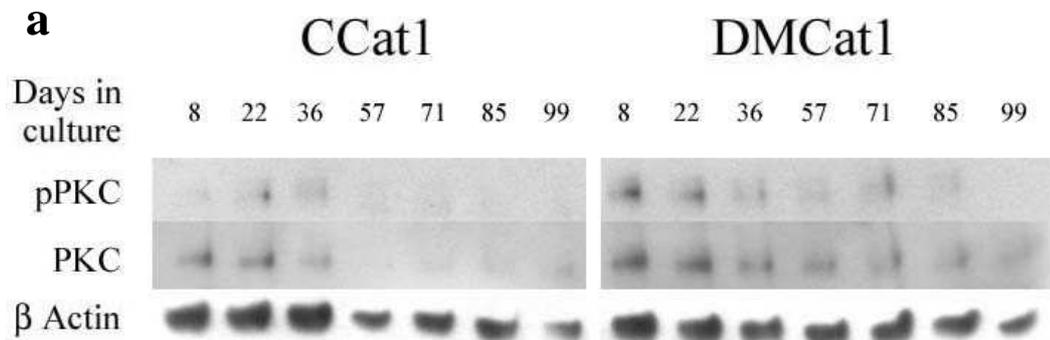


Figure 5.10 – Activation of PKC in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of pPKC and total PKC from a selection of samples. β actin is included as a loading control. **(b)** Bands were analysed densitometrically and levels of pPKC were normalised to total PKC. **(c)** Average pPKC levels show that DMCat1 has greater levels of pPKC compared to CCat1 but this is not significant ($n = 7$, data expressed as mean \pm SEM of seven independent experiments, statistical analysis was performed by Student's T test).

5.3.2 Signalling pathways activated in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS

To establish which signalling pathways are active under normal culture conditions, levels of the activated signalling molecules pAkt, pERK, pJNK, pp38 and pPKC were measured in CCat1 and DMCat1 cells grown in medium supplemented with 10% FCS, throughout the culture period shown in figure 3.9 using Western blotting.

As was seen with cells cultured in SF medium, the levels of pAkt in CCat1 cells decreased during the culture period, however, this was not as noticeable in DMCat1 cells (see figure 5.11). At all but one time point (85 days) the DMCat1 cells had greater levels of pAkt. Average levels throughout the culture period reveal significantly greater levels in DMCat1 cells compared to CCat1 cells, which was also observed in SF medium ($p < 0.01$).

Levels of pERK and ERK varied during the culture period in both cell types (see figure 5.12). After 57 days in culture, pERK was rarely detected in CCat1 cells, whereas pERK was found at each time point in DMCat1 cells. Average levels throughout the culture period show that DMCat1 cells have significantly greater levels of pERK compared to CCat1 cells ($p < 0.01$).

Levels of pJNK were significantly greater in DMCat1 cells compared to CCat1 cells ($p < 0.01$) (see figure 5.13). This was also observed in cells cultured in SF medium, however, activation of JNK appeared to be lower in both cell types when cultured in medium supplemented with 10% FCS. After 43 days in culture, activation of JNK was no longer seen in CCat1 cells, whereas pJNK was detected at each time point in DMCat1 cells.

In medium supplemented with 10% FCS, levels of pp38 were very low (see figure 5.14). The levels were greater at earlier time points in the culture period, which was also observed in SF medium, however, levels appeared to be much lower than those observed in SF medium. There was no difference in levels of pp38 between the two cell types.

In medium supplemented with 10% FCS, levels of pPKC were lower in DMCat1 cells compared to CCat1 cells, however, this was not significant (see figure 5.15). This was the opposite of what was observed in cells following addition of SF medium. Differences in the levels of activated signalling molecules in DMCat1 cells compared to CCat1 cells are summarised in table 5.1.

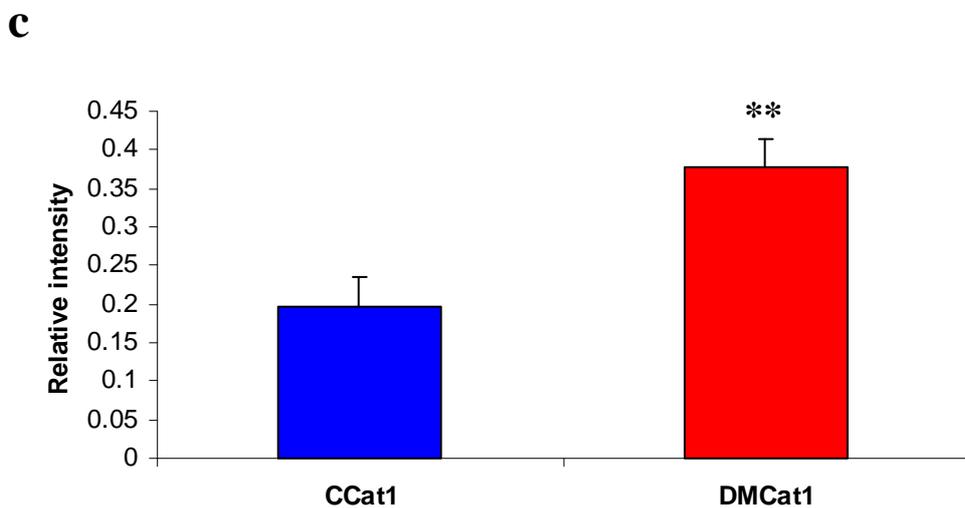
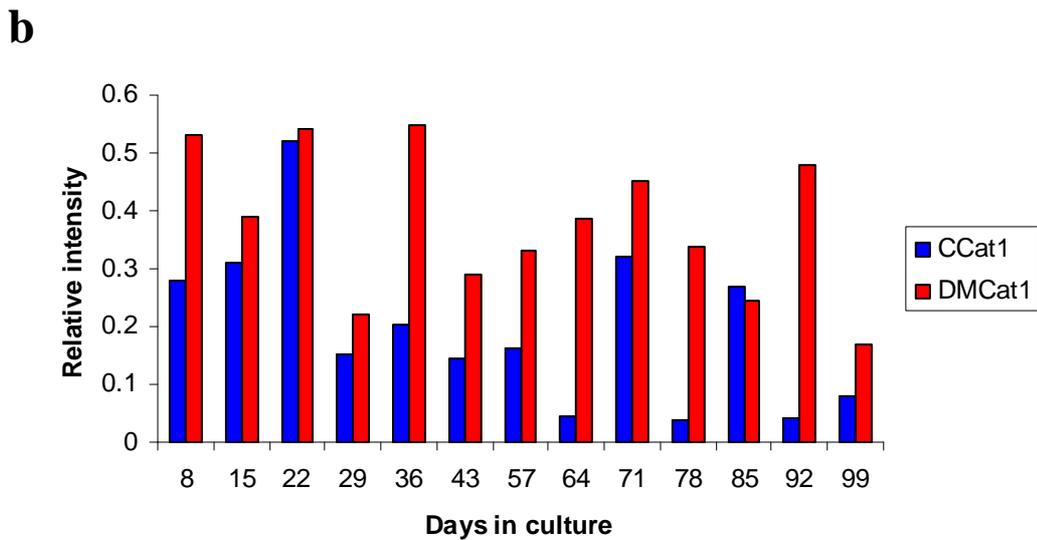
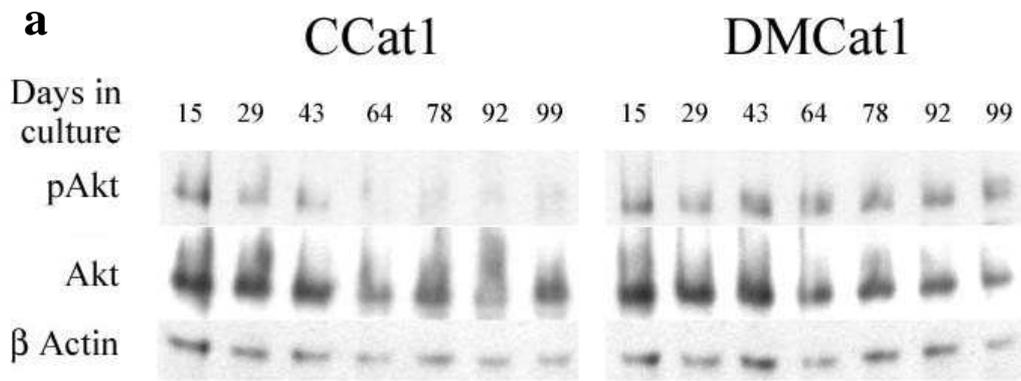


Figure 5.11 – Activation of Akt in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS. (a) An example Western blot showing levels of pAkt and total Akt from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pAkt were normalised to total Akt. (c) Average pAkt levels show that DMCat1 has significantly greater levels of pAkt compared to CCat1 ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test, ** $p < 0.01$ relative to CCat1).

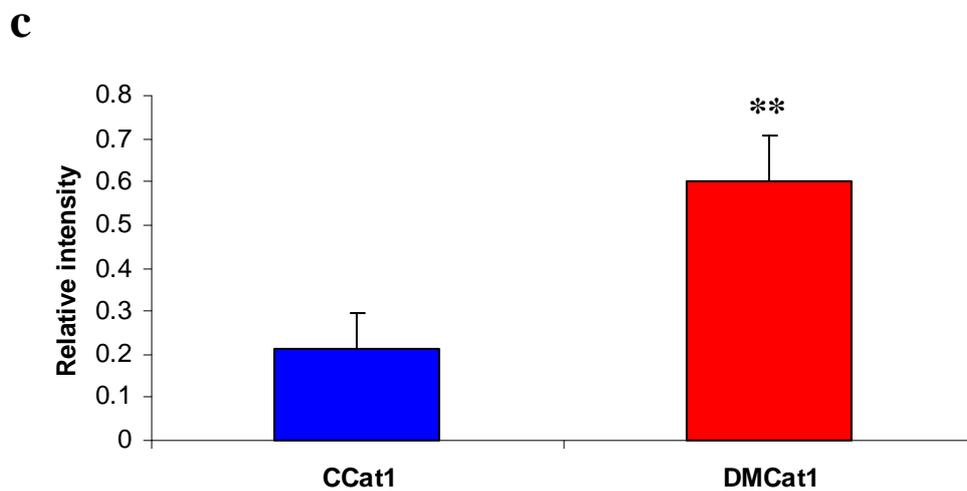
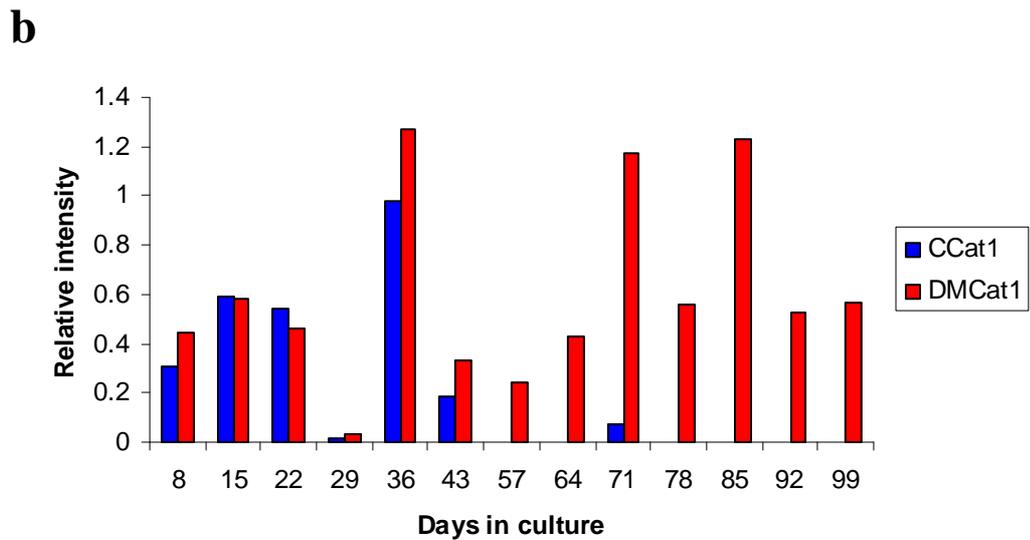
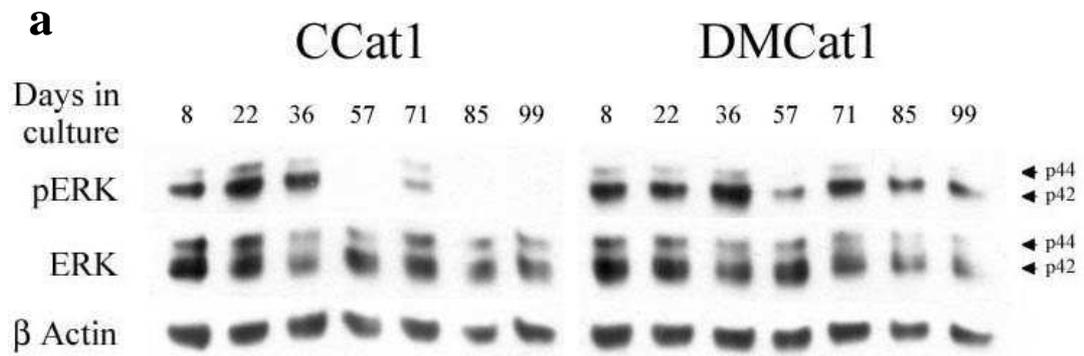


Figure 5.12 – Activation of ERK in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS. (a) An example Western blot showing levels of pERK and total ERK from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pERK were normalised to total ERK. (c) Average pERK levels show that DMCat1 has significantly greater levels of pERK compared to CCat1 (n = 13, data expressed as mean ± SEM of thirteen independent experiments, statistical analysis was performed by Student’s T test, ** p < 0.01 relative to CCat1).

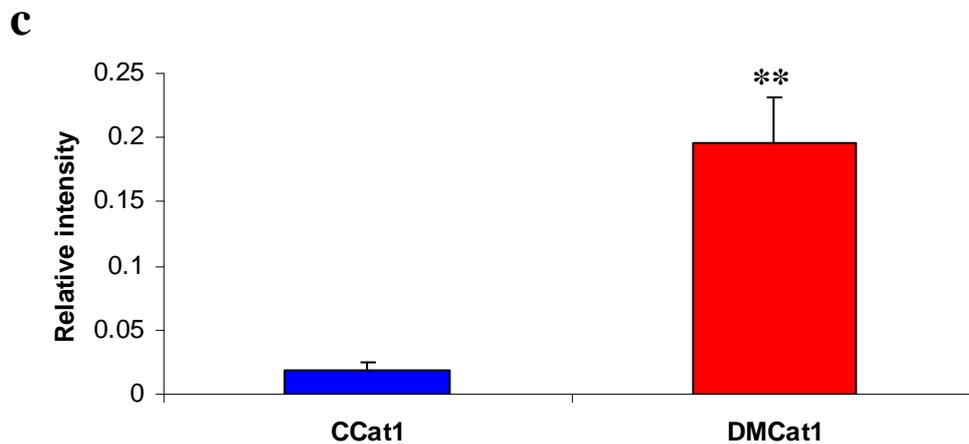
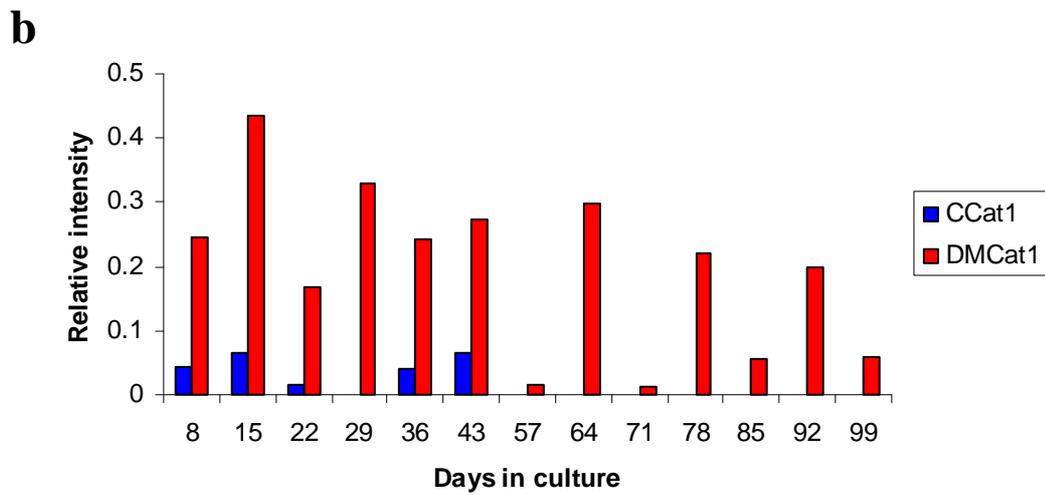
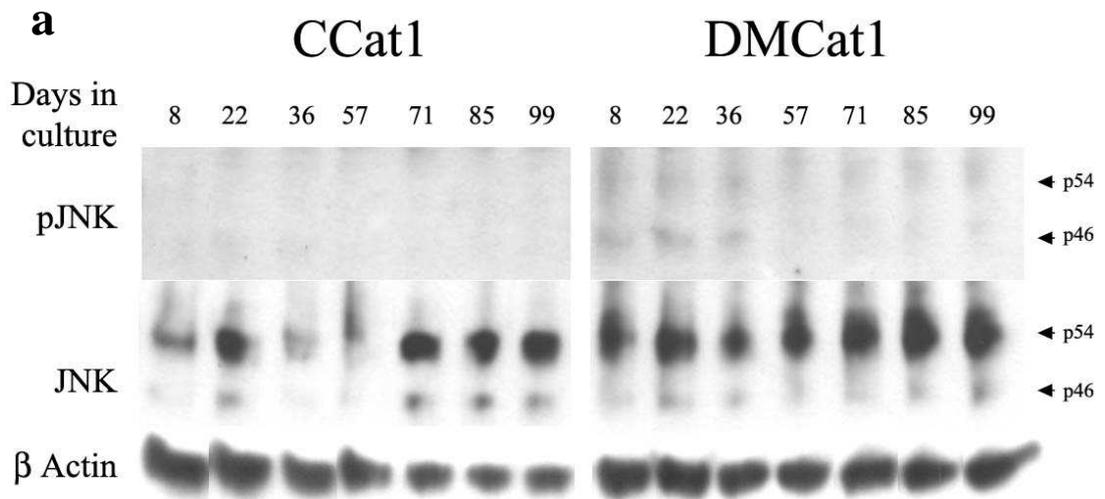


Figure 5.13 – Activation of JNK in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS. (a) An example Western blot showing levels of pJNK and total JNK from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pJNK were normalised to total JNK. (c) Average pJNK levels show that DMCat1 has significantly greater levels of pJNK compared to CCat1 (n = 13, data expressed as mean ± SEM of thirteen independent experiments, statistical analysis was performed by Student’s T test, ** p < 0.01 relative to CCat1).

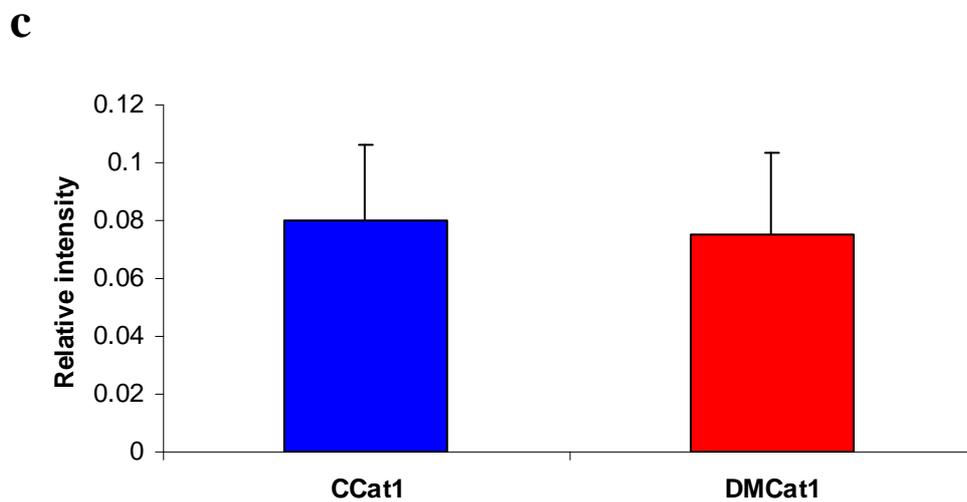
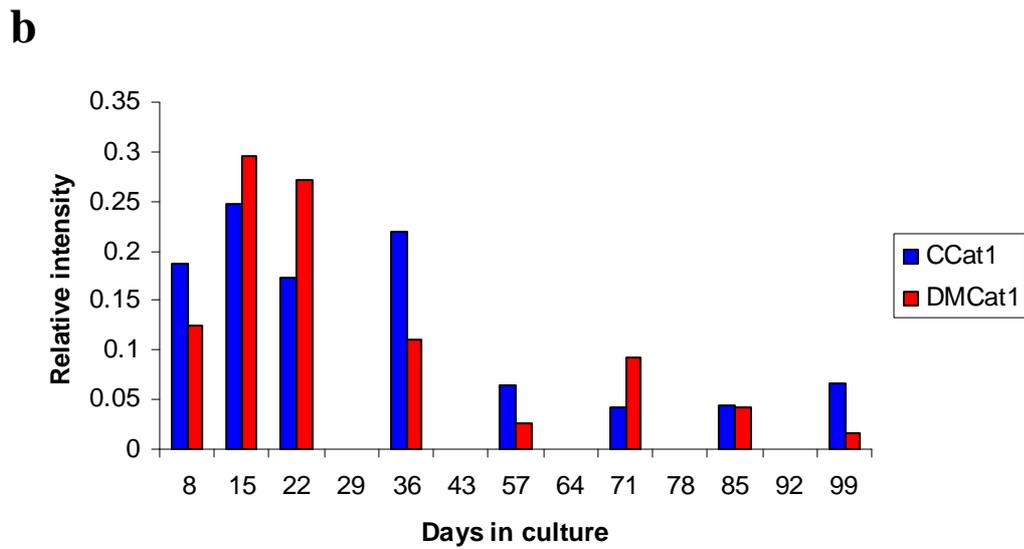
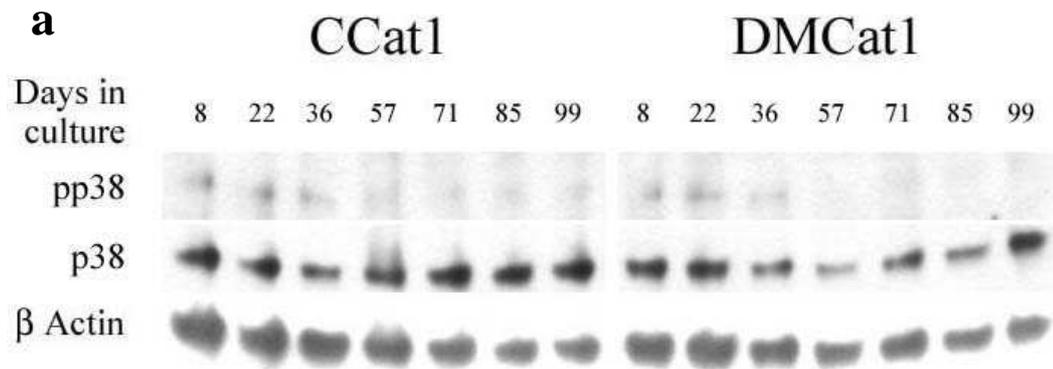


Figure 5.14 – Activation of p38 in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS. (a) An example Western blot showing levels of pp38 and total p38 from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pp38 were normalised to total p38. (c) Average pp38 levels reveal no significant difference between CCat1 and DMCat1 cells (n = 13, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test).

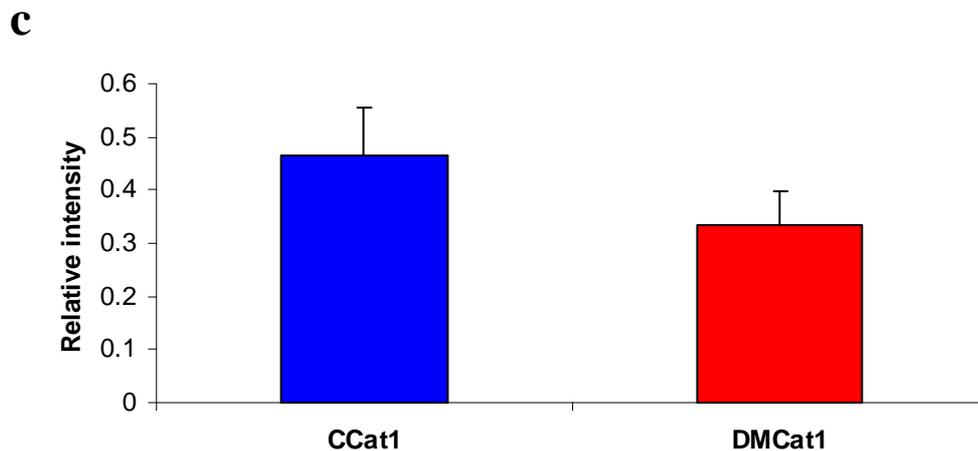
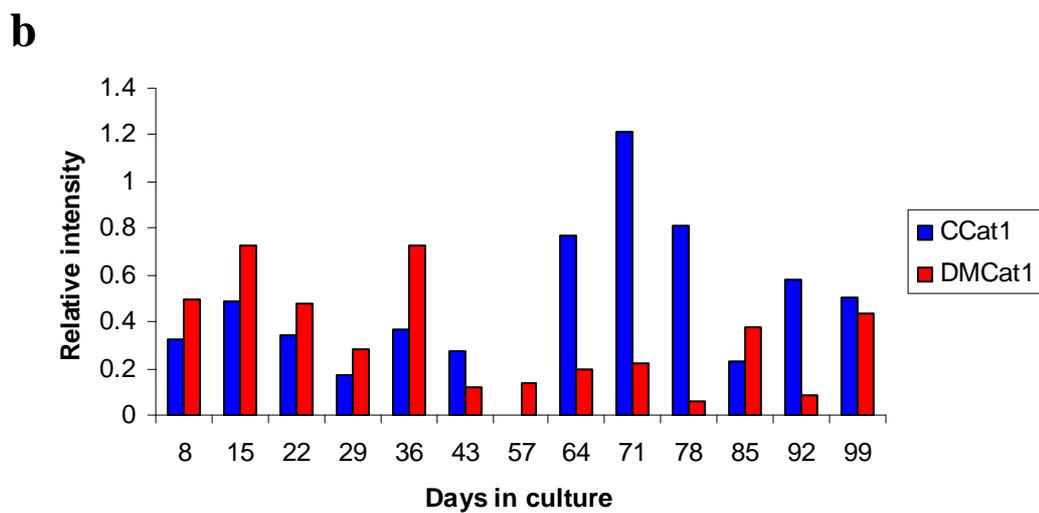
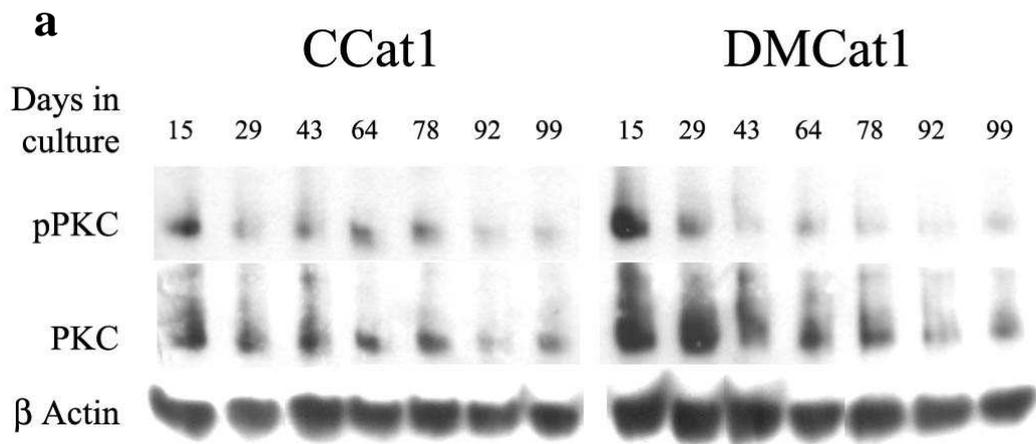


Figure 5.15 – Activation of PKC in DM1 lens epithelial cells cultured in medium supplemented with 10% FCS. (a) An example Western blot showing levels of pPKC and total PKC from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pPKC were normalised to total PKC. (c) Average pPKC levels show that DMCat1 has lower levels of pPKC compared to CCat1 but this is not significant ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test).

Signalling molecule	SF medium	Medium supplemented with 10% FCS
pAkt	↑**	↑**
pERK	↑	↑**
pJNK	↑**	↑**
pp38	-	-
pPKC	↑	↓

Table 5.1 – Differences in levels of activated signalling molecules in DMCat1 cells compared to CCat1 cells. ↑** = a significant increase ($p < 0.01$), ↑ = an increase, – = no difference, ↓ = a decrease.

5.3.3 Inhibition of signalling pathways in DM1 lens epithelial cells cultured in SF medium

In order to establish the role of particular signalling pathways in autocrine signalling in DM1 lens epithelial cells, inhibitors were applied to CCat1 and DMCat1 cells cultured in SF medium for 48 hours.

Signalling via the PI3K/Akt signalling pathway was inhibited using the PI3K inhibitor, LY294002 (25 μ M) (see figure 5.16). A significant reduction in growth was observed in both cell lines following addition of the inhibitor ($p < 0.01$), however, levels of cell growth were significantly lower in DMCat1 cells compared to CCat1 ($p < 0.01$). Despite the significant reduction in cell growth, no change was seen in levels of cell death measured using the LDH assay in CCat1 cells. Although levels of cell death were elevated in DMCat1 cells, this was not found to be statistically significant. Signalling via the MAPK/ERK signalling pathway was inhibited using the MEK1 inhibitor, PD98059 (10 μ M) (see figure 5.17). Neither cell growth nor cell death was significantly affected in either cell line following addition of the inhibitor. Signalling via the MAPK/JNK signalling pathway was inhibited using the JNK inhibitor, SP600125 (1 μ M and 10 μ M) (see figure 5.18). Levels of cell growth and death were not affected in either CCat1 or DMCat1 cells following addition of SP600125 (1 μ M), however, a significant reduction in growth was observed in CCat1 cells following addition of SP600125 (10 μ M) ($p < 0.05$). Although a reduction in growth was observed in CCat1 cells at this concentration, no significant effect was seen in levels of cell death.

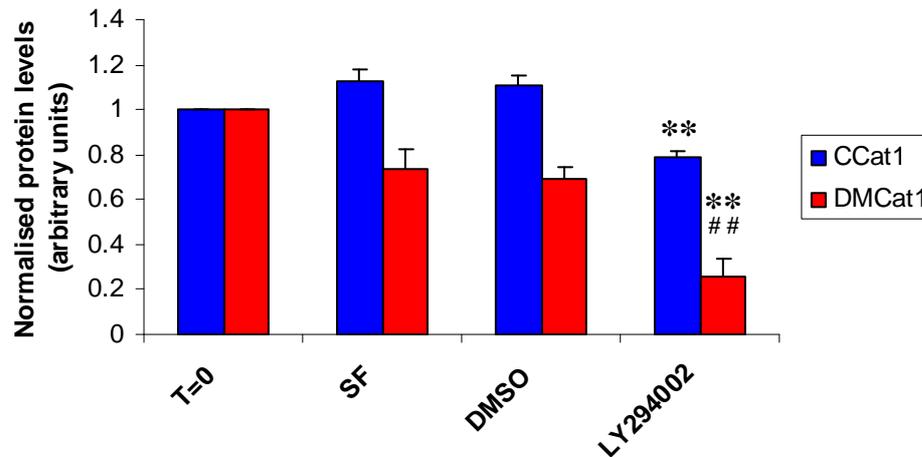
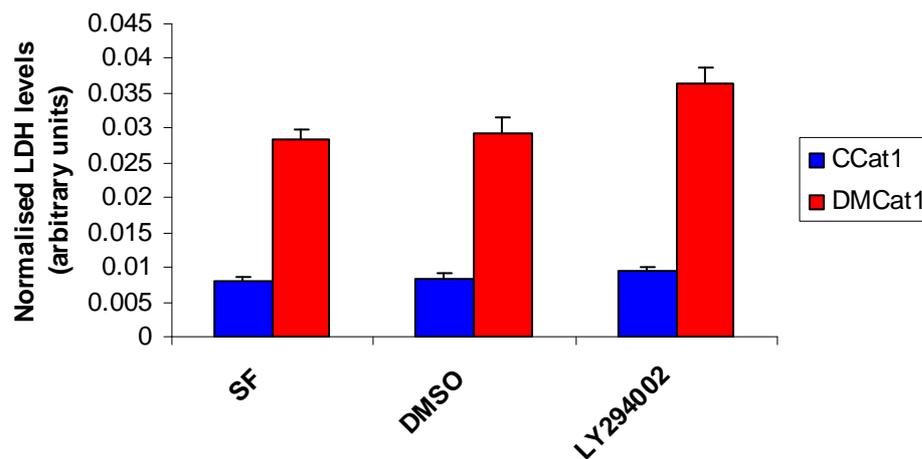
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Figure 5.16 - The effect of PI3K inhibition on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of LY294002 (25 μM) resulted in a significant decrease in cell growth in both CCat1 and DMCat1 cells, however, DMCat1 had significantly lower levels of cell growth following LY294002 addition compared to CCat1 ($n = 4$, data expressed as mean \pm SEM of four independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test, ** $p < 0.01$ relative to the relevant DMSO control, ## $p < 0.01$ relative to CCat1 treated with LY294002 (25 μM)). (b) LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of LY294002 resulted in an increase in cell death in DMCat1 cells but this was not statistically significant ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test).

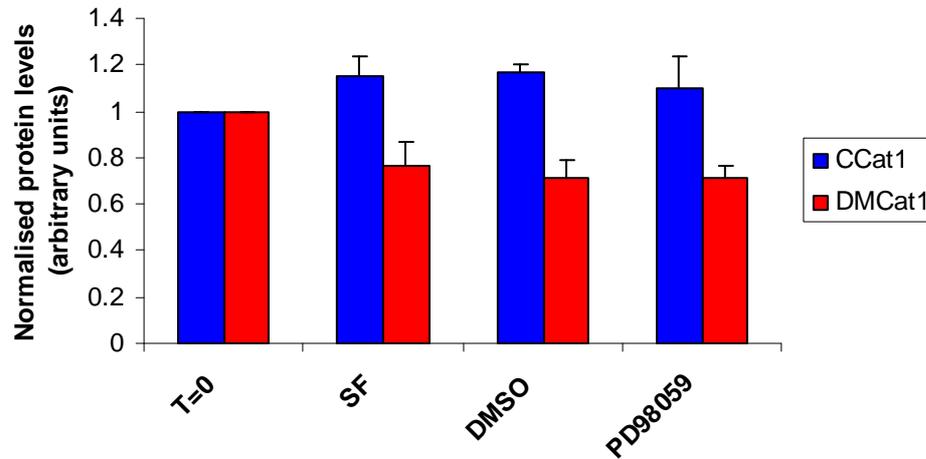
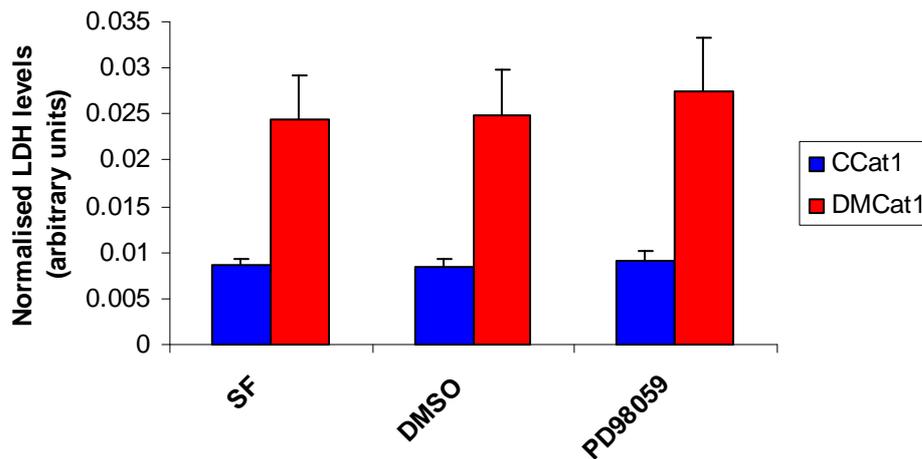
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Figure 5.17 - The effect of MEK1 inhibition on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of PD98059 (10 μM) had no effect on cell growth ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test). **(b)** LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of PD98059 had no significant effect on cell death ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test).

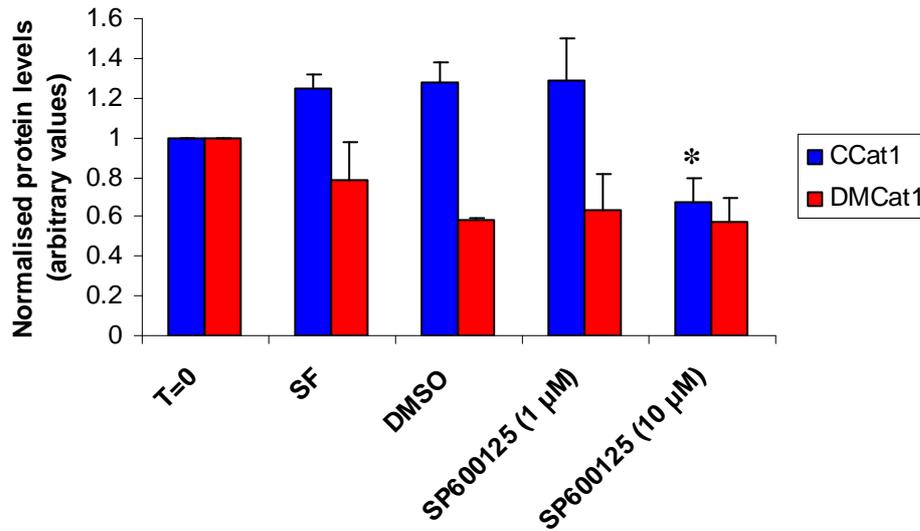
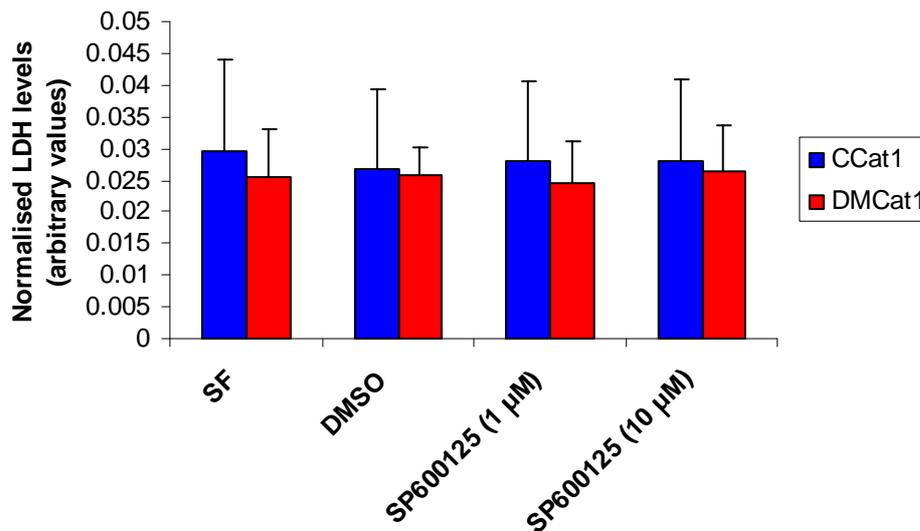
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Figure 5.18 - The effect of JNK inhibition on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of SP600125 (1 μM) had no effect on cell growth, however, addition of SP600125 (10 μM) significantly reduced growth in CCat1 cells (n = 3, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test, * p < 0.05 relative to CCat1 DMSO control). (b) LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of SP600125 (1 μM and 10 μM) had no effect on cell death (n = 3, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test).

To verify that LY294002, PD98059 and SP600125 inhibited signalling downstream of their targets, Western blots were performed on protein samples collected 24 hours after addition of the inhibitors. Levels of pAkt were shown to be markedly reduced following addition of LY294002 (25 μ M) as were levels of pERK following addition of PD98059 (10 μ M), however, levels of pJNK were not reduced by SP600125 (1 μ M and 10 μ M) at either concentration (see figure 5.19). At the greater concentration, SP600125 (10 μ M) was actually shown to increase levels of pJNK.

Figure 5.19 – The effects of PI3K, MEK1 and JNK inhibition on downstream signalling in DM1 lens epithelial cells after 24 hours. Levels of (a) pAkt (b) pERK1/2 and (c) pJNK were examined by Western blotting following addition of LY294002 (25 μ M), PD98059 (10 μ M) and SP600125 (1 μ M and 10 μ M) respectively. Bands were analysed densitometrically and graphs show the activated protein levels normalised to their total protein control. Addition of LY294002 and PD98059 inhibited activation of Akt and ERK1/2 respectively in both CCat1 and DMCat1 cells, however, SP600125 failed to inhibit the activation of JNK.

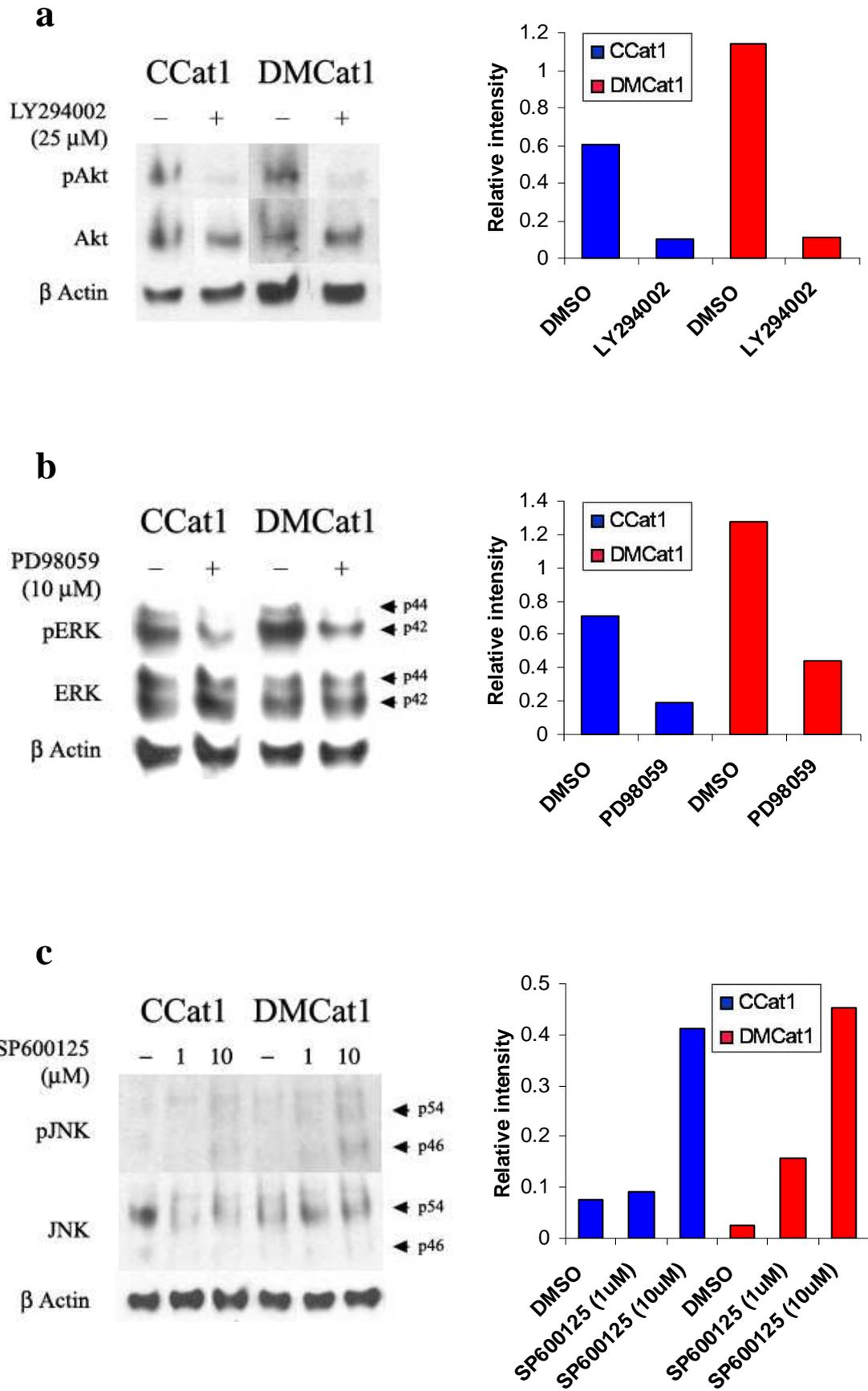


Figure 5.19 – see previous page for figure legend.

5.3.3.1 Apoptotic cell death following inhibition of signalling pathways in DM1 lens epithelial cells cultured in SF medium

Inhibition of the PI3K/Akt pathway caused a significant reduction in cell growth in both CCat1 and DMCat1 cells, however, no increase in cell death as measured using the LDH assay was observed in CCat1 cells and the increase in DMCat1 cells was not significant. We therefore used the more sensitive technique of the TUNEL assay to look specifically at levels of apoptotic cell death. The inhibitors, LY294002 (25 μ M) and PD98059 (10 μ M) were applied to CCat1 and DMCat1 cells for 24 hours before levels of apoptotic cell death were analysed.

Inhibition of the PI3K/Akt pathway using LY294002 resulted in a significant increase in the level of apoptotic cell death in DMCat1 cells from 17.0% \pm 0.9% in the DMSO control (2.5 μ l/ml) to 30.9% \pm 3.8% in LY294002 ($p < 0.05$) (see figure 5.20). Interestingly, there was no difference in the levels of apoptotic cell death in CCat1 cells with 12.2% \pm 4.7% apoptotic cells in the DMSO control compared to 11.5% \pm 4.1% in LY294002. This data indicates that inhibition of the PI3K/Akt pathway using LY294002 does increase levels of cell death in DMCat1 cells and that cell death occurs via apoptosis, however, it does not increase levels of cell death in CCat1 cells.

Levels of apoptotic cell death were also analysed following inhibition of the MAPK/ERK pathway using PD98059 (see figure 5.21). No significant difference in levels of apoptotic cell death was observed between the DMSO control (1 μ l/ml) and PD98059 in CCat1 or DMCat1 cells. Apoptotic cell death did increase in CCat1 cells following addition of PD98059 from 10.9% \pm 2.5% to 17.4% \pm 2.8%, however, the variability in the samples did not make this significant. In DMCat1 cells there were 16.7% \pm 0.7% apoptotic cells in the DMSO control compared to 15.4% \pm 2.4% in PD98059.

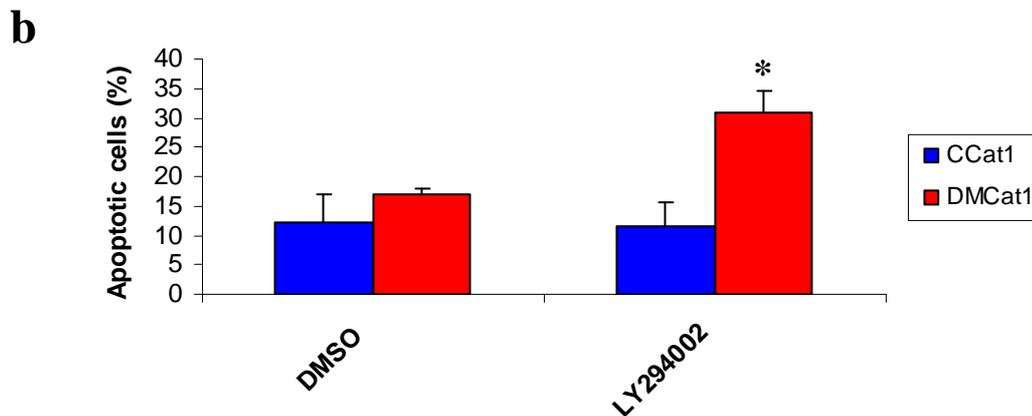
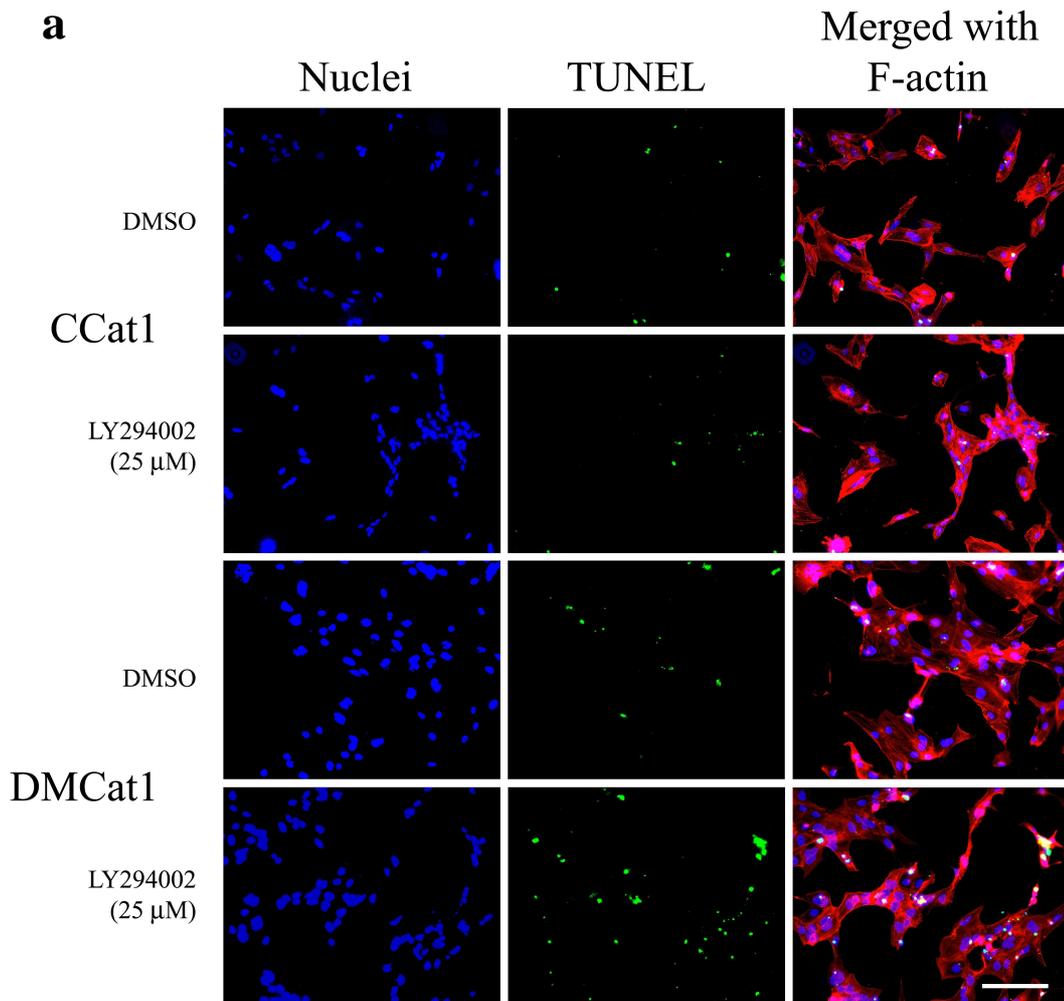


Figure 5.20 – Apoptotic cell death in DM1 lens epithelial cells following PI3K inhibition. CCat1 and DMCat1 cells were treated with DMSO (2.5 μ l/ml) or LY294002 (25 μ M) for 24 hours. (a) Fluorescent micrographs showing nuclei labelled with DAPI (blue), apoptotic cells labelled using a TUNEL assay (green) and F-actin labelled with Texas red-X phalloidin (red) (bar = 200 μ m). (b) Analysis of TUNEL positive apoptotic cells shows that addition of LY294002 significantly increases apoptosis in DMCat1 cells (n = 3, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by Student's T test, * p < 0.05 relative to DMSO control and CCat1 cells treated with LY294002).

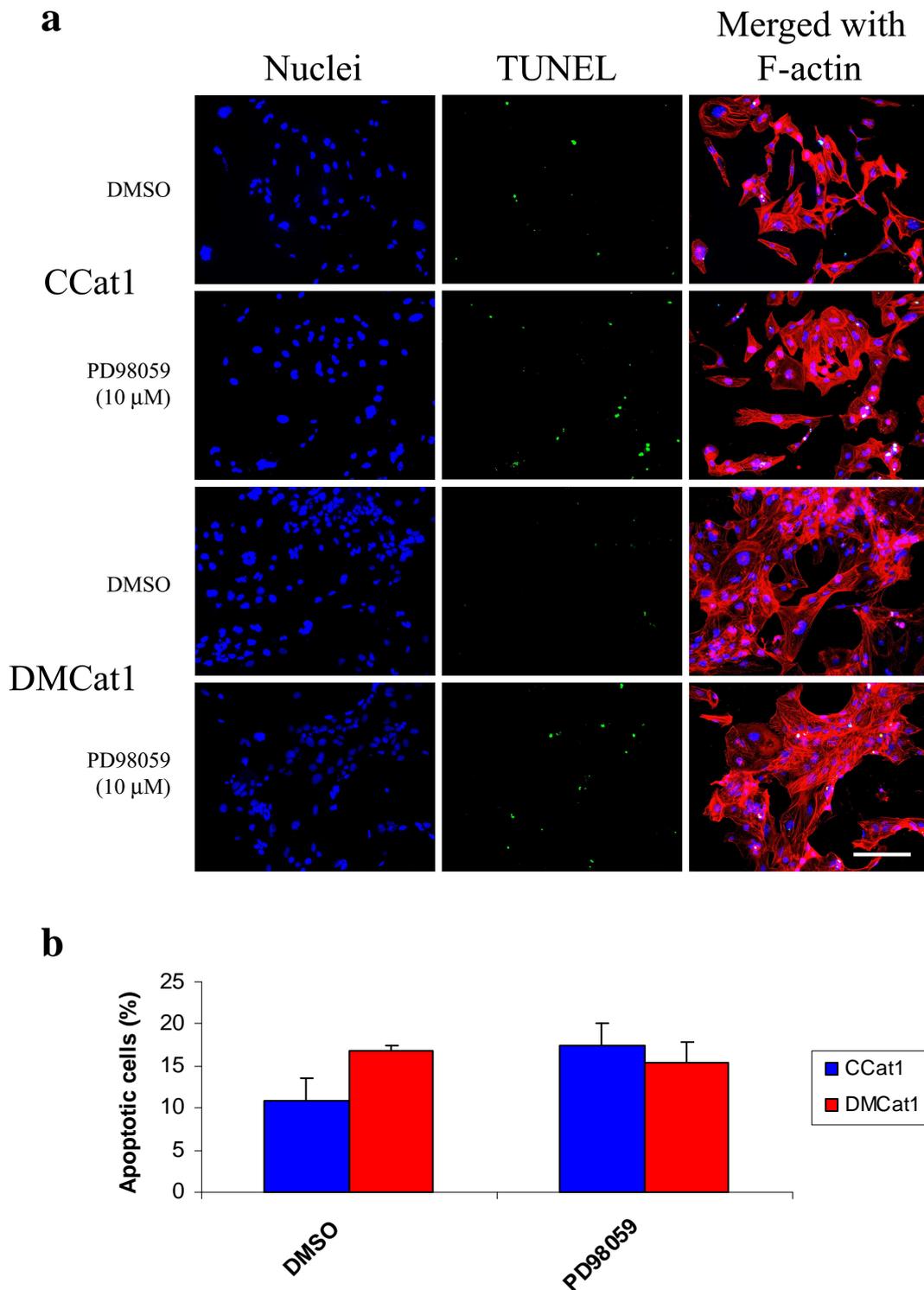


Figure 5.21 – Apoptotic cell death in DM1 lens epithelial cells following MEK1 inhibition. Cells were treated with DMSO (1 μl/ml) or PD98059 (10 μM) for 24 hours. (a) Fluorescent micrographs of cells showing nuclei labelled with DAPI (blue), apoptotic cells labelled using a TUNEL assay (green) and F-actin labelled with Texas red-X phalloidin (red) (bar = 200 μm). (b) Analysis of TUNEL positive apoptotic cells shows that addition of PD98059 has no significant effect on levels of apoptotic cell death (n = 3, data expressed as mean ± SEM of three independent experiments, statistical analysis was performed by Student’s T test).

5.3.4 Investigation of the Akt pathway in DM1 lens epithelial cells

The Akt pathway appears to be critical for survival in DM1 lens epithelial cells and was therefore investigated in more detail.

The PI3K activator, 740 Y-P (50 mg/ml), was applied to CCat1 and DMCat1 cells for 48 hours (see figure 5.22). A minor increase in protein levels was observed in DMCat1 cells indicating a slight increase in growth, however, the increase was not significant. No difference in protein levels was observed in CCat1 cells. LDH release was also measured and revealed a significant increase in cell death in both CCat1 and DMCat1 cells ($p < 0.01$).

Levels of the negative regulator of the PI3K/Akt pathway, PTEN, were analysed in DMCat1 and CCat1 cells following addition of SF medium throughout the culture period shown in figure 3.9 (see figure 5.23). Levels of PTEN remained relatively consistent throughout the culture period in DMCat1 cells, however, levels of PTEN increased considerably in CCat1 cells. Average levels of PTEN throughout the culture period revealed significantly lower levels of PTEN in DMCat1 cells compared to CCat1 cells ($p < 0.01$).

In order to establish whether DM1 LECs do have lower levels of PTEN or whether the previous result was just a consequence of the increase in levels seen in the control, the levels of PTEN were analysed in all eight of the cell lines at early time points (between days 29 and 36 of the culture period shown in figure 3.9) during the culture period (see figure 5.24). The data revealed that CCat1 cells actually had the lowest level of PTEN compared to the other control cell lines and that it was the only cell line with similar levels of PTEN to the DM1 cell lines. Average levels revealed that DM1 cell lines had lower levels of PTEN compared to the controls, however, this was not statistically significant due to the variability in the cell lines.

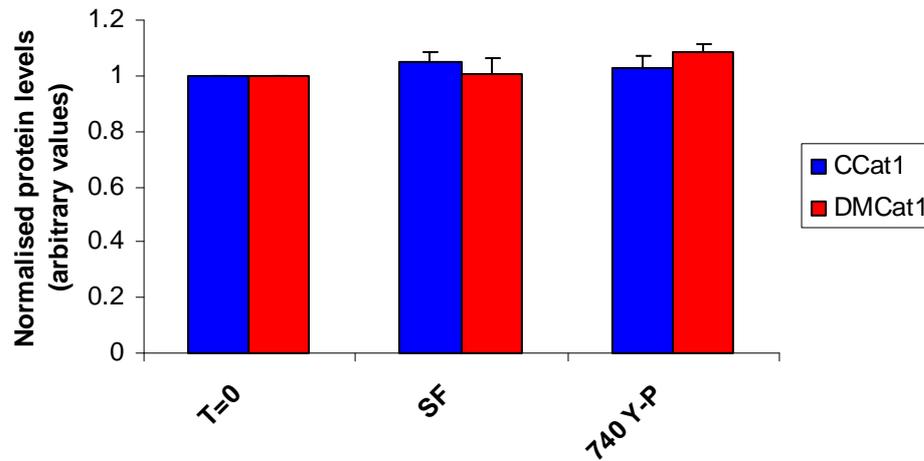
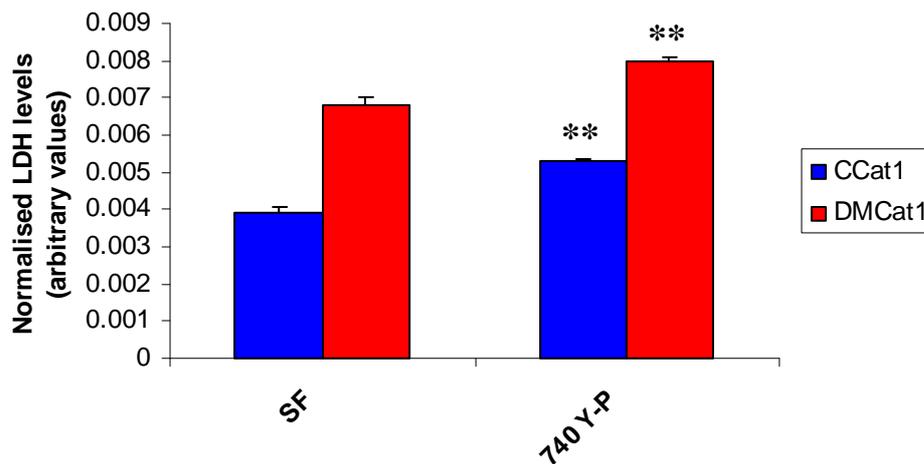
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Figure 5.22 - The effect of Akt activation on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of 740 Y-P (50 ng/ml) had no significant effect on cell growth (n = 4, data expressed as mean \pm SEM, statistical analysis was performed by one-way ANOVA with Tukey's test). (b) LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of 740 Y-P significantly increased cell death in both CCat1 and DMCat1 cells (n = 4, data expressed as mean \pm SEM, statistical analysis was performed by one-way ANOVA with Tukey's test, ** p < 0.01 relative to SF control).

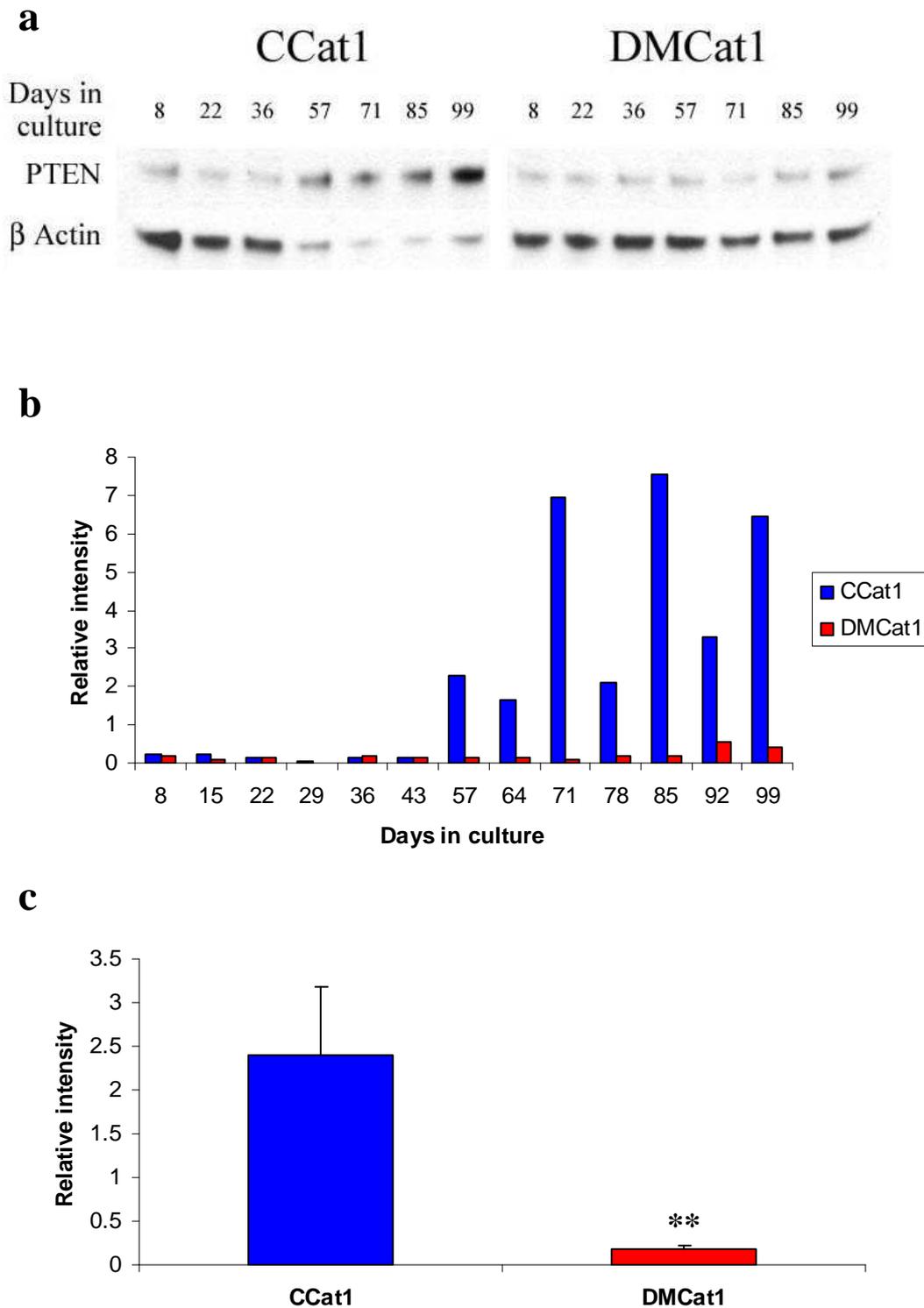


Figure 5.23 – Levels of PTEN in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of PTEN from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of PTEN were normalised to β actin. (c) Average PTEN levels revealed that DMCat1 cells had significantly lower levels of PTEN compared to CCat1 cells ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test, ** $p < 0.01$ relative to CCat1).

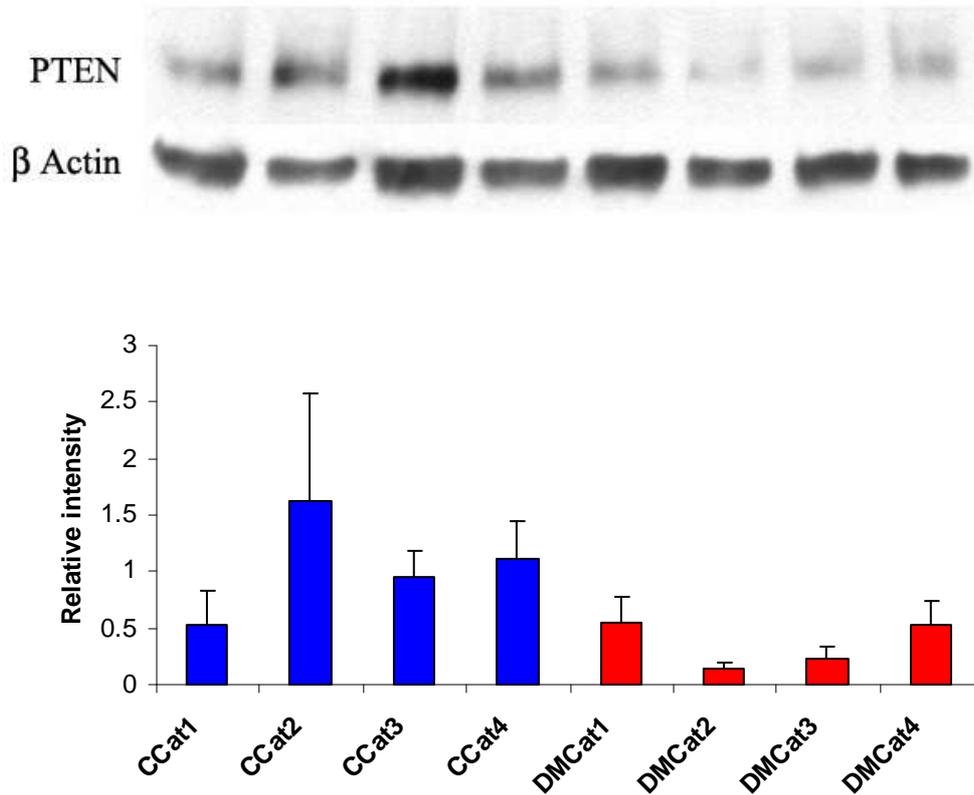


Figure 5.24 – Levels of PTEN in DM1 lens epithelial cells. An example Western blot is shown with β actin included as a loading control. Bands were analysed densitometrically and levels of PTEN were normalised to β actin. PTEN levels were greater in the majority of the control cell lines, however, this was not statistically significant ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by Student's T test).

5.3.4.1 Protein synthesis downstream of Akt in DM1 lens epithelial cells

Akt activates mTORC1, which is a key regulator of protein synthesis (see figure 5.1). The signalling pathway downstream of Akt which is involved in protein synthesis was inhibited using the mTOR inhibitor, Rapamycin (25 nM and 50 nM), which blocks the activity of mTORC1 (see figure 5.25). Levels of cell growth decreased in both CCat1 and DMCat1 cells following addition of the inhibitor, however, neither was statistically significant. Levels of cell death were not significantly affected in either CCat1 or DMCat1 cells following addition of Rapamycin (25 nM and 50 nM).

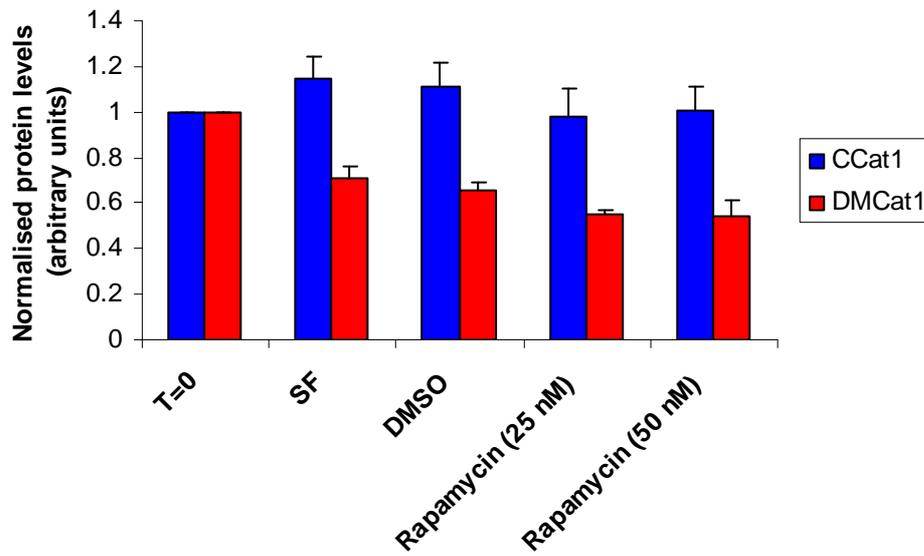
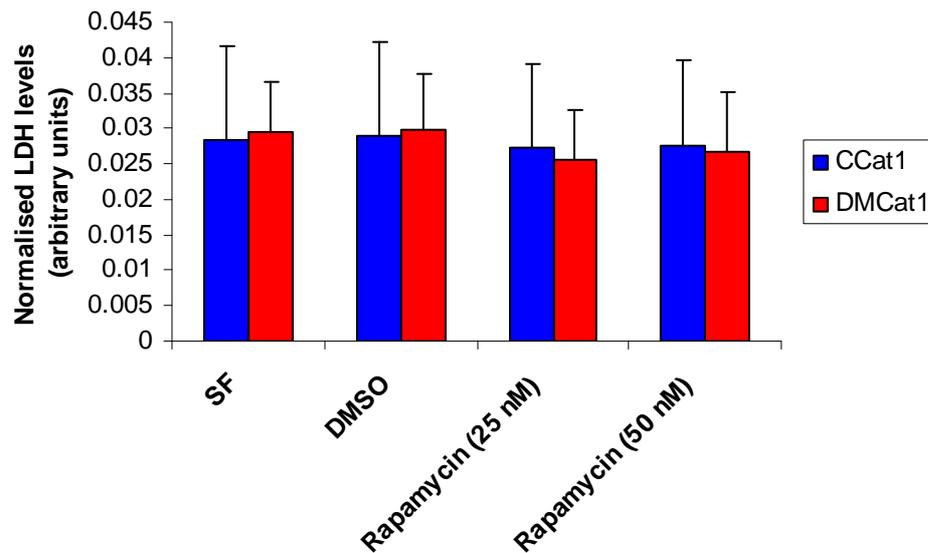
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Figure 5.25 - The effect of mTOR inhibition on DM1 lens epithelial cells over 48 hours. (a) Cell growth was measured by calculating the total protein ($\mu\text{g/ml}$) in cell lysates after 48 hours. Protein values were normalised to the T = 0 value for each cell line and averages were calculated. Addition of Rapamycin (25 nM and 50 nM) had no significant effect on cell growth ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test). **(b)** LDH release was measured to analyse cell death and levels were normalised to the total protein levels at T = 0. Addition of Rapamycin (25 nM and 50 nM) had no effect on cell death ($n = 3$, data expressed as mean \pm SEM of three independent experiments, statistical analysis was performed by one-way ANOVA with Tukey's test).

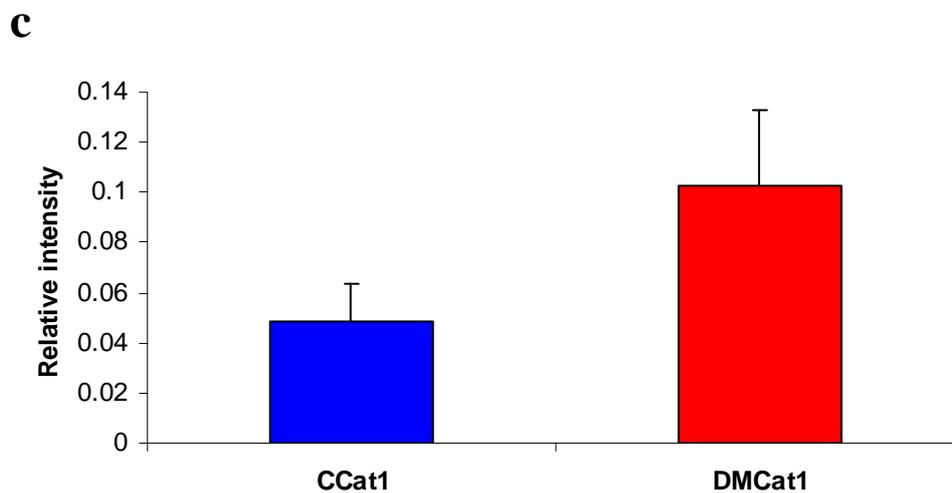
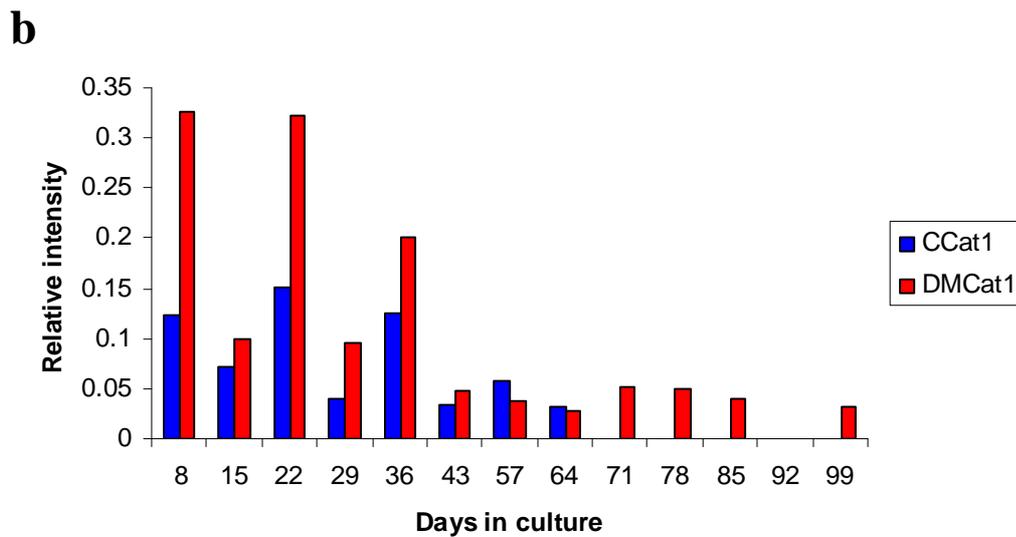
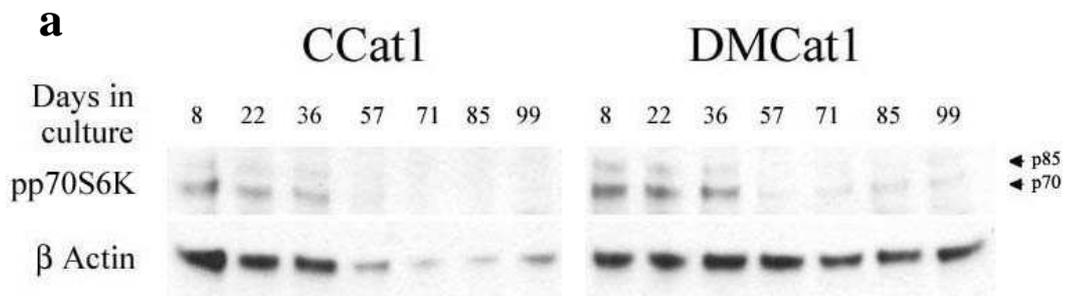


Figure 5.26 – Levels of pp70S6K in DM1 lens epithelial cells cultured in SF medium. (a) An example Western blot showing levels of pp70S6K from a selection of samples. β actin is included as a loading control. (b) Bands were analysed densitometrically and levels of pp70S6K were normalised to β actin. (c) Average pp70S6K levels show that DMCat1 has greater levels of pp70S6K compared to CCat1, however, this is not statistically significant ($n = 13$, data expressed as mean \pm SEM of thirteen independent experiments, statistical analysis was performed by Student's T test).

Levels of activated p70S6K (pp70S6K), which is found downstream of mTORC1, were analysed in DMCat1 and CCat1 cells following addition of SF medium throughout the culture period shown in figure 3.9 (see figure 5.26). Levels of pp70S6K were higher at early time points during the culture period. After 64 days pp70S6K could no longer be detected in CCat1 cells, however, low levels were still found in DMCat1 cells. Average levels throughout the culture period revealed that DMCat1 had greater levels of pp70S6K, however, the difference was not significant.

To confirm that inhibition of mTORC1 using Rapamycin inhibited the activation of p70S6K, levels of pp70S6K in CCat1 and DMCat1 cells treated with Rapamycin (50 nM) for 24 hours were analysed using Western blotting. Rapamycin completely inhibited the activation of p70S6K (see figure 5.27).

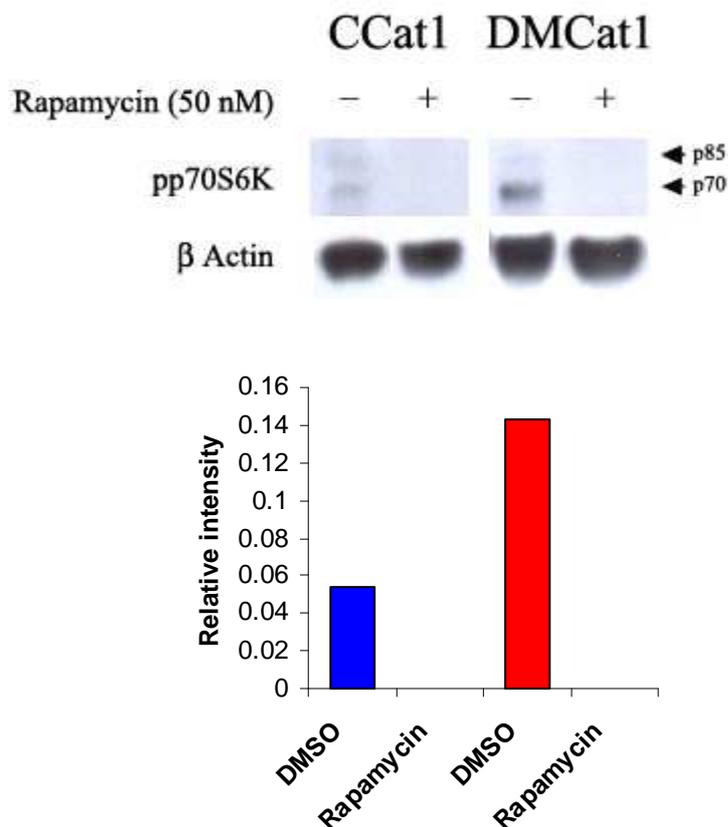


Figure 5.27 – Levels of pp70S6K in DM1 lens epithelial cells following inhibition of mTOR. Protein levels of pp70S6K were examined by Western blotting and analysed densitometrically. Graph shows the activated protein levels normalised to β actin. Addition of Rapamycin (50 nM) for 24 hours completely inhibited the activation of p70S6K.

5.4 Discussion

Analysis of activated signalling pathways in both SF medium and medium supplemented with 10% FCS revealed not only differences between the two cell lines, but also differences during the culture period within each cell line (see figures 5.6-5.15). Interestingly, there was relatively little difference in levels of activated proteins between cells cultured in SF medium and those cultured in medium supplemented with 10% FCS. Under both conditions the DM1 cell line, DMCat1, had significantly greater levels of activated Akt and JNK compared to the control cell line, CCat1. Levels of pERK were also greater in DMCat1 cells under both conditions, however, the difference was only significant in medium supplemented with 10% FCS. In SF medium, levels of pPKC were greater in DMCat1 cells compared to CCat1 cells, but this was not significant. The opposite was found in medium supplemented with 10% FCS, as levels of pPKC were lower in DMCat1 cells compared to CCat1 cells, but this was also not significant. No difference was found between pp38 levels in CCat1 and DMCat1 cells under either condition. Surprisingly, the pattern of activated protein levels appeared similar between the two culture conditions (see figures 5.7 and 5.12 for an example). Only active levels of p38 and JNK appeared to be reduced when cultured in medium supplemented with 10% FCS compared to SF medium. As p38 and JNK are involved in stress signalling it is likely that levels would be higher in the absence of added serum as this has been shown to induce apoptosis (see figure 3.15).

In the majority of cases, levels of both activated protein and total protein decreased during the culture period despite levels of β actin remaining relatively stable. This could indicate that the expression of cell signalling proteins decreases as the cells age. Expression of other proteins, such as the receptors known to activate the signalling pathways that have been analysed, including FGFR-2 and IGFR-1, have previously been shown to decrease in the human lens in later life (Bhuyan *et al.*, 2000). The response of LEC explants to bFGF was also shown to reduce with age in rats, with those from older rats undergoing a slower progression through the differentiation process in response to bFGF (Lovicu & McAvoy, 1992). Reduced expression of signalling molecules in our cell lines could therefore result from a reduction in expression of the receptors which

trigger their activation or could reveal a general trend in protein expression which reduces with age. Studies analysing gene expression in the human lens have revealed that epithelial cells from lenses with age-related cataract have significantly reduced gene expression compared to those of clear lenses (Zhang *et al.*, 2002; Ruotolo *et al.*, 2003). The first study used RT-PCR to show that expression of ribosomal proteins was reduced in LECs from lenses with age-related cataract. Ribosomal proteins are required for the translation of mRNA into proteins, indicating that protein synthesis would also be reduced in cataractous lenses (Zhang *et al.*, 2002). A further study used microarrays to analyse expression of over 4000 genes and found that 262 were downregulated in LECs from lenses with age-related cataract. Interestingly, the largest class of genes found to be downregulated were those involved in signal transduction (Ruotolo *et al.*, 2003). A reduction in protein expression can therefore be linked to both ageing and cataract development. Despite this, the CCat1 cells which showed the greatest variability in levels of activated proteins with some being undetectable halfway through the culture period, had relatively constant growth rates throughout culture, indicating that a reduction in cell signalling did not affect the ability to proliferate (see figure 3.9a). This data highlights the variability in cell lines and the requirement to analyse levels throughout the culture period, as is seen here, in order to obtain the most accurate results.

Signalling via the PI3K/Akt pathway appears to be critical to the survival of DMCat1 cells. Firstly, the DM1 cells release factors capable of activating the PI3K/Akt pathway in FHL124 cells (see figure 4.7), secondly, DMCat1 cells have been shown to have significantly greater levels of pAkt when compared to CCat1 cells (see figures 5.6 and 5.11), and thirdly, when signalling via the PI3K/Akt pathway is blocked a significant decrease in cell growth is observed (see figure 5.16) and the DMCat1 cells were shown to have significantly greater levels of apoptotic cell death (see figure 5.20). This data reveals that signalling via the PI3K/Akt pathway is necessary for survival in DMCat1 cells. Interestingly, levels of pAkt were significantly higher in DMCat1 cells cultured in both SF medium and medium supplemented with 10% FCS. This data could indicate that autocrine signalling via the PI3K/Akt pathway is not only important for survival during serum deprivation, but may also be required for survival in the presence of added growth factors and serum. Further experiments inhibiting signalling via the

PI3K/Akt pathway in the presence of serum would be required to confirm this. However, in the presence of the many growth factors found in serum, it is unlikely that additional autocrine signalling would be required, and therefore this could indicate that activation of Akt is independent of the addition or absence of extracellular signalling molecules and is instead somehow more directly linked to the triplet repeat mutation.

Akt requires phosphorylation of S473 by mTORC2 and T308 by PDK1 for full activation (see figure 5.1). In this study we detected phosphorylation at S473 as a marker of Akt activity. Phosphorylation of this site is therefore not directly linked to signalling via PI3K, however, inhibition of PI3K using the specific inhibitor, LY294002, blocks phosphorylation of S473, indicating that it is also regulated by PI3K activity (see figure 5.19a). This agrees with other studies which have also shown that phosphorylation of Akt by mTORC2 at S473 is regulated by PI3K activity (Sarbasov *et al.*, 2005). This data shows that signalling via both mTORC2 and PI3K could be responsible for increased levels of Akt activation in DM1 LECs. The mechanisms by which mTORC2 activity is stimulated and regulated is largely unknown, however, PI3K is activated mainly by nutrient and growth factor stimulation, but may also be activated by some GPCRs (Franke, 2008). If the triplet repeat mutation triggers Akt activation, this could occur via a more direct mechanism intracellularly or via the release of factors which activate the pathway extracellularly. A number of intracellular regulators of the PI3K/Akt pathway exist, however, the main intracellular regulator is PTEN.

PTEN is a tumour suppressor which functions by negatively regulating the PI3K/Akt pathway. PTEN dephosphorylates PIP₃ and converts it back to PIP₂, preventing the activation of Akt by PDK1. DMCat1 cells were shown to have significantly lower levels of PTEN compared to CCat1 cells and analysis of all eight cell lines also revealed reduced levels in the DM1 cell lines, however, this was not significant (see figures 5.23 and 5.24). This data is interesting as DMCat1 was shown to have greater levels of pAkt compared to CCat1 and this could therefore be due to a reduction in the levels of PTEN. PTEN levels increased in CCat1 cells during the culture period, which coincided with a decrease in pAkt levels and underlines PTEN's role as a key regulator of the PI3K/Akt pathway. PTEN levels could be reduced in DM1 cells in order to increase PI3K/Akt signalling and improve survival rates. PTEN itself is regulated both

transcriptionally and post-translationally. Analysis of PTEN expression levels using QRT-PCR may help to elucidate whether the reduction in PTEN protein is due to a reduction in transcription of the gene. PTEN is subject to many types of post-translational regulation, including interactions with other proteins, phosphorylation, ubiquitination, oxidation and acetylation, which can affect its activity, breakdown and turnover (Wang & Jiang, 2008). Further experiments would be required to assess any possible role that these modifications may play in the reduction of PTEN in DM1 LECs. As CM from DM1 cell lines was shown to increase signalling via Akt in the non-virally transformed cell line, FHL124, it is unlikely that a reduction in PTEN alone is responsible for the increase in pAkt in DMCat1. A factor must also be released which activates the PI3K/Akt pathway extracellularly, however, the identity of this factor is unknown.

Activation of the PI3K/Akt pathway using the activator 740 Y-P (50 mg/ml) caused a significant increase in cell death in both CCat1 and DMCat1 cells (see figure 5.22). This was a surprising result as inhibiting PI3K using LY294002 (25 μ M) was shown to cause a significant decrease in growth in both CCat1 and DMCat1 cells and a significant increase in apoptotic cell death in the DMCat1 cells. This data reveals that signalling via the PI3K/Akt pathway could play roles in both survival and apoptosis in human lens epithelial cells. Addition of the PI3K activator may have caused an increase in the activation of Akt which could have switched downstream signalling from survival to apoptosis via crosstalk with other signalling pathways. Alternatively, 740 Y-P may not be a specific activator of PI3K and may therefore interact with other proteins to activate or inhibit other pathways which may be responsible for the increase in cell death (Derossi *et al.*, 1998).

Inhibition of PI3K resulted in a significant decrease in levels of total protein in both CCat1 and DMCat1 cells which may be due to a reduction in protein synthesis or in cell number. A reduction in cell number could either be due to increased levels of cell death or decreased levels of proliferation. Levels of LDH revealed an increase in cell death in the DMCat1 cells, but not in the CCat1 cells, although this was not significant (see figure 5.16b). As the LDH assay did not appear to be sensitive enough to find a significant difference in the levels of cell death, we used the more sensitive technique of TUNEL to specifically analyse levels of apoptotic cell death. Levels of apoptosis were subsequently shown to be

significantly increased in the DMCat1 cells following addition of the PI3K inhibitor, LY294002 (see figure 5.20). This data indicates that signalling downstream of PI3K/Akt is primarily involved in survival in DMCat1 cells and that the decrease in levels of total protein following PI3K inhibition was therefore due to a reduction in cell number due to increased levels of cell death. This could be confirmed by analysing the phosphorylation of downstream proteins involved in the survival pathway such as BAD. BAD is a pro-apoptotic protein which targets the anti-apoptotic proteins Bcl-2 and Bcl-xL in the mitochondria and inactivates them. Phosphorylation of BAD by Akt creates a binding site for 14-3-3 proteins which bind to and sequester BAD in the cytoplasm. This results in the release of Bcl-2 and Bcl-xL and therefore promotes survival (Manning & Cantley, 2007). As increased levels of cell death does not appear to underlie the decrease in levels of total protein following PI3K inhibition in CCat1 cells, signalling must therefore be involved in either protein synthesis or proliferation in normal LECs (see figure 5.1).

The signalling pathway downstream of Akt which is involved in protein synthesis signals via mTORC1 and p70S6K (see figure 5.1). This pathway was inhibited using the mTOR inhibitor, Rapamycin, which binds to and inhibits the mTORC1 complex. Surprisingly, no significant decrease in total protein was observed in either CCat1 or DMCat1 cells, indicating that blocking the pathway did not affect cell growth (see figure 5.25). Inhibition of mTORC1 by Rapamycin was shown to prevent the activation of p70S6K which would inhibit the activation of S6 Kinase and should therefore have blocked protein synthesis (see figure 5.27). A more direct method to analyse levels of protein synthesis, such as addition of radioactive amino acids and measuring their incorporation into newly synthesised proteins, may have given a more accurate measure of the effects of Rapamycin and may therefore have shown that the PI3K/Akt pathway is involved in protein synthesis in human LECs. However, the data presented here suggests that signalling via the PI3K/Akt pathway does not significantly affect protein synthesis in either cell type.

Inhibition of PI3K signalling resulted in a significant decrease in levels of total protein extracted from dishes of both CCat1 and DMCat1 cells. In DMCat1 cells this was shown to be due to increased levels of apoptosis, therefore indicating that downstream signalling of the PI3K/Akt pathway was primarily

driven via the survival pathway. However, this was not the case in CCat1 cells, which did not show increased levels of cell death. Signalling via the downstream pathway involved in protein synthesis was also shown to have no significant role in the CCat1 cells. This leaves the downstream pathway involved in proliferation as the only remaining possible cause for reduced levels of total protein in CCat1 cells following PI3K inhibition. This pathway was not investigated in the cell lines and therefore cannot conclusively be described as the pathway by which PI3K/Akt primarily signals via in the CCat1 cells, however, it is likely that inhibition of PI3K prevented the inhibition of the cell cycle regulators p21^{Cip1/Waf1} and p27^{Kip1} by Akt and these would therefore continue to block cell cycle progression. Analysis of levels of phosphorylated p21^{Cip1/Waf1} and p27^{Kip1} following PI3K inhibition would confirm the role of the cell proliferation pathway downstream of Akt in CCat1 cells. Interestingly, expression of p21^{Cip1/Waf1} and p27^{Kip1} has been shown to be downregulated in DM1 myoblasts (Timchenko *et al.*, 2001b; Salisbury *et al.*, 2008). Downregulation of p21^{Cip1/Waf1} expression was found to result from increased levels of pAkt, which phosphorylate CUG-BP1 and prevent it from upregulating translation of p21^{Cip1/Waf1} mRNA (Salisbury *et al.*, 2008). Downregulation of p21^{Cip1/Waf1} and p27^{Kip1} prevents DM1 myoblasts from exiting the cell cycle and initiating differentiation. It would therefore also be interesting to analyse levels of total p21^{Cip1/Waf1} and p27^{Kip1} in DM1 LECs. If p21^{Cip1/Waf1} and p27^{Kip1} are found to be downregulated in DM1 LECs, signalling via Akt would be less important for the prevention of cell cycle inhibition and this could explain why signalling is primarily involved in survival in DM1 LECs but not in control LECs.

As well as pAkt, levels of pERK (see figures 5.7 and 5.12) and pJNK (see figures 5.8 and 5.13) were also found to be elevated in DMCat1 cells. The MAPK/ERK pathway is mainly associated with proliferation and differentiation in the lens (Lovicu & McAvoy, 2001; Iyengar *et al.*, 2006; Wang *et al.*, 2009b). Inhibition of the MAPK/ERK pathway had no significant effect on levels of total protein or cell death as shown by LDH release and the TUNEL apoptosis assay in DMCat1 or CCat1 cells (see figures 5.17 and 5.21). MAPK/JNK signalling in the lens has previously been associated with survival (Long *et al.*, 2004; Seomun *et al.*, 2005). Interestingly, inhibition of signalling via JNK did not affect survival in DMCat1 cells and only affected cell growth and/or proliferation in CCat1 cells

when added at a much greater concentration (10 μM) than its IC_{50} value (see table 2.1 and figure 5.18). However, at greater concentrations ($> 10 \mu\text{M}$) the JNK inhibitor SP600125 can also inhibit the activity of other signalling molecules such as isoforms of ERK, p38 and PKC, which could therefore have caused the decrease in cell growth and/or cell proliferation (Bennett *et al.*, 2001). Levels of pJNK were analysed following addition of SP600125 in both cell lines and revealed that JNK activation was not inhibited and at higher concentrations SP600125 actually increased pJNK levels (see figure 5.19c). This could either indicate that the inhibitor does not block JNK activation or that the cells are able to overcome its effects through, for example, the breakdown or removal of the inhibitor. SP600125 is an adenosine triphosphate (ATP)-competitive inhibitor and therefore competes with ATP to prevent the activation of JNK. If ATP levels are greater in LECs compared to other cell types tested using the inhibitor, then greater concentrations may be required to prevent JNK activation. The role of increased levels of pJNK in DMCat1 cells is therefore unknown and requires further investigation with a more specific inhibitor or greater concentrations of SP600125. The JNK pathway is associated with cellular stress as it is activated in response to stressors such as UV radiation, cytokines, growth factor deprivation and DNA damaging agents. The JNK pathway can promote both survival and apoptosis, with these opposing effects thought to be mediated by the length and strength of the activating signals (Krishna & Narang, 2008). Significantly increased levels of pJNK in DM1 cells could indicate increased exposure to cellular stress which may be linked to the triplet repeat mutation. Although JNK activation in the lens has been associated with survival following exposure to UV radiation and oxidative stress, it is possible that its sustained activation results in increased levels of apoptosis in the DM1 LECs (Long *et al.*, 2004; Seomun *et al.*, 2005). Further experiments are required to investigate the role of JNK signalling in DM1 LECs.

Analysis of signalling pathways activated in cells grown in SF medium and medium supplemented with 10% FCS revealed that signalling via JNK, p38 and PKC appeared to be greater under conditions of serum deprivation. This is not surprising as activation of both the MAPK/JNK and MAPK/p38 pathways is associated with cellular stress. We did not find any differences between SF medium and medium supplemented with 10% FCS in levels of activation of Akt

or ERK which would indicate autocrine activation. Although levels of pPKC were greater in DMCat1 cells in SF medium and lower in medium supplemented with 10% FCS, neither was significantly different from CCat1 cells. Further experiments are required to investigate any possible role that the PLC/PKC signalling pathway may play in increased levels of apoptotic cell death in DM1 LECs in SF medium. As signalling via PLC results in a rise in intracellular Ca^{2+} levels, leading to PKC activation, the role of increased Ca^{2+} in the DM1 cells would also need to be investigated. Rhodes *et al.* (2006) observed increased levels of cell death in the DM1 LECs used in this study following addition of the Ca^{2+} ionophore, ionomycin, when compared to controls. Ionomycin results in prolonged high levels of intracellular calcium, which the DM1 LECs were more susceptible to (Rhodes *et al.*, 2006). Elevated levels of active PKC have also been linked to the presence of expanded CUG repeats in DM1 and were shown to be responsible for the increased activation of CUG-BP1 observed in the disease (Kuyumcu-Martinez *et al.*, 2007). Inhibition of PKC in a heart-specific mouse model was shown to increase survival rates by reducing CUG-BP1 steady-state levels, which ameliorated the cardiac conduction defects and contraction abnormalities observed in the mouse model (Wang *et al.*, 2009a). Increased levels of apoptotic cell death could therefore result from increased signalling via PLC leading to Ca^{2+} release and elevated levels of activated PKC, however, this requires further investigation. Levels of pp38 increased in DMCat1 cells in SF medium, however, this was also observed in CCat1 cells resulting in no significant difference in pp38 levels. Active p38 was also only detected early in the culture period when both cell types were growing well, indicating that signalling via p38 does not play a role in apoptotic cell death. Levels of pJNK increased in DMCat1 cells in SF medium and were significantly higher compared to CCat1 cells, indicating that increased activation of JNK may be responsible for the increased levels of apoptotic cell death in DM1 cells when cultured in SF medium.

From the data described in this chapter, it is clear that signalling via the PI3K/Akt pathway is critical to the survival of DMCat1 cells, but it is not as important for survival in CCat1 cells. In the absence of PI3K/Akt signalling, DMCat1 cells undergo apoptosis. During culture, the levels of both Akt and pAkt were shown to decline, which coincides with a reduction in cell populations

observed in DMCat1 cells. It is possible that as the DMCat1 cells age, they are no longer able to maintain the level of signalling via Akt that is required to maintain survival. The reduced population doubling times and shorter lifespan of DM1 LECs may result from sustained activation of JNK and a reduction in the activation of Akt during culture respectively.

DM1 cataract patients have been shown to have reduced LEC densities, with larger cells maintaining coverage of the epithelium over the lens (Abe *et al.*, 1999). As the lens epithelium maintains the balance of ions and water which is vital to lens transparency, a loss of cells from this layer could result in loss of transparency and cataract development. Cataracts in DM1 patients form in later life and even in congenitally affected patients they can take over a decade to develop. It is possible that as the cells age they express lower levels of either the receptors that are responsible for Akt activation, or of Akt itself, resulting in a reduction in pAkt levels and a subsequent increase in apoptotic cell death in DM1 LECs. Increased levels of cellular stress causing sustained activation of JNK may also contribute to increased levels of apoptotic cell death. Greater levels of apoptosis caused by reduced levels of pAkt and sustained activation of JNK could, therefore, underlie the formation of cataracts in DM1.

CHAPTER 6

GENERAL DISCUSSION

6.1 General discussion

The development of pre-senile cataracts is one of the most distinctive and key features of DM. The characteristic, iridescent opacities present in patients affected both minimally and congenitally. Despite the prominence of cataracts in the disease, very little is known about the mechanisms behind their development. Three main hypotheses have been proposed to explain the development of DM1 in general: firstly, a reduction in *DMPK* expression; secondly, a reduction in expression of the neighbouring genes, *SIX5* and *DMWD*; and thirdly a gain of function by the mutant *DMPK* mRNA. In the lens, the hypothesis with the greatest support to explain cataract development was reduced expression of *SIX5*. In two separate studies using knock-out mice, partial (*Six5*^{+/-}) or complete (*Six5*^{-/-}) loss of *Six5* resulted in the development of cataracts and the incidence and severity of lens opacities increased with decreasing *Six5* dosage (Klesert *et al.*, 2000; Sarkar *et al.*, 2000). However, the cataracts which formed did not have the characteristic features of DM1 cataracts as no iridescent, dust-like opacities were found and the cataracts formed in the nucleus as opposed to the posterior subcapsular region where they are found in DM1 patients. Further to this, similar cataracts are also found in DM2, which is caused by a repeat expansion mutation found at a different gene locus and the *SIX5* gene is unaffected in this form of the disease. As no conclusive mechanism had been identified to explain the development of cataracts in DM1, this study aimed to investigate the underlying mechanism in the lens using human LECs derived from DM1 cataract patients.

Previous work in this laboratory characterised the human lens epithelial cell lines used in this study and showed that the *DMPK* gene contained large triplet repeat expansions only in the DM1 cell lines. *DMPK* was shown to be expressed in the control and DM1 LECs as well as *SIX5* and *DMWD*. *SIX5* expression was found to be significantly reduced in the DM1 cell lines compared to the controls (Rhodes *et al.*, 2006). In this study we employed two further

control cell lines and found that *SIX5* expression was also low in these cell lines, indicating that the expression of this gene is highly variable in LECs and the haploinsufficiency of *SIX5* that has been observed previously in DM1 may not be a true result of the DM1 mutation (see figure 3.2). This data adds further weight to the argument against *SIX5* haploinsufficiency as the cause of DM1 cataract. In this study we also found expression of *DMPK* in all of the cell lines and revealed that the transcripts from the mutant allele in DM1 cells formed foci which are trapped within the nucleus (see figures 3.3 and 3.4). This is a significant finding because the formation of nuclear foci of *DMPK* transcripts is a common feature in cells affected by DM1 and is linked to the sequestration of MBNL proteins and the increased activation of CUG-BPs which are involved in the regulation of splicing events (Timchenko *et al.*, 2001a; Fardaei *et al.*, 2002). Although foci were shown to form and the LECs were found to express *MBNL1*, *MBNL2*, *CUG-BP1* and *CUG-BP2* (see figures 3.5 and 3.6), we found no difference in levels of alternatively spliced isoforms of transcripts commonly affected in DM1. Alternative splicing has been used to explain many of the symptoms of DM1, for example, the altered splicing of the chloride channel *ClC-1* is thought to cause the myotonia which is a characteristic feature of the disease and altered splicing of *IR* is likely to underlie insulin resistance and diabetes which is often observed in DM1 (Savkur *et al.*, 2001; Mankodi *et al.*, 2002). As no aberrant splicing patterns were observed in the DM1 LECs used in this study, the symptom of cataract does not appear to result from this mechanism. The triplet repeat mutation in *DMPK* must therefore exert its effects via a different mechanism in the lens.

The repeat regions that cause DM are unstable and biased towards expansion. The repeats not only expand when passed between generations, but also expand in the somatic cells of an individual during their lifetime. This is known as somatic mosaicism and the level of expansion varies depending on cell and tissue type, for example, cells in skeletal muscle, heart and brain have significantly larger repeat expansions than leukocytes (Thornton *et al.*, 1994). Lens cells from DM1 cataract patients have also been shown to have larger repeats than those found in peripheral blood samples from the same patient (Abe *et al.*, 1999). The cultured human DM1 LECs used in this study have also previously been shown to contain large repeats (> 1400) even at the earliest passage sampled and these were found to expand during culture (Rhodes *et al.*,

2006). The size of the triplet repeat in the lens could explain the development of cataracts even in mildly affected patients, as larger repeats would make the lens more susceptible to their downstream effects. Cataracts do not develop in mildly affected patients until later in life and do not develop in congenitally affected patients until at least 10 years of age (Harper, 2001). The accumulation of repeats in the lens during the lifetime of an individual could explain why this is observed.

An interesting study by Khajavi *et al.* (2001) aimed to investigate the mechanism behind the expansion of the triplet repeat in DM1 and showed that the repeat containing alleles of DM1 lymphoblastoid cells derived from blood, shifted towards expansion via multiple 'step-wise' mutations and also through rarer 'gross' mutations. Rare gross mutants which gained large repeats (40-290 repeats) were shown to eventually replace the progenitor allele population. It was hypothesised that this was due to a growth advantage of the mutant with the larger CTG repeat size over the progenitor population. The authors showed that this was the case by mixing cell lines containing different sizes of the expanded repeat which consistently resulted in survival of the cell line containing the larger repeat. The growth advantage was attributed to the increased proliferation rate of the cells containing larger repeat expansions, which was found to be due to downregulation of p21^{Cip1/Waf1} expression. As p21^{Cip1/Waf1} inhibits cell cycle progression, lower levels result in an increased proliferation rate. Repeat number was therefore shown to correlate inversely with cell doubling times (Khajavi *et al.*, 2001). In this study we found similar population doubling times in all DM1 LECs which did not appear to correlate with the previously published repeat sizes for these cell lines (summarised in figure 3.1a) which contradicts the data from Khajavi *et al.* (2001) (Rhodes *et al.*, 2006). Khajavi *et al.* (2001) only compared the growth rates of DM1 cell lines with varying repeat sizes and did not show whether DM1 cell lines proliferated faster than controls, however, the lifespan of DM1 lymphoblastoid cell lines was shown to be significantly shorter than controls as DM1 cell lines reached an average of 10.5 passages compared to controls which reached over 25 passages (Khajavi *et al.*, 2001). In this study we have shown that the control cell lines proliferate at a faster rate than the DM1 cell lines as the controls have shorter population doubling times compared to DM1 LECs. We have also shown that the lifespan of our DM1 lens epithelial cell lines is significantly shorter than the controls, as DM1 LECs survived for an average of

79.5 days in culture compared to control LECs which survived for over 101 days (see figure 3.9). Furling *et al.* (2001a) have also shown that the lifespan of satellite cells derived from muscle biopsies of congenital DM1 fetuses was also reduced. Lifespan was measured by counting the number of divisions the cells could go through before reaching growth arrest, which was found to be between 50 and 60 divisions for control cells but a maximum of only 31 divisions was observed in DM1 cells. The number of divisions was also shown to be lower in cells derived from a foetus showing more severe signs of the disease (Furling *et al.*, 2001a).

DM1 has been described as a premature ageing disorder as many of its symptoms are often commonly associated with ageing, such as cataract, erectile dysfunction, frontal balding, insulin resistance, cardiac arrhythmias and muscle weakness (Harper, 2001; Antonini *et al.*, 2009). If expansion of the CTG repeat confers a proliferative advantage, as shown by Khajavi *et al.* (2001), this may indicate that DM1 cells proliferate faster and could therefore reach growth arrest earlier which could underlie the suggestions of premature ageing and senescence observed in the disease. However, this is unlikely as it would lead to greater cell numbers which has not been observed in the disease, unless cell death is also greater. A further study using satellite cells from congenital DM1 fetuses showed that neither proliferation nor cell death was greater in DM1 cells compared to controls. A 59% increase in loss of telomere length per cell division was, however, observed and DM1 cells stopped dividing with longer telomeres than controls. This was shown to result in the premature senescence of the cells which could be overcome by blocking the p16 cyclin-dependent kinase inhibitor. p16 is a key regulator of replicative senescence and can be upregulated in response to telomere shortening and stress mechanisms including DNA damage and oxidative stress. p16 was activated earlier in DM1 cells compared to controls, however, increased levels of telomere shortening was shown to be independent of p16 activation and was suggested to result from increased susceptibility to reactive oxygen species in DM1 cells as this has previously been shown to correlate with CTG repeat length (Usuki & Ishiura, 1998; Bigot *et al.*, 2009).

A number of studies have reported that DM1 cells and cells containing expanded CTG repeats have a reduced tolerance to stress (Usuki & Ishiura, 1998; Usuki *et al.*, 2000; O'Coilain *et al.*, 2004; Rhodes *et al.*, 2006; Usuki *et al.*,

2008). A recent study has shown that myoblasts from DM1 patients have increased levels of stress. This was shown to be due to the activation of PKR by CUG repeats which inactivates eukaryotic initiation factor 2 α (eIF2 α) and prevents translation of downstream mRNAs, resulting in formation of stress granules (Huichalaf *et al.*, 2010). Transgenic mice expressing over 25 extra copies of a complete *DMPK* gene with normal sized repeats revealed DM1-like symptoms upon ageing. The accumulation of *DMPK* transcripts was shown to cause severe cellular distress which affected cardiac, skeletal and smooth muscles. Symptoms included workload intolerance, hypertrophic cardiomyopathy and myotonic myopathy, which are all characteristic of DM1 (O'Coilain *et al.*, 2004). Rhodes *et al.* (2006) showed that DM1 LECs have increased expression levels of the Ca²⁺-activated K⁺ channel, SK3, which led to increased levels of cell death in conditions of calcium overload. Mouse myoblasts transfected with *DMPK* containing varying numbers of CTG repeats, showed CTG repeat number-dependent susceptibility to oxidative stress, resulting in apoptosis (Usuki & Ishiura, 1998; Usuki *et al.*, 2000). In this study, we have shown that DM1 LECs are less able to withstand the stress of being grown in SF medium compared to control LECs. SF medium contains no added growth factors and therefore the cells rely on autocrine signalling to maintain survival. We have shown that DM1 cells have significantly increased levels of apoptotic cell death when cultured in SF medium compared to controls, indicating that autocrine signalling was somehow impaired in these cells leading to increased susceptibility to stress (see figure 3.15). Reduced lifespan, premature senescence and increased susceptibility to stress can all result in a reduction in cell number in DM1 tissues which could contribute to some of the symptoms observed in the disease, such as muscle atrophy and cataract formation.

Lens epithelial cell density has been shown to decrease with age and as the incidence of cataract increases with age, this could indicate a link between the two (Konofsky *et al.*, 1987; Guggenmoosholzmann *et al.*, 1989). It has been suggested that LECs in vivo accumulate damage from agents to which the lens is exposed to throughout life, such as UV radiation and hydrogen peroxide, and this damage impairs their function and leads to cell death (Harocopos *et al.*, 1998). Studies have shown that cataractous lenses contain greater numbers of apoptotic cells and that cell death precedes cataract development (Konofsky *et al.*, 1987; Li *et al.*,

1995; Li & Spector, 1996). Analysis of the lens epithelium in DM1 cataract patients revealed reduced population densities of lens epithelial cells (Abe *et al.*, 1999). The decrease in cell density appeared to be dependent on the age of onset of disease symptoms, with lower densities observed in patients with an earlier onset. The lens epithelium contained larger cells to maintain coverage of the lens; however, fewer cells may not be capable of maintaining homeostasis in levels of ions and water and may not offer enough protection to the underlying fibre cells from damaging agents such as UV radiation and oxidative insults, which could lead to the development of cataract. Whether decreased levels of proliferation or increased levels of cell death caused the reduced cell densities in lenses from DM1 cataract patients is unknown. However, if the CTG repeats in DM1 LECs make them more susceptible to stress, then it could be hypothesised that exposure of the lens to normal levels of damaging agents may cause cell death earlier than in normal controls.

Increased levels of cell death could underlie cataract formation in DM1, however, the mechanisms that lead to increased cell death are unknown. In this study we have shown that DM1 LECs have greater levels of cell death compared to controls when grown in both SF medium and medium supplemented with 10% FCS (see figure 3.12). We have also shown that DM1 LECs have greater levels of apoptosis when cultured in SF medium (see figure 3.15). We hypothesised that autocrine signalling is impaired in DM1 LECs leading to increased levels of apoptotic cell death when cultured in SF medium. We investigated the release of autocrine signalling factors in DM1 LECs by collecting conditioned medium (CM) and applying it to the non-virally transformed lens epithelial cell line, FHL124. We found that DM1 CM significantly increased growth and/or survival in FHL124 cells and activated signalling pathways resulting in greater levels of Akt activation compared to controls (see figures 4.5 and 4.7). We investigated the role of the FGF and IGF signalling pathways in autocrine signalling in DM1 LECs as both pathways have been shown to activate signalling via PI3K and Akt in the lens (Chandrasekher & Bazan, 2000; Chandrasekher & Sailaja, 2003, 2004; Wang *et al.*, 2009b). Inhibition of FGFR1 in DM1 LECs cultured in SF medium resulted in a significant decrease in growth, however, no difference was found in levels of cell death (see figure 4.14). No difference was found in either cell growth or cell death following IGFR-1 inhibition (see figure 4.15). Inhibition of FGFR1 in

FHL124 cells during DM1 CM addition resulted in a decrease in growth and levels of pAkt, however, both were still elevated compared to addition of SF medium, indicating that other factors were also released by the DM1 cells (see figures 4.11 and 4.12). Low levels of bFGF were found in the DM1 CM, however, addition of greater levels of bFGF to FHL124 cells did not increase growth and/or survival or levels of pAkt to the same extent as was observed following DM1 CM addition (see figures 4.5, 4.8 and 4.12). This data indicates that other factors are responsible for the increase in growth and levels of pAkt in FHL124 cells. As DM1 cells were shown to release factors capable of increasing growth and/or survival, this would indicate that their responses to these factors are impaired as cell death is increased in these cells compared to controls. In order to investigate this, we analysed the activation of signalling pathways in DM1 LECs which are known to be involved in growth, proliferation, survival and apoptosis within the lens.

Studies have shown that CTG repeats can lead to the aberrant activation and/or regulation of signalling pathways in DM1 cells (Hernandez-Hernandez *et al.*, 2006; Usuki *et al.*, 2008; Befly *et al.*, 2010). Usuki *et al.* (2008) showed that aberrant activation of the MAPK/JNK pathway in cells containing large triplet repeats instead of the MAPK/ERK pathway led to cell death rather than survival when exposed to oxidative stress. Befly *et al.* (2010) showed that sustained activation of the MAPK/ERK pathway along with reduced activation of the MAPK/p38 pathway prevented differentiation in myoblasts from congenitally affected DM1 fetuses compared to controls, which could play a role in the impaired muscle development observed in congenital DM1. The study also showed that enlarged vacuoles formed in the DM1 cells, which were not observed in controls. These were found to be autophagic vacuoles, which are double-membraned vesicles which fuse with lysosomes to degrade cellular components. Autophagy is regulated by mTOR and is suppressed by activation of the PI3K/Akt signalling pathway, which could also indicate that regulation of the PI3K/Akt signalling pathway is disturbed in these cells (Glick *et al.*, 2010). A further study has also identified disturbances in the PI3K/Akt pathway in a neuronal cell line expressing 90 CUG repeats (CTG90 cells) (Hernandez-Hernandez *et al.*, 2006). Hernandez-Hernandez *et al.* (2006) showed that the failure of CTG90 cells to differentiate in response to nerve growth factor was likely due to the altered

activity of microtubule-associated protein tau (τ) and the impaired activation of glycogen synthase kinase 3 β (GSK3 β) which regulates τ phosphorylation. GSK3 β activity is negatively regulated by phosphorylation at residue S9 by pAkt. Levels of pAkt were found to be reduced in the CTG90 cells leading to an increase in GSK3 β activity and hyperphosphorylation of τ , which could be linked to symptoms observed in the CNS in DM1, such as mental retardation. Aberrant activation of signalling pathways could therefore play a role in some of the symptoms observed in DM1.

To investigate whether aberrant activation of signalling pathways led to increased levels of apoptosis in DM1 cells cultured in SF medium, we analysed pathways activated in response to both SF medium and medium supplemented with 10% FCS. Signalling via the MAPK/JNK, MAPK/p38 and PLC/PKC pathways appeared to increase in SF medium in DM1 LECs compared to levels observed in medium supplemented with 10% FCS (see figures 5.8 to 5.10 and 5.13 to 5.15). As the MAPK/JNK and MAPK/p38 pathways are activated under stress conditions, it is not surprising that activation is higher during serum deprivation. Active levels of p38 were only detected early in the culture period and as levels of p38 also increased in control LECs in SF medium, no difference was found in the levels observed in DM1 LECs compared to controls. Active levels of JNK, however, were found throughout the culture period and were significantly higher in DM1 LECs compared to controls. Sustained activation of JNK can lead to apoptosis and therefore requires further investigation in DM1 LECs as it may play a role in the increased levels of apoptotic cell death in SF medium compared to controls. Although levels of active PKC were found to be higher in DM1 LECs when cultured in SF medium compared to medium supplemented with 10% FCS, levels were not significantly different to those observed in the control LECs. Signalling via PLC activates PKC by increasing levels of DAG and intracellular Ca²⁺. Elevated levels of intracellular Ca²⁺ have previously been shown to cause increased levels of cell death in human DM1 LECs (Rhodes *et al.*, 2006). Transgenic mice which express 25 extra copies of the *DMPK* gene with normal repeat sizes also demonstrated intracellular Ca²⁺ overload which promoted nuclear translocation of transcription factors responsible for changes in gene expression leading to hypertrophic cardiomyopathy (O'Coilain *et al.*, 2004). Increased signalling via PLC could therefore also play a

role in apoptotic cell death in DM1 LECs cultured in SF medium via increased levels of intracellular Ca^{2+} and pPKC, however, this requires further investigation.

Cell death was also greater in DM1 cells cultured in medium supplemented with 10% FCS compared to controls (see figure 3.12), however, the increase in apoptotic cell death was not significant (see figure 3.15). Analysis of activated signalling pathways in medium supplemented with 10% FCS revealed that DM1 LECs had significantly increased activation of the PI3K/Akt and MAPK/JNK pathways compared to controls, which was also observed in SF medium (see figures 5.6, 5.8, 5.11 and 5.13). Increased activation of the PI3K/Akt pathway in DM1 LECs was shown to encourage their survival as inhibiting the pathway led to increased levels of apoptosis (see figure 5.20). Increased activation of the MAPK/JNK pathway could have the opposite effect as this can lead to increased levels of apoptosis, however, the role of signalling via the MAPK/JNK pathway was not elucidated as the JNK inhibitor, SP600125, was found not to inhibit the MAPK/JNK pathway in this study. The sustained activation of JNK indicates that the DM1 LECs are exposed to increased levels of cellular stress, which is known to activate the pathway, even when cultured in medium with serum. Signalling via the MAPK/JNK pathway could therefore be responsible for the reduced population doubling times observed in DM1 LECs by increasing cell death (see figure 3.9).

As no single pathway was shown to be significantly upregulated in only SF medium in DM1 LECs compared to controls, it appears unlikely that the increase in apoptotic cell death in SF medium is due to the aberrant activation of signalling pathways. However, the PI3K/Akt and MAPK/JNK pathways are aberrantly activated in DM1 LECs in both SF medium and medium supplemented with 10% FCS. The JNK signalling pathway has been shown to be activated by serum deprivation and we found that levels of pJNK increased in both cell types in SF medium (Huang *et al.*, 1997). As DM1 LECs had greater levels of pJNK in medium supplemented with 10% FCS, it is likely that serum deprivation caused a further increase in signalling via the MAPK/JNK pathway which resulted in greater levels of cell death when compared to control LECs.

Although an increase in signalling via the PI3K/Akt pathway appeared to be protective in DM1 LECs, this may not be the case in other cell types. Increased levels of pAkt have also been demonstrated in both cultured and primary DM1

myoblasts (Salisbury *et al.*, 2008). Activated Akt was shown to phosphorylate CUG-BP1 at serine residue S28, which increased the interactions of CUG-BP1 with mRNA of the cell cycle regulator, cyclin D1. Translation of cyclin D1 mRNA is increased by interactions with CUG-BP1, resulting in increased levels of proliferation. CUG-BP1 was also shown to be phosphorylated by cyclin D3-cdk4/6 at serine residue S302, which increases CUG-BP1 binding with p21^{Cip1/Waf1} and CCAAT-enhancer-binding protein β (C/EBP β) mRNAs. An increase in translation of p21^{Cip1/Waf1} and C/EBP β by CUG-BP1 is required to block cell cycle progression and enable differentiation in myoblasts. Therefore, phosphorylation of CUG-BP1 can play an important role in regulating both proliferation and differentiation in myoblasts. Levels of cyclin D3 were found to be lower in DM1 myoblasts and along with elevated levels of pAkt, which increased phosphorylation of CUG-BP1 at S28 and encouraged CUG-BP1 to bind to cyclin D1 mRNA rather than p21^{Cip1/Waf1} and C/EBP β mRNAs, these resulted in a block in differentiation in DM1 myoblasts. Increased levels of pAkt could therefore contribute to the impaired differentiation of skeletal muscle in DM1 patients. If the triplet repeat mutation is responsible for increasing Akt activity in other cell types affected by DM1, then the response will be cell type specific. In LECs, increased levels of pAkt increase survival which confers an advantage, whereas in myoblasts it leads to impaired differentiation which is detrimental.

Here we have shown that DM1 LECs require signalling via the PI3K/Akt pathway for survival in culture. However, we have also shown that levels of both total Akt and active Akt in control and DM1 LECs reduced over time during a four month culture period (see figures 5.6 and 5.11). Levels of other downstream signalling molecules such as ERK and PKC also appeared to decrease (see figures 5.7, 5.10, 5.12 and 5.15). Interestingly, studies have shown that expression of receptors such as FGFR-2 and IGFR-1 decrease in the human lens in later life and the response of rat LEC explants to bFGF also reduces with age (Lovicu & McAvoy, 1992; Bhuyan *et al.*, 2000). Expression of proteins involved in signal transduction have also been found to be lower in lenses with age-related cataract (Ruotolo *et al.*, 2003). This data could therefore indicate that either protein expression in general decreases with age in the lens, or that a reduction in levels of receptors that respond to growth factors results in a reduction in protein levels

of downstream signalling molecules. Either way, a reduction in signalling molecules could prevent the signals from growth and survival factors reaching their destination. As DM1 LECs appear to rely on signalling via the PI3K/Akt pathway for survival, this could explain the reduction in population doubling times observed during the culture of DM1 LECs and their limited lifespan compared to controls (see figure 3.9).

Although the cause of increased activation of Akt and JNK in DM1 LECs is unknown, it appears to result from the presence of the triplet repeat. However, we could speculate a possible mechanism which would link the triplet repeat mutation to the activation of Akt and JNK in the disease. Transcription of the mutant *DMPK* allele found in DM1 results in the production of RNA containing CUG repeats. The expanded CUG repeats have been shown to form stable hairpin structures where the repeat tract folds back on itself and results in formation of dsRNA with G•C and C•G base pairs separated by U•U mismatches. These hairpin structures have been shown to activate PKR, which is a protein kinase activated by dsRNA of over 30 bp in length which is usually only found in cells following infection by viruses which contain dsRNA (Tian *et al.*, 2000). PKR initiates anti-viral responses by phosphorylating eIF-2 α which prevents its recycling and results in a block in protein synthesis (Garcia *et al.*, 2006). PKR can also regulate translation, cell cycle progression and apoptosis via interactions with other proteins including protein phosphatase 2A, nuclear factor κ B (NF- κ B) and p53. Interestingly, PKR has also been shown to regulate the activity of Akt, ERK, JNK and p38 (Iordanov *et al.*, 2000; Takada *et al.*, 2007; Alisi *et al.*, 2008). Signalling induced by the cytokine, tumour necrosis factor (TNF) has been shown to be regulated by PKR which was required for TNF induced activation of Akt and JNK, however, it was shown to negatively regulate activation of ERK and p38 (Takada *et al.*, 2007). In a separate study, the activation of JNK by dsRNA was shown to require PKR-mediated inhibition of protein synthesis by a mechanism which activated the upstream kinase, MKK4 (Iordanov *et al.*, 2000). PKR has also been shown to directly interact with Akt and p38 to regulate their activity, with the interaction with Akt encouraging its activation and the interaction with p38 resulting in its inhibition. Through its interactions with Akt and p38, PKR was shown to play a role in regulating muscle differentiation, which could also link to the muscle symptoms observed in DM1 (Alisi *et al.*, 2008). Beffy *et al.* (2010)

showed that DM1 myoblasts had reduced activity of MAPK/p38 which could be responsible for impaired muscle differentiation in the disease. Activation of PKR by the expanded CUG repeats found in DM1 could therefore underlie the reduction in p38 that is observed in DM1 myoblasts from congenitally affected foetuses (Beffy *et al.*, 2010). A recent study has confirmed that PKR levels are elevated in primary DM1 myoblasts and found that this led to increased levels of phosphorylated eIF2 α , resulting in cellular stress (Huichalaf *et al.*, 2010). If the unusual hairpin structures that are formed by the expanded CUG repeats are confirmed to activate PKR in DM1 LECs, then through these interactions PKR could cause the increase in pAkt and pJNK levels, with pAkt encouraging survival and pJNK likely leading to increased levels of apoptotic cell death. It is also possible that the hairpin structures formed by the CUG repeats may activate other sensors of dsRNA that are present in cells. These include receptors such as toll-like receptor 3 (TLR3), which is located in endosomal compartments, and retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA5) which are cytoplasmic sensors. These sensors activate signalling pathways which result in the nuclear translocation of NF- κ B and interferon regulatory factor 3 (IRF3), resulting in the production of the type I interferons (IFNs), IFN α and IFN β . IFNs are cytokines which are released by cells and induce autocrine and paracrine signalling via the type I interferon receptor (IFNAR) which results in transcription of IFN stimulated genes (ISGs) (Baum & Garcia-Sastre, 2010). ISGs regulate many cellular processes to protect the host cell from infection and prevent the spread of viruses, for example, by inhibiting cell growth and proliferation, and by activating apoptosis (Sen, 2001). IFNs have been shown to activate the PI3K/Akt pathway resulting in mTOR activation, which was found to be essential for translation of some ISG mRNAs and IFNs have also been shown to induce sustained activation of the MAPK/JNK pathway leading to apoptosis (Yanase *et al.*, 2005; Kaur *et al.*, 2008). IFN activation of the PI3K/Akt pathway has also been shown to promote survival via activation and nuclear translocation of NF- κ B, which counteracts the apoptotic effects of IFN in some cell types (Yang *et al.*, 2001). An increase in levels of pAkt in the DM1 LECs may therefore be induced by IFN to counteract increased levels of pJNK and maintain survival. If the hairpin structures formed by the CUG repeats are found to stimulate IFN production, this could also be found to underlie increased levels

of cell death in DM1 LECs. Release of IFN could also be responsible for the effects observed following addition of DM1 CM to FHL124 cells as growth and/or survival and levels of pAkt were found to increase. If DM1 CM contains IFN, addition of DM1 CM to FHL124 cells could promote survival via Akt, however, long term addition could result in increased levels of cell death via the sustained activation of JNK, which may not have been observed in the short term experiments performed in this study. The role of PKR and other dsRNA sensors in DM1 lens epithelial cell death, and in DM1 in general, requires further investigation.

DM1 cataractous lenses have reduced LEC density (Abe *et al.*, 1999). A reduction in cell number could result from increased levels of cell death or reduced levels of proliferation. From this study, we have shown that DM1 LECs are subject to increased levels of cell death compared to control LECs. As cataracts form later in life in minimally affected DM1 patients and take at least a decade to form in congenitally affected patients, this suggests that the underlying cause of cataract formation takes a while to develop or exert its effects. If levels of active signalling pathways are age dependent, as is observed in the cultured human LECs, then a reduction in active signalling molecules could underlie DM1 cataract. An increase in levels of cell death, and specifically apoptosis, as a consequence of reduced signalling via PI3K/Akt and sustained activation of the MAPK/JNK pathway throughout the life of DM1 patients could therefore result in cataract development in the disease.

6.2 Summary of major conclusions

The data presented in this thesis has revealed some novel and important findings that may help to further the field of research into cataract formation in DM1. We have shown that *SIX5* levels are not affected by the triplet repeat mutation and that *DMPK* is expressed in the lens where transcripts form foci in the nuclei of DM1 LECs. This data may help to shift the emphasis of research into DM1 cataracts away from the current hypothesis of *SIX5* haploinsufficiency and towards possible mechanisms downstream of mutant transcript production. Although we found no evidence for alternative splicing in the proteins that we investigated, there are many more that have been observed in other tissues in

DM1 that we did not analyse, but could be potential causes of cataract formation in DM1, such as the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA). Two isoforms of SERCA, SERCA1 and SERCA2 are alternatively spliced in DM1 muscle cells and have been linked to impaired Ca^{2+} homeostasis in the disease (Kimura *et al.*, 2005). The SERCA isoforms SERCA2 and SERCA3 are present in the lens and have been linked to cataract formation due to their roles in Ca^{2+} homeostasis (Liu *et al.*, 1999). Analysis of alternative splicing of these isoforms in DM1 LECs could therefore be very interesting. This study has also highlighted the role of signalling pathways in the survival of DM1 LECs. We have shown that the DM1 LECs release factors into the medium which are capable of activating the Akt pathway and increasing growth in FHL124 cells. Activation of Akt was found to be upregulated in DM1 LECs which was critical to their survival. PTEN, the negative regulator of the Akt pathway was also found to be downregulated. During culture the levels of pAkt declined in the cell lines which coincided with a decline in cell numbers. A decline in cell number in the lens could lead to cataract formation due to a loss of homeostasis in levels of ions and water.

6.3 Future directions

In order to build upon this project, further replicates are required for a number of the experiments and confirming the results in all eight cell lines would be of great benefit. Many of the interesting results could then be followed up and investigated further. Establishing the identity of the factor(s) released by DM1 LECs which activates the Akt pathway and increases growth in FHL124 cells is a key area for future studies. Cytokines, such as interferons, are strong candidates and these could be investigated using multianalyte ELISAs. Identification of the pathways which lead to the release of factors by the DM1 LECs is also of great importance. Microarrays could be used to examine expression levels of thousands of genes at once which could highlight pathways that are aberrantly activated in DM1 LECs. Pathways that are either up or downregulated in the DM1 LECs compared to controls may be direct or indirect effects of the triplet repeat mutation and may therefore cause or contribute to the symptoms of the disease. This project has laid the groundwork for a new focus for DM1 cataract research.

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