INVESTIGATIONS OF DNA DAMAGE AND REPAIR IN THE HUMAN LENS

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Abstract

Purpose: Cataract is the leading cause of blindness worldwide, affecting millions each year. Not only is cataract a debilitating disease for the individual sufferer, it also places a huge burden on healthcare budgets. Developing a greater understanding of the underlying causes could lead to potential therapies or preventative treatments being elucidated. Oxidative stress is a key factor in the formation of cataract, of which one consequence is DNA damage. The current study investigates the role of DNA damage and repair in cataract formation. The present body of work has three main aims. Firstly, to develop a model to study the effects of oxidative stress in human lens cells; secondly, investigate the role of non-homologous end joining (NHEJ) in the lens in respect to oxidative stress and cataract formation; thirdly, investigate the role of poly(ADP-ribose) polymerase-1 (PARP-1) in human lens cell responses to oxidative stress and cataract.

Methods: The FHL124 human lens epithelial cell line and a whole human lens culture system were used as the experimental models. Oxidative stress was induced via exposure to hydrogen peroxide (H_2O_2). Oxidative stress-induced changes in FHL124 cell viability/cytotoxicity and apoptosis were assessed with the ApoTox-Glo Triplex Assay and DNA strand breaks determined by the alkaline comet assay. Lactate dehydrogenase levels in culture medium were assayed as a marker of cell death in both FHL124 cells whole human lenses. Targeted siRNA was used to deplete expression of the NHEJ protein Ku80 and PARP-1 with protein expression evaluated by western blot and immunocytochemistry. Chemical inhibition of PARP-1 activity was also undertaken.

Results: A 24 hour exposure to hydrogen peroxide induced a concentration-dependent decrease in cell viability and increase in cytotoxicity and apoptosis. A 24 hour exposure to 1mM H_2O_2 induced significant decreases in loss of visual quality and cataract in whole human lenses; these changes corresponded to increased cell death. Depletion of Ku80 rendered FHL124 cells more susceptible to H_2O_2-induced DNA strand breaks and whole human lenses to significant decreases in visual quality compared to match-paired controls. Chemical inhibition of PARP-1 and its depletion by siRNA rendered FHL124 cells more susceptible to H_2O_2-induced DNA strand breaks. Conversely, chemical inhibition of PARP-1 protected FHL124 cells from H_2O_2-induced cell death and protected whole human lenses from oxidative stress-induced decreases in visual quality and cataract.

Conclusions: The experimental model developed allows for the study of oxidative stress-induced changes in lens cell line and the whole organ system. The NHEJ protein Ku80 is required for efficient responses to oxidative stress-induced DNA strand breaks; thus, Ku80 expression is important to protecting the human lens against opacity formation induced with H_2O_2. PARP-1 has multiple roles in both cell death and survival, with its depletion or inhibition rendering FHL124 cells susceptible to DNA strand breaks whilst conversely protecting against cell death and formation of opacity in whole human lens cultures.

Abbreviations

•OH	Hydroxyl radical
8-OHdG	8-hydroxyl 2-deoxyguanosine
ADP	Adenine diphosphate
AIF	Apoptosis inducing factor
APE1	apurinic/apyrimidinic endonuclease-1
ATM	Ataxia telangiectasia mutated
ATP	Adenine triphosphate
ATR	Ataxia telangiectasia and Rad3 related
BCA	Bicinchonic acid
BER	Base excision repair
CCD	Charge coupled device
BSA	Bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled H_2O
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
DNA-PKcs	DNA protein kinase catalytic subunit
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagle's minimum essential medium
FCS	Foetal calf serum
FGF	Fibroblast growth factor

FHL124	Foetal human lens cell 124
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2O_2	Hydrogen peroxide
HR	Homologous recombination
HRP	Horse radish peroxidase
IOL	Intraocular lens
LDH	Lactate dehydrogenase
Lig4	Ligase IV
MMR	Mismatch repair
MMS	methylmethane sulfonate
MNNG	Methylnitronitrosoguanidine
NAD+	Nicotinamide adenine dinucleotide
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
O_2•-	Superoxide radical
PAR	Poly (ADP-ribose)
PARG	Poly (ADP-ribose) glycohydrolase
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCO	Posterior capsule opacification
PIKK	Phosphatidylinositol 3-kinase-related kinase

PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SCID	Severe combined immunodeficiency
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	Short interfering ribonucleic acid
SOD	Superoxide dismutase
TEMED	Tetramethylethylenediamine
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1

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Chapter 1 General Introduction

1.1 The eye

The human eye is a sense organ essential for facilitating vision and it is comprised of many interrelated components to achieve this. The outer layer consists of the sclera and cornea, the latter of which allows light to enter the eye and focus on the lens, which itself is located behind the aqueous humour and iris. The iris controls how much light can enter the pupil by expanding and contracting. The role of the lens is to focus light through the vitreous humour onto the retina which relays electrical impulses to the brain via the optic nerve (Figure 1.1) (Roberts, 2001).

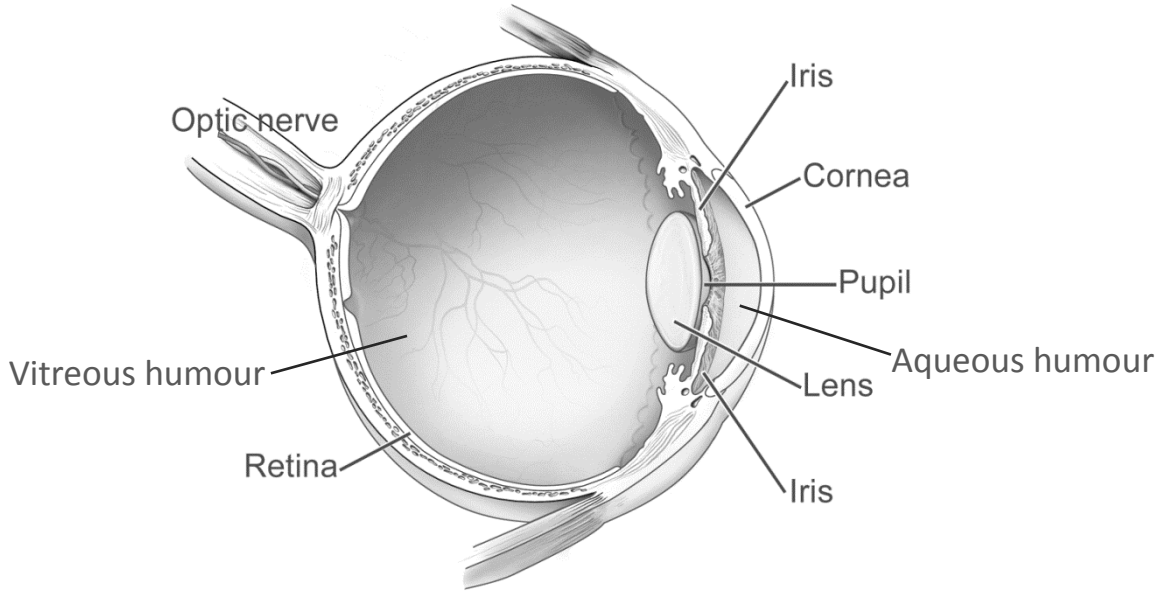


Figure 1.1. A diagram of the human eye. Adapted from www.nei.nih.gov/photo/eyean/images/NEA08_72.jpg
National Eye Institute, National Institutes of Health Ref#: NEA08

1.2 The lens

The lens is a refractive organ of the eye which, together with the cornea is required to focus light onto the retina to facilitate vision. In humans, it is located in the anterior portion of the eye behind the cornea, iris and aqueous humour in front on the vitreous humour. The lens is non-innervated and has no blood supply and thus obtains all its nutritional needs from the aqueous and vitreous humours in which it is bathed (Figure 1.1) (Wormstone et al., 2006).

The lens is comprised of two distinct cell types, epithelium and fibre cells, with the whole organ enveloped by a thick basement membrane, the lens capsule, which separates the cells of the lens from the rest of the eye (Figure 1.2). The lens epithelium is a monolayer of cuboidal cells located on the anterior surface of the organ required for the regulation of normal lens homeostasis (Roberts, 2001; Wormstone et al., 2006) and from which all the cells of the lens originate. The bulk of the organ is comprised of fibre cells which are essentially an inactive mass of denucleated, elongated cells packed full of transparent, water soluble proteins called crystallins (Andley, 2007; Tholozan and Quinlan, 2007).

All the cells of the lens originate from the proliferation and differentiation of the lens epithelium which occurs at a specific location within this population of cells known as the equator (Figure 1.2) (Wormstone et al., 2006). Cells of the central lens epithelium are essentially a non-dividing population. The lens equator is located at the boundary between the aqueous and vitreous humours, immediately above which cell proliferation occurs, followed by a downward migration below the equator before differentiating into fibres as they move inwards adding to the mass of the lens (Tholozan and Quinlan, 2007). This process is driven

and influenced by growth factors contained within the aqueous and vitreous humours and involves a concentration gradient of fibroblast growth factors (FGF). In the vitreous, below the equator, a higher concentration of FGF promotes differentiation, whereas above the equator, the concentration is lower and thus proliferation is favoured. (Lovicu and McAvoy, 2005; Tholozan and Quinlan, 2007).

The process of differentiation progresses as cells migrate inwards towards the centre of the lens and involves an increased production of key structural proteins (crystallins), cellular elongation and a complete loss of organelles – all of which contribute to a decrease of light scattering properties and promote lens transparency (Tholozan and Quinlan, 2007). A unique feature of the lens is that it continues to grow throughout life without any turnover (Andley, 2007). Indeed, this growth occurs in a linear fashion from the age of 10 to 80 years (Duncan et al., 1997). Thus, as epithelial cells are produced and differentiate into fibres, older cells become increasingly packed into the centre of the lens, with new layers of fibres being added to its surface (Augusteyn, 2010). This tight packing of the lens fibres gives rise to a highly organised regular arrangement of cells which, along with a decrease in cell thickness and extracellular space aids transparency (Michael and Bron, 2011).

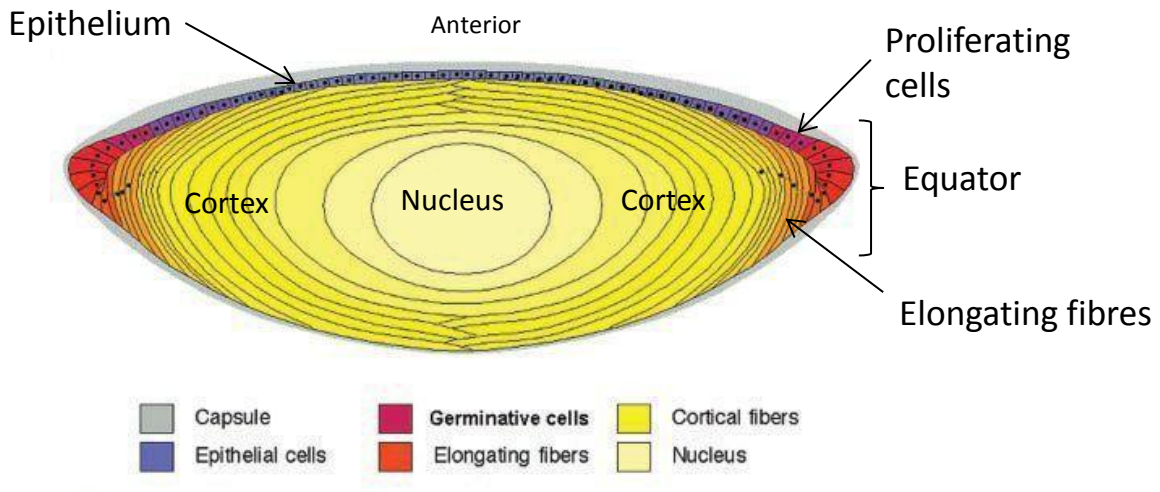


Figure 1.2. A diagram of a cross-section through the human lens (Image adapted from Maidment et al, 2004).

Also key to the transparency of the lens is the extremely high concentration of the structural proteins of the lens, the crystallins (α, β and γ), production of which is greatly upregulated during fibre cell differentiation. These proteins account for 90% of all lens proteins (Sharma and Santhoshkumar, 2009), are highly water soluble and form durable long lasting structures. The endurance of the crystallins is critical since the differentiation process (loss of organelles) renders the cells unable to produce new protein (Bloemendal et al., 2004). Therefore, longevity of the crystallins is essential to maintaining transparency of the lens when considering that the proteins of the central lens (the nucleus) are as old as the individual.

The lens epithelium plays an essential protective and supportive role to the fibres and any disruption in this relationship can render proteins of the lens susceptible to damage and lens as a whole susceptible to a loss of transparency, termed cataract.

1.3 Cataract

1.3.1 Prevalence

Cataract is predominantly an age-related disease, although forms of congenital cataract exist. It is the most common causes of blindness worldwide (Wormstone et al., 2006) with current World Health Organisation statistics stating that cataract accounts for 51% of all blindness worldwide, accounting for approximately 20 million people (WHO, 2014). Cataract is defined as any opacity, or clouding of the lens and this may encompass part of or the entire organ. When this opacity is situated on the visual axis a loss of vision occurs.

1.3.2 Classification/types of cataract

There are several forms of age-related cataract and classification is based on their location within the lens and they may occur individually or in combination (Figure 1.3). The three most common forms are nuclear, cortical and posterior sub-capsular cataract. Nuclear cataract occurs in the centre of the lens, or nucleus. It is generally associated with post-translational modification to the structure of the crystallins, which causes these otherwise transparent proteins to lose solubility and form light scattering protein aggregates (Vrensen, 1995). Additionally, other changes occur that manifest in the formation of a brown/yellow pigment within the lens over time.

Cortical cataract occurs in the lens cortex which is comprised of the newer fibres that overlay the nucleus. These fibres are younger than those found in the nucleus but with age changes in membrane permeability occur causing osmotic imbalance, cell swelling and protein modification (Michael and Bron, 2011). One consequence is an increase in intracellular Ca^{2+} which has been suggested to promote protein aggregation (Duncan et al., 1989).

Posterior sub-capsular cataracts occur at the rear of the lens and are associated with abnormal fibre cell production resulting in dysplastic cells that migrate and accumulate at the posterior pole of the lens (Al-ghoul et al., 1998).

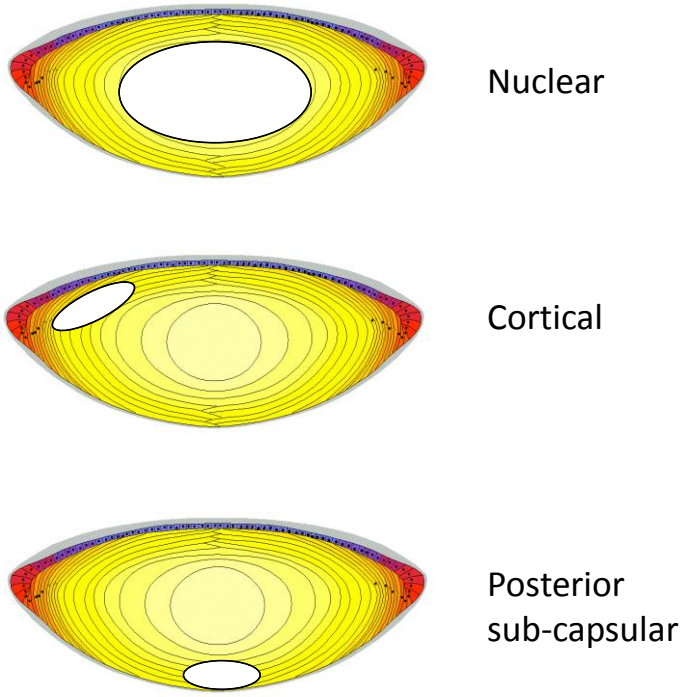


Figure 1.3. Types and location of cataracts in the human lens. Clear areas highlight the region of cataract.

1.3.3 Risk factors associated with cataract formation.

The overwhelming risk factor associated with cataract formation is age; indeed, there is an exponential increase in cataract above the age of 50 years (Duncan et al., 1997). As we age, older fibres of the lens are not replaced and so structural changes to the fibres occur which cannot be reversed by the production of newer fibres. Such changes accumulate and the result is a reduction in transparency. Additionally, as we age there is a build-up of yellow/brown pigment which results in a decrease in the ability of light to pass freely through the lens (Allen and Vasavada, 2006). Geographical and socioeconomic factors also influence cataract formation with the disease being more prevalent in the developing world, with malnutrition, exposure to sunlight and poorly developed healthcare systems playing a role. It has also been suggested that females are more susceptible to cataract (Asbell et al., 2005), but that males have increased incidence compared to females below the age of 50, above this however incidence is greater in females. This suggests a hormonal role, since women undergoing hormone replacement therapy have a decreased incidence (Duncan et al., 1997). Lifestyle factors have also been implicated in cataract formation with smoking, sunlight exposure and alcohol use all linked as well as common medical problems such as obesity, diabetes, hypertension, diarrhoea and dehydration. Certain risk factors have also been linked to formation of specific types of cataract, for example systemic corticosteroid use and posterior sub-capsular cataract and diabetes with both cortical and posterior sub-capsular cataract (Asbell et al., 2005).

1.3.4 Management of cataract – surgery.

Currently, the only means of treating cataract is by surgical intervention (Wormstone et al., 2009). Perhaps not surprisingly owing to the prevalence of the disease, cataract surgery is a

widely performed procedure with 32 million operations estimated to be performed each year by 2020 (Liu et al., 2013).

Modern cataract surgery involves phacoemulsification to break up and remove the cataractous fibre cell mass. A small incision of approximately 2 millimetres is made at the edge of the cornea to allow access to the lens capsule, in which a capsularexis, a small circular tear (approximately 5 millimetres in diameter), is made allowing access to the lens fibres. An ultrasonic probe is inserted, which breaks up and removes the cataractous lens to create a capsular bag consisting of the posterior capsule and the remaining anterior capsule minus the capsularexis into which an artificial intraocular lens (IOL) is inserted. This restores refraction and free passage of light to the retina (Asbell et al., 2005; Wormstone et al., 2009).

1.3.5 Posterior capsule opacification.

A common complication of cataract surgery is posterior capsule opacification (PCO). PCO causes a secondary loss of vision and is a wound healing response. Epithelial cells that are not eliminated from the remaining anterior capsule during surgery grow rapidly, stimulated by the trauma of surgery, invade the visual axis covering the normally cell-free posterior capsule causing it to wrinkle or contract causing light scattering (Figure 1.4) (Wormstone, 2002; Wormstone et al., 2006; Wormstone et al., 2009). Although steps have been made to attempt to avert this secondary loss of vision, which occurs in up to 41% of patients (Wormstone, 2002), such as improvements in IOL design or in the surgical procedure itself, quite often expensive and potentially risky corrective laser surgery is necessary (Ranta and Kivela, 1998).

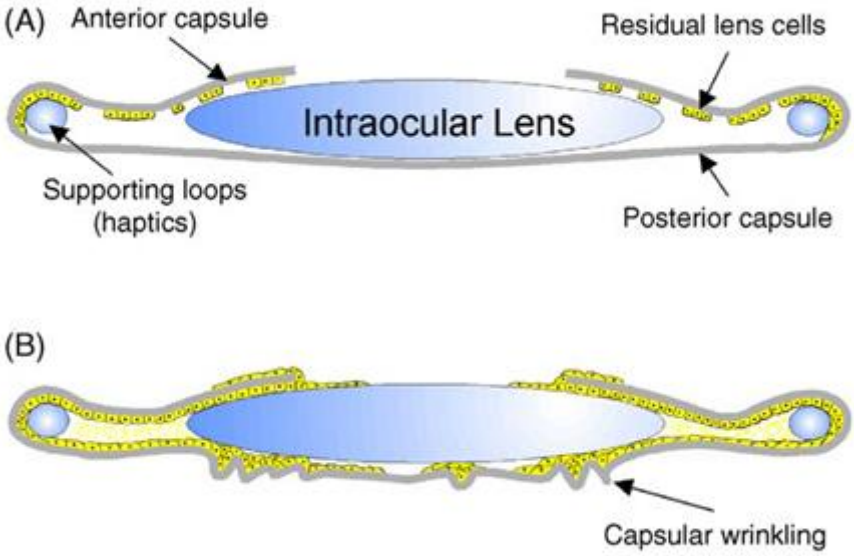


Figure 1.4. Diagram illustrating PCO. (A) During cataract surgery the cataractous lens is removed creating a capsular bag into which an IOL is inserted, restoring free passage of light. However, not all epithelial cells are removed. (B) remaining epithelial cells grow and invade the normally cell free posterior capsule causing contraction or wrinkling producing light scattering and secondary loss of vision (adapted from Wormstone et al, 2002).

1.4 Oxidative stress and cataract

1.4.1 Oxidative stress

Oxidative stress plays a key role in the formation of cataract (Truscott, 2005; Vinson, 2006). Oxidative stress can be defined as an imbalance between pro- and anti-oxidants in favour of the former (Evans et al., 2004). When this imbalance occurs and antioxidant defence systems become overwhelmed, there is a potential for oxidative damage to proteins, lipids and DNA. Reactive oxygen species (ROS) are the mediators of oxidative stress; they are continuously generated and can arise through endogenous processes such as cellular metabolism or exogenously via processes such as UV-induced photochemical reactions or exposure to ionizing radiation (Cooke et al., 2003; Evans et al., 2004). Major cellular ROS include the superoxide radical ($O_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) (Spector, 1995).

Fortunately, the cell has evolved a number of defences to counter ROS and prevent the damaging state of oxidative stress. Such defences are either enzymatic or non-enzymatic. Non-enzymatic antioxidants present in the cell include vitamins C and E which act effectively to neutralise ROS preventing damage to the cell. Reduced glutathione (GSH), another reducing compound, is particularly important in the lens and is present in high concentrations (Giblin, 2000). Such compounds represent the simplest forms of antioxidant. However, enzymatic antioxidant systems such as superoxide dismutase (SOD) and catalase are particularly efficient defences against ROS. Superoxide and H_2O_2 are both products of cellular respiration. SOD catalyses the reduction of $O_2^{\bullet-}$ to the less reactive H_2O_2, which in turn is converted by catalase to water and oxygen. A further complication is that H_2O_2, via

interaction with intracellular iron, can produce the highly reactive $^{\bullet}OH$ radical (Sorg, 2004; Spector, 1995).

1.4.2 Antioxidants in the lens

Lens cells, as with other cell types, are constantly exposed to oxidative stress. High levels of ROS, either due to their increased production, or due to a decrease in the cell's ability to eliminate them, leads to the state of oxidative stress and subsequent cellular damage. The lens possesses an array of antioxidant defences to protect it from oxidative damage and these include membrane bound vitamin E, cytosolic reduced glutathione (GSH), vitamin C, cysteine, methionine, glutathione reductase (GR), glutathione peroxidase, thioredoxin and thioltransferase (Michael and Bron, 2011).

Of the antioxidant systems present in the lens, GSH is perhaps the most important. It is produced by the epithelium, from where it is circulated to the rest of the lens, it is present in high (millimolar) amounts (Giblin, 2000; Michael and Bron, 2011; Truscott, 2005). As GSH is oxidised (to GSSG) it rapidly becomes recycled by GR using NADPH as a reducing agent. With age, however, production of GSH and its reduction by GR declines, potentially rendering proteins of the lens susceptible to oxidative modification, with the ultimate consequence of formation of opacities of the lens. GSH plays a key role in protection of the lens crystallins, and also in protecting other targets of oxidation such as Na^+/K^+ ATPase and Ca^{2+} ATPase ion pumps and other proteins essential for the transport of nutrients, water and importantly antioxidants throughout the lens (Michael and Bron, 2011).

1.5 DNA damage and DNA repair

The genomes of all cells are constantly exposed to and damaged by a variety of DNA damaging stimuli. These can arise exogenously, such as exposure to ultraviolet light or ionizing radiation, or endogenously such as ROS generated during cellular metabolism, an example being H_2O_2.

Within the human cell, approximately 10,000 DNA lesions arise per day (Lindahl, 1993). Key to the prevention of disease such as cancer and the delayed onset of ageing is the cell's ability to repair this DNA damage through a variety of DNA repair mechanisms, since any lesions that go unrepaired may become mutations following replication; if they are able to be replicated they cannot be detected by the DNA damage response. Another consequence of DNA damage may be cell death (Hoeijmakers, 2007; Schumacher et al., 2008).

Various DNA damage repair pathways are available for the cell to utilise depending on the type of lesion present. These include base excision repair (BER), nucleotide excision repair (NER), mis-match repair (MMR), homologous recombination (HR), non-homologous end joining (NHEJ) and direct reversal of DNA damage (Friedberg, 2003; Hoeijmakers, 2001).

The BER pathway is responsible for the repair of DNA base damage caused by oxidation, hydrolysis and alkylation of DNA, which leads to lesions that include oxidised bases, abasic sites and single-strand breaks (Wilson and Bohr, 2007). Oxidative stress-induced DNA damage is a common mode of DNA damage; however, some ROS do not react directly with

DNA. For example, H_2O_2 does not react directly with DNA and must first be converted to $^{\bullet}OH$ via the Fenton reaction. The $^{\bullet}OH$ radical is then able to react with DNA bases by addition or removal of hydrogen. A common and mutagenic lesion, 8-hydroxyguanine, is produced by addition to guanine. Oxidative damage can also lead to the formation of abasic sites and also single and double strand breaks (although the latter are not repaired by BER) via damage to deoxyribose residues (Marnett, 2000; Powell et al., 2005).

Two sub-pathways of BER exist: Short-patch and long-patch, replacing one or multiple nulceotides (up to 13 nucleotides) respectively (Hakem, 2008). Short-patch BER involves the removal of the damaged base by a glycosylase (specific to the type of base modification) to produce an abasic site, strand excision by an endonuclease APE1 and incorporation of nucleotides by DNA polymerase β. Strand ligation is performed by a complex of ligase-III and XRCC1 (Wilson and Bohr, 2007). Long-patch BER follows a different pathway whereby several nucleotides are replaced, and as such utilises different proteins to process the DNA, including DNA polymerases ε or δ, proliferating cell nuclear antigen (PCNA) and a flap endonuclease, FEN1, and ligase I to produce a similar outcome (Wilson and Bohr, 2007). The BER repair pathways may also be initiated in response to single strand breaks. To stabilise the strand break PARP binds with XRCC1, which is followed by the production of 5'phosphate and 3'OH by polynucleotide kinase (PNK). The remaining repair is predominantly carried out via short-patch BER (Figure 1.5) (Hoeijmakers, 2001; Wilson and Bohr, 2007).

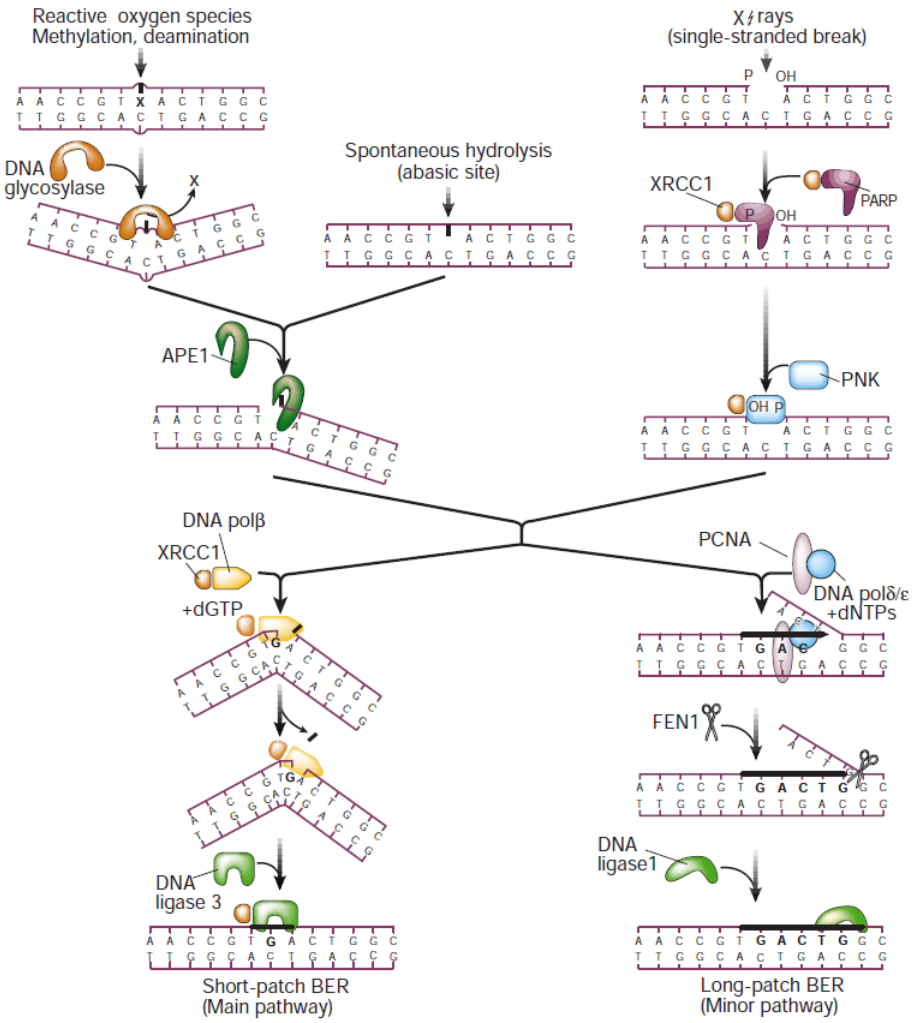


Figure 1.5. Base excision repair. Base damage or single strand breaks are either repaired by short-patch or long patch BER depending on the number of nucleotides to be replaced. A damaged base (x) is removed by a DNA glycosylase to produce an abasic site, which can also be generated by spontaneous hydrolysis. Strand excision is performed by APE1 (or a DNA strand break). PARP and XRCC1 bind to stabilise the exposed DNA ends. In the case of short-patch BER nucleotides are incorporated by DNA polymerase β, with strand ligation carried out by DNA ligase 3. In the case of long patch BER, DNA polymerases δ/ε, PCNA and FEN1 process the damage with ligation performed by DNA ligase I (Image adapted from Hoeijmakers, 2001).

NER, again comprising of two sub-pathways, is responsible to the repair of bulky DNA adducts such as those caused by ultraviolet light. Global genome NER (operating throughout the genome) and transcription coupled NER (operating to repair actively transcribed genes) utilise common proteins, except during damage recognition. A multi-protein complex recognises the lesion in both instances (XPC and XPE proteins in the case of global genome NER, and CSA and CSB in the case of transcription coupled NER). Subsequent steps are consistent for both sub-pathways; DNA helicases XPB and XPD unwind the DNA either side of the lesion with RPA stabilising the single stranded DNA and XPA aiding in recognising the lesion. Endonucleases XPG and XPF then act to remove the lesion by producing a single strand break to each side, with the resulting gap filled and sealed by a DNA polymerase and a DNA ligase respectively (Jackson and Bartek, 2009; Lombard et al., 2005).

The MMR pathway serves to replace insertions and deletions that occur during DNA replication (i.e. errors of DNA replication that elude the proof-reading activity of DNA polymerase). Mismatches are first recognised by a protein complex comprising of MSH2 and either MSH6 or MSH3, which possess differing specificities to various mismatches (Christmann et al., 2003; Martin et al., 2010). Following recognition, a second protein complex, MLH1-PMS2 binds, which is thought to direct the initiation of excision of the lesion by exonuclease 1, with repair completed by a DNA polymerase (possibly α or β) and a ligase (Martin et al., 2010).

DNA double strand breaks can be generated from exogenous sources of DNA damage such as ionizing radiation and endogenous stimuli such as ROS produced during cellular metabolism (Costantini et al., 2007). They are repaired via two independent mechanisms

operating at different phases of the cell cycle. The major pathway for the repair of DNA double strand breaks is NHEJ (Figure 1.6), which accounts for the repair of approximately 90% of double strand breaks and is predominant during G1. By contrast, HR repairs approximately 10% of DNA double strand breaks and is active during S and G2 phases of the cell cycle. In addition to its role in the repair of DNA double strand breaks, NHEJ is also required for the re-joining of double strand breaks produced during V(D)J recombination (Hakem, 2008; Weterings and Chen, 2008).

NHEJ does not require a template strand and involves the re-ligation of DNA double strand breaks without consideration for sequence. Several key proteins are involved; Ku70, Ku80, ligase IV, XRCC4, DNA-PKcs and Artemis. Briefly, Ku70 and Ku80 bind as a heterodimer to the DNA either side of the break, where their presence facilitates the recruitment and binding of DNA-PKcs (DNA dependent kinase catalytic subunit) which together with the ku70/ku80 heterodimer forms DNA-PK (DNA protein kinase). DNA-PK phosphorylates and activates itself and other proteins of NHEJ such as Artemis, a nuclease which processes DNA ends unable to be directly re-joined. Activated DNA-PK is also auto-phosphorylated, promoting the release of DNA-PKcs from the Ku70/80 heterodimer allowing for subsequent re-joining by the ligase IV-XRCC4-XLF/Cernnunos complex (Costantini et al., 2007; Jeggo et al., 2011; Lombard et al., 2005). Figure 1.6 illustrates NHEJ. Importantly, since NHEJ does not rely on a template strand, as it generates simple re-joining of double strand breaks without consideration for any sequence, it is error prone (Hakem, 2008; Weterings and Chen, 2008).

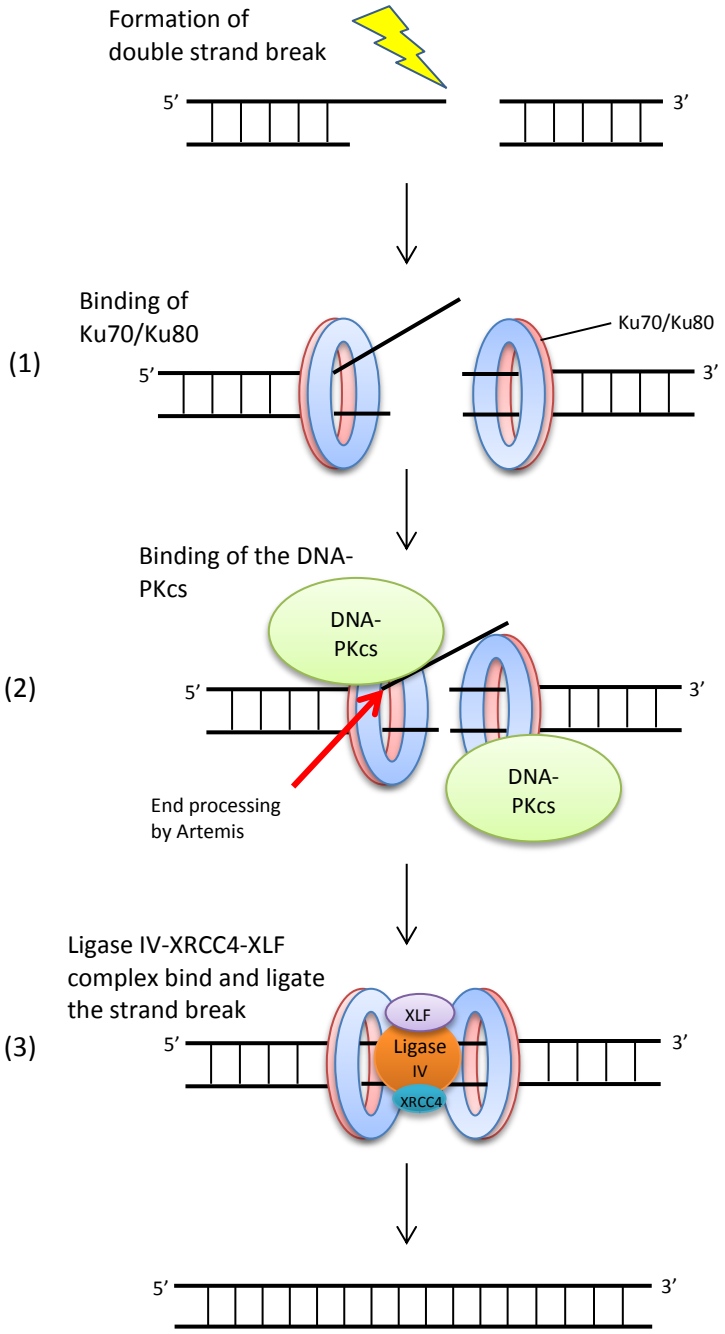


Figure 1.6. A summary of Non-homologous end joining. Following the formation of a DNA double strand break a heterodimer of Ku70 and Ku80 recognises and bind to either side of the lesion (1) and recruits the DNA-PKcs which binds to form DNA-PK. DNA-PK phosphorylates itself and other proteins of NHEJ involved in end processing such as Artemis (2). DNA-PK autophosphorylation may allow for dissociation of DNA-PKcs from the Ku heterodimer, allowing for binding the of Ligase IV-XRCC4-XLF complex which ligates the remaining strand break (3).

Importance of NHEJ is highlighted by phenotypes observed in disorders characterised by defects in NHEJ proteins, with cells devoid of functioning NHEJ exhibiting hypersensitivity to DNA double strand break-inducing stimuli (Kerzendorfer and O'Driscoll, 2009). Ligase IV syndrome is a disease in which patients display symptoms which include radiosensitivity, reduced stature, abnormal brain development and compromised immunity and is caused by hypomorphic mutations in DNA ligase IV. Frank et al (Frank et al., 2000) further highlight the importance of ligase IV, observing that ligase IV null mice (rather than the hypomorphic mutation observed in human ligase IV syndrome patients) display embryonic lethality and neuronal apoptosis, probably as a result of p53-dependent response to unrepaired DNA damage. Other syndromes associated with defective NHEJ include Artemis-dependent SCID and XLF-Cernnunos-SCID (severe combined immunodeficiency), with both characterised by defective immunity and radiosensitivity (Hakem, 2008; Kerzendorfer and O'Driscoll, 2009).

No human patients have been described with mutations in either Ku protein (Seluanov et al., 2004; Wang et al., 2009a), however, a study of Ku80 knockout mice by Vogel and colleagues (Vogel et al., 1999) highlights a role for Ku80 in the ageing process. They reveal an early onset of age-specific changes including osteopenia, atrophic skin, hepatocellular degeneration and shorter life-span in addition to an earlier onset of cancer when compared to wild type mice. Interestingly however, Ku proteins have been implicated in the ageing process within human fibroblasts. End joining by NHEJ has been shown to decrease in efficiency within senescing cells (Seluanov et al., 2004) and levels of Ku70 and Ku80 have been demonstrated to decrease in senescing cells (Seluanov et al., 2007).

HR, unlike NHEJ is relatively error free and occurs during the S or G2 phases of the cell cycle, using the undamaged sister chromatid as a template. This pathway involves multiple proteins with the initial step being processing of the double strand break by nuclease activity to generate single stranded DNA by proteins of the MRN (MRE11-RAD50-NBS1) complex. Single stranded DNA is stabilised by RPA and a protein complex including RAD51, BRCA1 and BRCA2 directs the invasion of the single strand into the undamaged sister chromatid template, with DNA polymerases and ligases completing the repair (Jackson and Bartek, 2009; Lombard et al., 2005).

The most simple of the DNA damage responses is the direct reversal of DNA damage, which occurs without the need to excise the damaged base. This is mediated by a single enzyme specific to the damaged base in question and is used to repair modified bases such as O^6-alkylguanine. O^6-alkylguanine is repaired by O^6-methylguanine DNA methyltransferase which simply removes the alky group from guanine, thus reversing the damage (Hakem, 2008).

1.5.1 Poly (ADP-ribose) polymerase and DNA damage repair

Poly (ADP-ribose) polymerases (PARPs) are a group of proteins consisting of approximately 20 members that are increasingly implicated in the DNA damage response (Sousa et al., 2012). They catalyse the post translational modification of their target or acceptor proteins by the addition of ADP-ribose polymers which are produced and added to these proteins using nicotinamide adenine dinucleotide (NAD+) as a substrate (Figure 1.7). The poly(ADP-ribose) (PAR) polymer possesses a negative charge and the result of this binding to target proteins is

an alteration in their structure and function. (Heeres and Hergenrother, 2007; Sousa et al., 2012). In addition to their role in post translational modification target proteins, PAR polymers are also able to act as free PAR, potentially acting as signalling molecules (Heeres and Hergenrother, 2007; Sousa et al., 2012). The process of poly (ADP-ribosyl)ation is a reversible process with PAR polymers, which can reach up to hundreds of units in length, rapidly degraded by poly (ADP-ribose), glycohydrolase (PARG) (Krishnakumar and Kraus, 2010; Sousa et al., 2012).

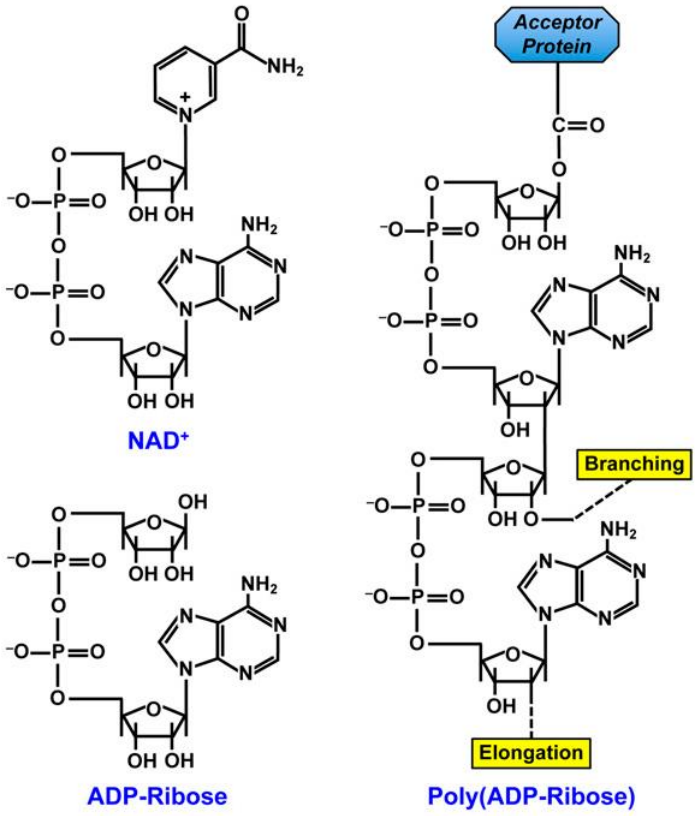


Figure 1.7. PARP catalyses the addition of poly(ADP-ribose) (PAR) chains onto acceptor proteins using NAD+ as a source of ADP-ribose. PAR is attached to aspartate or glutamate residues on acceptor proteins and can form long branched structures able to alter structure and function (adapted from Luo and Kraus, 2012).

The major stimulus for PARP activation and PAR production is DNA damage (Langelier and Pascal, 2013). PARP-1 is the most abundant of the PARP family of proteins and is the predominant PARP generating PAR in response to DNA damage (Sousa et al., 2012). PARP-1 is approximately 113 kDa and contains three functional domains; a DNA binding domain, an automodification domain and a catalytic domain (Hong et al., 2004; Virag et al., 2013). Upon DNA damage, including both single and double DNA strand breaks, production of PAR is increased up to 500 times (Heeres and Hergenrother, 2007).

PARP-1 has a high affinity for DNA strand breaks, and once bound to the strand break catalyses its auto PARylation which is thought to aid the DNA damage response allowing recruitment of proteins involved in several DNA repair pathways (Malanga and Althaus, 2005). PARP-1 and the PAR polymer have been shown to interact with a large variety of proteins involved in the DNA damage response including base excision repair proteins APE1, DNA polymerase β, DNA ligase IIIα and FEN1; NHEJ proteins Ku70, Ku80, DNA ligase IV and DNA-PK; and homologous recombination proteins MRE11 and NBS (Sousa et al., 2012).

PARP-1 also functions to allow DNA repair proteins access to DNA lesions in a process whereby histones are acceptor proteins of PAR allowing chromatin remodelling permitting a relaxation of DNA facilitating an ease of access to the site of damage (Althaus, 1992; Poirier et al., 1982).

1.6 Studies of DNA damage in the lens

Cataract is a disease of which oxidative stress is a key causative factor (Truscott, 2005), one consequence of oxidative stress is DNA damage. Surprisingly, very few studies to date have investigated the role of DNA damage in cataract formation despite the fact that apoptosis (which may result from DNA damage) in the lens epithelium has been linked to cataract formation (Li et al., 1995).

Ates and colleagues (Ates et al., 2010) investigated DNA damage in lymphocytes isolated from cataract patients and compared them to healthy subjects assaying levels of 8-hydroxy 2-deoxyguanosine (8-OHdG) as a marker of oxidative DNA damage. Significantly greater levels of oxidative DNA damage were noted in cataract patients compared to healthy control subjects, suggesting a greater vulnerability to DNA damage in cataract patients.

Kleiman and Spector (Kleiman and Spector, 1993) and Sorte et al (Sorte et al., 2011) have both investigated levels of DNA strand breaks in lens epithelial samples obtained from cataract patients following surgery with comparisons made to control subjects. Kleiman and Spector found levels of DNA strand breaks to be elevated in approximately 50% of patient samples whereas Sorte and colleagues reported significant increases in cataract patients compared to control subjects. The latter also made comparisons between levels of DNA strand breaks in various cataract types concluding levels of DNA strand breaks in cortical cataracts were greater than in nuclear and posterior sub-capsular cataracts.

Animal studies have also been performed. In one, Reddy and colleagues (Reddy et al., 1998) investigated the effect of the concentration of ascorbic acid in the aqueous humour of rats and guinea pigs on levels of UV-induced DNA damage. They found that high levels of ascorbic acid protected against UV-induced DNA strand breaks, concluding that lenses in diurnal animals such as guinea pigs (which have naturally higher levels of ascorbic acid in the aqueous) were probably protected against UV-induced cataracts in this manner. In contrast, DNA strand breaks in rats (which are nocturnal and have naturally lower levels of aqueous ascorbic acid) following UV irradiation were higher, but these levels could be reduced with injection of ascorbic acid into the aqueous.

Sasaki et al (Sasaki et al., 1998) explored the ability of TEMPOL (a compound that mimics nitroxide free radical and superoxide dismutase) to protect against X-ray induced DNA strand breaks and cataract in rabbits. They found that lenses cultured in TEMPOL and X-rayed in vitro had significantly reduced levels of DNA strand breaks compared to control lenses. They also discovered that levels of X-ray-induced DNA strand breaks in vivo following TEMPOL injection into the aqueous humour were reduced and that this treatment afforded protection from cataract.

A more recent study by Mesa and Bassnett (Mesa and Bassnett, 2013) investigated UV-B-induced DNA damage and repair in the mouse lens, noting that modest doses of UV-B were sufficient to induce photolesions in the lens epithelium of mice. Some photolesions were found to persist and the authors suggested somatic mutations which may arise as a result could contribute to cortical cataract formation.

DNA damage and repair in the lens is evidently and surprisingly a much neglected area of investigation in cataract research. These previous studies certainly indicate a potential role for DNA damage in the formation of cataract and therefore this provides scope for further investigation.

1.7 Aims

Cataract is a disease that affects millions worldwide. Reducing the occurrence of this disease would be beneficial not only to the individual sufferer, but also significantly decrease the worldwide burden on healthcare budgets. The current study aims to identify contributing factors underlying the formation of cataract.

Cataract is primarily an age-related disease and the cells of the lens are constantly exposed to oxidative stress. One consequence of oxidative stress is DNA damage; the cells of the lens are exposed to such stressors over long time frames. The incidence of cataract increases as we age and ageing has been associated with a decrease in the ability to repair DNA damage. It is therefore proposed that the ability to manage DNA damage by DNA repair pathways is crucial for the delayed onset of cataract.

The aims of this thesis are:

(1) Establish a model for studying the effects of oxidative stress on human lens cells.
(2) Investigate the role of non-homologous end joining DNA damage repair in human lens cells in respect to elucidating the role of this pathway in lens cell responses to oxidative stress and cataract formation.
(3) Investigate the possible role of the nuclear protein poly(ADP-ribose) polymerase-1 in human lens cell responses to oxidative stress and cataract formation.

Chapter 2 Materials and Methods

2.1 Cell culture

The FHL124 human lens epithelial cell line is a non-virally transformed cell line generated from human capsule-epithelial explants (Reddan et al., 1999). FHL124 cells demonstrate a 99.5% homology to native lens epithelium in transcript profile (Wormstone et al., 2004). FHL124 cells were cultured at 35°C with 5% CO_2, 95% air in Eagle's Minimum Essential Medium (EMEM) (Gibco, Paisley, UK) containing 5% v/v foetal calf serum (FCS) (Gibco) and 50 μg/ml gentamicin (Sigma-Aldrich, Dorset, UK).

FHL124 cells were seeded onto 96 well plates (5000 per well for ApoTox-Glo Triplex Assay), 35 mm tissue culture dishes (35,000 per dish for alkaline comet assay and western blot) or 18 x 18 mm glass coverslips contained with 35 mm tissue culture dishes (10,000 per coverslip for immunocytochemistry). 24 hours prior to addition of experimental conditions, cell culture medium was replaced with EMEM without FCS.

2.2 FHL124 cell treatments

2.2.1 Hydrogen peroxide treatments

FHL124 cells were grown until approximately 70% confluent and placed in serum-free EMEM for 24 hours. H_2O_2 (Sigma-Aldrich) was freshly diluted in serum-free EMEM and

added to culture dishes to produce appropriate concentrations. For the alkaline comet assay, at time points post-treatment FHL124 cells were washed free of treatment medium with ice-cold phosphate buffered saline (PBS) (Thermo Scientific, Basingstoke, UK), harvested, counted with a haemocytometer and re-suspended in PBS containing 10% dimethyl sulfoxide (DMSO) (Fisher Scientific, Loughborough, UK) and frozen at -80°C until the alkaline comet assay was performed.

For ApoTox-Glo Triplex Assay experiments, FHL124 cells were seeded onto 96 well plates and placed in serum-free EMEM for 24 hours prior to addition of experimental conditions. Serum-free EMEM was removed from wells and replaced with H_2O_2 freshly diluted to appropriate concentrations in serum-free EMEM.

For LDH assay experiments cells were placed into serum-free EMEM 24 hours prior to H_2O_2 addition. H_2O_2 was either freshly diluted in serum-free EMEM and added to culture medium to produce appropriate concentrations or serum-free EMEM was removed from cells and replaced with H_2O_2 freshly diluted in serum free EMEM.

In experiments where transfections were performed, H_2O_2 was freshly diluted in Opti-MEM (Invitrogen, Paisley, UK) and added to cultures to produce appropriate concentrations.

2.2.2 Chemical inhibition of Poly(ADP-ribose) polymerase (PARP)-1

PARP-1 was chemically inhibited with AG14361 (Selleck Chemicals, Houston, TX, USA), a potent and specific inhibitor of PARP-1 (Calabrese et al., 2004; Smith et al., 2005). AG14361 was diluted in DMSO (Fisher Scientific, Loughborough, UK) upon delivery to produce a 25 mM stock which was frozen at -80°C. All experiments therefore also contained a DMSO control diluted to the same concentration. AG14361 was freshly diluted in serum-free EMEM and added to cultures to give a final concentration of 1 μM.

2.3 Cell viability and cell death assays

2.3.1 ApoTox-Glo Triplex Assay

Cells were seeded onto 96 well plates and 24 hours prior to addition of experimental conditions culture medium was replaced with serum-free EMEM. Serum-free EMEM was replaced with experimental conditions and plates incubated at 35°C with 5% CO_2, 95% air. The ApoTox-Glo Triplex Assay (Promega, Madison, WI, USA) was performed according to the manufacturer's instructions to measure FHL124 cell viability, cytotoxicity and apoptosis and based upon previous work within our laboratory (Liu et al., 2013; Wang et al., 2012). Viability and cytotoxicity are measured by fluorescent signals produced upon cleavage of two substrates by either live-cell proteases or dead cell proteases. GF-AFC, a fluorogenic peptide substrate, can enter viable cells and is cleaved by live-cell proteases (which become inactive upon cell death), whereas bis-AAFR110, another fluorogenic peptide substrate, which is unable to enter live cells, is cleaved by dead-cell proteases released when membrane integrity is compromised. Fluorescence produced upon cleavage of GF-AFC or bis-AAFR110 is proportional to viability or cytotoxicity respectively, with each product having different

excitation and emission spectra. Apoptosis is measured by the addition of another substrate, a luminogenic caspase-3/7 (Caspase-Glo 3/7) substrate, cleaved in cells undergoing apoptosis to produce a luminescent signal.

A FLUOstar Omega plate reader (BMG LabTech, Ortenberg, Germany) was used to measure fluorescence at $380_{Ex}/510_{Em}$ (viability), $485_{Ex}/520_{Em}$ (cytotoxicity) and luminescence. Data was analysed as a percentage change in FHL124 cell viability, cytotoxicity or apoptosis from untreated samples.

2.3.2 Lactate Dehydrogenase (LDH) Assay

At experimental end points, culture medium (of FHL124 cells or whole human lenses) was sampled and assayed for LDH content as a measure of cell death using a Cytotoxicity Detection Kit (LDH) (Roche, Mannheim, Germany) following the manufacturer's instructions. LDH is a cytosolic enzyme released by damaged cells lacking membrane integrity and thus a marker of dead or dying cells when found in the culture medium. The level of LDH is assessed by an enzymatic test in two steps. Kit reagents are added, and firstly, NAD^+ is reduced to $NADH/H^+$ in the LDH-catalyzed conversion of lactate to pyruvate. Secondly, a catalyst (diaphorase) transfers H/H^+ to $NADH/H^+$ to a tetrozolium salt which becomes reduced to a formazan product which is read on a plate reader at 490 nm. The amount of LDH present in culture medium is proportional to the amount of dead or dying cells in a population. Data was analysed as a percentage change of absorbance at 490 nm from untreated FHL124 cells or whole human lenses.

2.3.3 Coomassie blue protein stain

At experimental end points FHL124 cultures were fixed with 4% formaldehyde for 30 minutes, formaldehyde was removed and cells washed with PBS 3 times for 5 minutes. To each culture 1 ml of coomassie blue stain (1g coomassie brilliant blue G (Sigma-Aldrich), 200 ml methanol (Sigma-Aldrich), 40 ml glacial acetic acid, 100 ml dH_2O) was added for 30 minutes. Coomassie blue stain non-specifically binds proteins and therefore can be used as a marker of cells present within a population, and thus viable cells. After 30 minutes, excess coomassie blue was removed by several washes in PBS and culture dishes photographed for presentation. To each culture dish 1 ml of 70% ethanol (Sigma-Aldrich) was added for 30 minutes to de-stain cultures, and 200 µl added to wells of a 96 well plate. Absorbance at 550 nm was measured on a plate reader (BMG LabTech). Data was analysed as a percentage change in absorbance at 550 nm from untreated samples.

2.4 Alkaline comet assay

2.4.1 Hydrogen peroxide treatment of FHL124 cells

FHL124 cells were seeded onto 35 mm plastic culture dishes and grown until approximately 70% confluent and placed into serum-free EMEM for 24 hours prior to addition of experimental conditions. Cultures were incubated at 35°C with 5% CO_2, 95% air for the experimental duration.

Hydrogen peroxide (Sigma-Aldrich) was freshly diluted in serum-free EMEM and added to culture dishes to produce appropriate concentrations. At time points post-treatment FHL124

cells were washed free of treatment medium with ice-cold PBS, harvested, counted with a haemocytometer and re-suspended in PBS containing 10% DMSO (Fisher Scientific) and frozen at -80°C until the alkaline comet assay performed.

2.4.2 Background and procedure

The alkaline comet assay (or single cell gel electrophoresis) is a simple, sensitive rapid and relatively cheap technique for analyzing and quantifying levels of DNA strand breaks in individual cells (Collins, 2004). The alkaline method employed measures a combination of both single and double strand breaks as well as alkaline labile sites (such as abasic sites produced as intermediates of base excision repair which are converted to single strand breaks under alkaline conditions) (Collins, 2004).

The method described here is based upon that developed by Singh and colleagues (Singh et al., 1988) and adapted and applied to lens cells (Liu et al., 2013). Procedures were performed under conditions of low light. FHL124 cells previously frozen were defrosted and immediately placed on ice. Approximately 20,000 cells per sample were centrifuged at 3000 rpm for 5 minutes at 4°C. Cell pellets were re-suspended in 0.6% low melting point agarose (Sigma-Aldrich), with 80 µl added in duplicate onto glass microscope slides (pre-coated in normal melting point agarose (Sigma-Aldrich)) and allowed to set on ice, with a glass coverslip placed on top to produce even, consistent gels. After approximately 5 minutes to allow gels to set, coverslips were removed and slides transferred into ice-cold lysis buffer (100 mM disodium EDTA (Fisher Scientific), 2.5 M NaCl (Fisher Scientific), 10 mM Tris-HCl (Sigma-Aldrich, Fisher Scientific), pH 10.0 with 1% triton-X-100 added immediately

prior to use) for 1 hour. Slides were washed twice for 10 minutes with ice-cold dH_2O, before being transferred to a flatbed electrophoresis tank and immersed in freshly prepared ice-cold electrophoresis buffer (300 mM NaOH (Fisher Scientific), 1 mM disodium EDTA, pH 13) for 30 minutes. Samples underwent electrophoresis in the same buffer at 21 V (1 V/cm) for 30 minutes.

Slides were drained of electrophoresis buffer and submerged in neutralisation buffer (0.4 M Tris-HCl, pH 7.5) for 30 minutes, followed by twice washing in dH_2O for 10 minutes. Slides were left to dry overnight at 37°C and stained with SYBR Green I nucleic acid (Sigma-Aldrich) stain diluted 1 in 10,000 in 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) for 5 minutes protected from light at room temperature. Slides were subsequently drained and dried at room temperature prior to visualisation and image capture by fluorescence microscopy (widefield microscope Zeiss AxioPlan 2ie, Zeiss, Gottingen, Germany). For each sample 100 comets (50 per gel) were randomly analysed and comets scored using Comet Assay IV Lite analysis software (Perceptive Instruments, Haverhill, UK). An overview of the alkaline comet assay is illustrated in Figure 2.1.

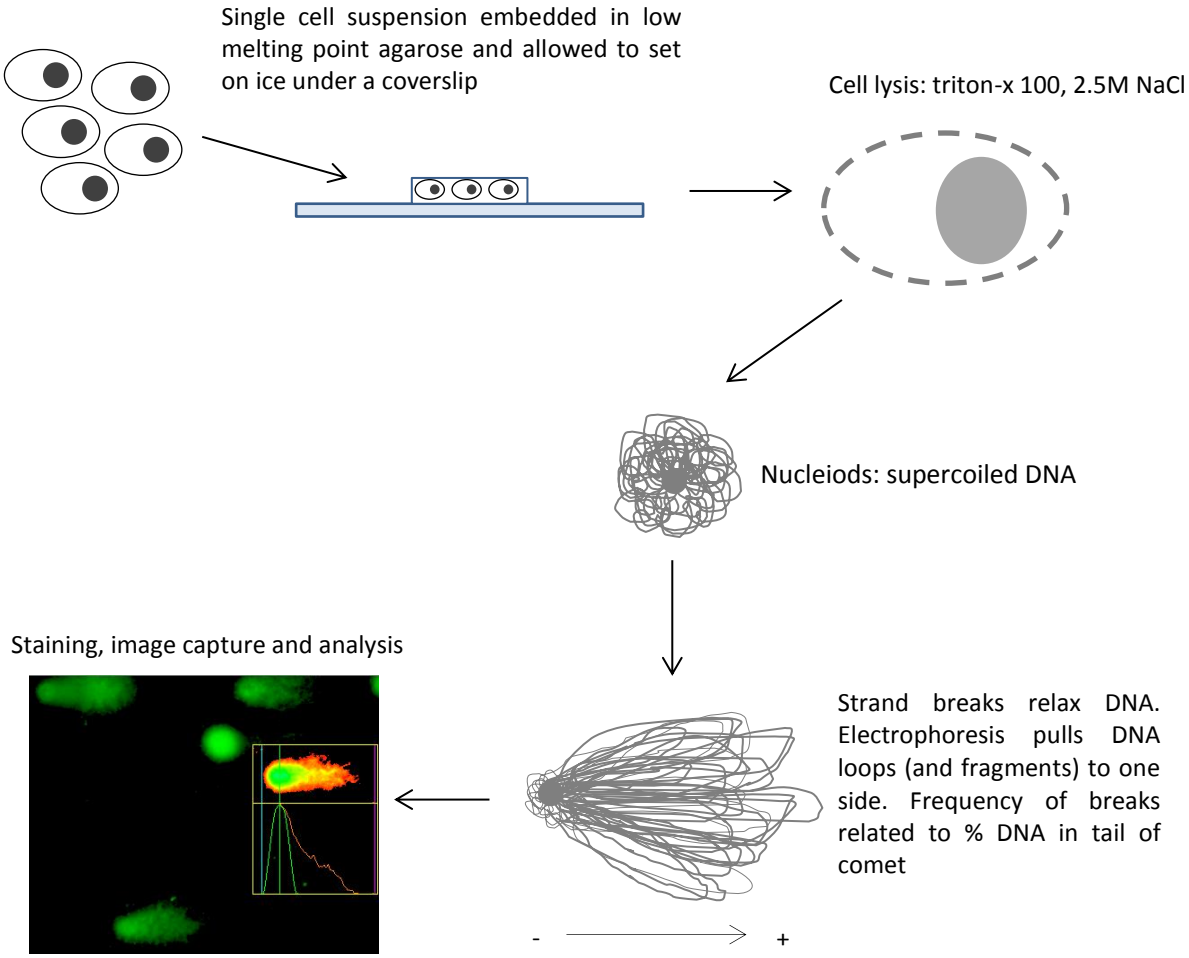


Figure 2.1. An overview of the alkaline comet assay. Cells are suspended in low melting point agarose and allowed to set under a class coverslip on ice. Coverslips are removed and slides submerged in lysis buffer to remove membranes and protein, leaving the nucleoids or supercoiled DNA. Where strand breaks are present, relaxation of DNA coiling occurs. Under electrophoresis relaxed loops of DNA migrate. Slides are stained, visualised and image analysis is performed with % DNA in the tail corresponding to levels of DNA strand breaks present within the cell.

2.5 Western blot analyses

2.5.1 Protein extraction

FHL124 cells were seeded onto 35 mm culture dishes and, 24 hours prior to addition of experimental conditions, cells were placed into serum-free EMEM. Culture medium was removed and cells washed with ice-cold PBS. Cells were then lysed by the addition of 350 µl M-PER buffer (mammalian protein extraction reagent) (Thermo Scientific) containing phosphatase and protease inhibitors and 0.5 M EDTA (Thermo Scientific) added at 10 µl/ml immediately prior to use, for 5 minutes. Cells were scraped from the culture dish with a cell scraper and centrifuged at 4°C for 10 minutes at 13,000 rpm. The supernatant was collected and cell lysates frozen at -20°C until required.

2.5.2 Bicinchoninic acid (BCA) assay

Protein content was determined with the BCA assay (Pierce, Thermo Scientific) performed according to manufacturer's instructions. The BCA assay is a colourimetric assay based on the reduction of Cu^{2+} to Cu^{+} by protein, producing a product which can be analysed at 562 nm on a plate reader. The amount of product is proportional to amount of protein present, which can be determined via the preparation of a standard curve using known amounts of protein.

A range of protein standards was produced by the dilution of bovine serum albumin (BSA) (Sigma-Aldrich) (0-1000 µg/ml) in M-PER. 10 µl of known standard (in triplicate) and unknown lysate sample (in duplicated) was added to wells of a 96 well plate and made up to 50 µl by the addition of dH_2O. Supplied reagents A and B were mixed in a ratio of 50:1 with

200 µl added to each well and the plate incubated for 1 hour at 37°C. The plate was allowed to cool and absorbance read at 562 nm on a plate reader (BMG LabTech). A standard curve was produced and protein concentration of each lysate sample determined.

2.5.3 Sample preparation

Samples were diluted to a constant concentration with dH_2O. Loading buffer was added to each sample (4% SDS (Melford Laboratories, Ipswich, UK), 0.01% bromophenol blue (Sigma-Aldrich), 30% glycerol (Sigma-Aldrich), 12.5% β-mercaptoethanol (Sigma-Aldrich) and 160 mM tris pH 6.8). Samples were centrifuged at 13,000 rpm for 2 minutes and heated to 100°C for 5 minutes.

2.5.4 SDS-PAGE

Protein samples were loaded into SDS-PAGE gels for electrophoresis. The upper gel consisted of 5% acrylamide (made by mixing dH_2O, 40% acrylamide solution (Bio-Rad Laboratories, Hempstead, UK), 4X upper gel buffer (0.5M Tris, 0.4% w/v SDS, pH 6.8), ammonium persulphate and TEMED (Sigma-Aldrich)). The lower running gel was made at 10% acrylamide (dH_2O, 40% acrylamide solution, 4X lower gel buffer (1.5M Tris, 0.4% w/v SDS, pH 8.8), ammonium persulphate and TEMED. Gels were run at a constant current of 0.03 A at 4°C.

2.5.5 Protein transfer

SDS-PAGE gels were incubated in transfer buffer (48 mM Tris, 39 mM glycine (Fisher Scientific), 4% v/v methanol (Fisher Scientific), 0.0375% w/v SDS, pH 8.3) for 30 minutes at room temperature. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer Life Sciences, Boston, MA, USA) (previously activated by immersion in 100% methanol for 30 seconds) by sandwiching gel and PVDF membrane between thick blotting paper which was then placed on a trans-blot semi-dry transfer cell (Bio-Rad) at a constant 15V and current of 0.3A per gel for 30 minutes.

2.5.6 Immunoblotting and development

Gels were discarded and PVDF membranes washed 3 times for 5 minutes in PBS. Membranes were blocked for 1 hour in PBS-T (5% w/v reduced fat milk powder (Marvel), 0.1% v/v Tween-20 (Fisher Scientific) in PBS). PBS-T was removed and membranes placed in PBS-T containing primary antibody (anti-Ku80, 1:1000; anti-PARP-1, 1:1000; anti-β-actin, 1:1000 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C.

Membranes were washed 6 times for 5 minutes in PBS-T before incubation in PBS-T containing secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase (HRP), 1:1000) (GE Healthcare, Little Chalfont, UK) for 1 hour at room temperature. Membranes were washed 6 times for 5 minutes in PBS-T and once for 10 minutes in PBS containing 0.1% v/v Tween-20.

Proteins were detected with ECL Plus Western Blotting Dectection System (GE Healthcare) protected from light for 5 minutes at room temperature. ECL Plus solution was drained from the membrane, which was then placed in a film cartridge. In a dark room, membranes were exposed to Amersham Hyperfilm ECL photographic paper (GE Healthcare) which was then passed through Kodak GBX developing solution (Kodak, Rochester, NY, USA), stop solution (SB80 photosol, Basildon, UK) and fixing solution (Hypam fixer, Ilford, Cheshire, UK). Bands were scanned and intensity measured using ImageJ 1.45s (Wayne Rasband, National Institutes of Health, USA) image analysis software, with β-actin used as a loading control.

Table 2.1. Summary of antibodies used for western blot analysis.

Antibody	**Description**	**Mw (KDa) (target protein)**	**Dilution**	**Company**
1°Ab				
Anti-Ku80	Rabbit monoclonal	86	1:1000	Cell Signaling Technology
Anti-PARP	Rabbit monoclonal	116, 89	1:1000	Cell Signaling Technology
Anti-β-actin	Rabbit monoclonal	45	1:1000	Cell Signaling technology
2°Ab				
Anti-Rabbit IgG HRP conjugate	Donkey secondary	-	1:1000	GE Healthcare

2.6 Immunocytochemistry

Culture or treatment medium was removed from coverslips or tissue samples by aspiration and samples fixed with 4% v/v formaldehyde in PBS for 30 minutes. Samples were then washed 3 times in PBS for 5 minutes and permeabilsed with 0.5% v/v Triton-x-100 (Sigma-Aldrich) for 30 minutes. Three washes in PBS containing 0.02% w/v BSA and 0.05% v/v IGEPAL (Sigma-Aldrich) were performed before blocking for non-specific binding sites with normal donkey serum (diluted 1:50 in 1% w/v BSA in PBS) for 1 hour. Primary antibodies were diluted in 1% w/v BSA and applied overnight at 4°C (Ku80, DNA-PK, PARP-1 (all Cell Signalling Technology) and Ligase IV (Sigma Aldrich) 1:200).

Table 2.2. Summary of antibodies used for immunocytochemistry.

Antibody	Description	Mw (KDa) (target protein)	Dilution	Company
1°Ab				
Anti-Ku80	Rabbit monoclonal	86	1:200	Cell Signaling Technology
Anti-Ligase IV	Rabbit polyclonal	~100	1:200	Sigma-Aldrich
Anti-DNA-PK	Rabbit monoclonal	450	1:200	Cell Signaling Technology
Anti-PARP-1	Rabbit monoclonal	116, 89	1:200	Cell Signaling Technology
2°Ab				
Anti-Rabbit Alexa488-conjugate	Donkey secondary	-	1:100	Invitrogen

Coverslips or tissue samples were washed 3 times for 5 minutes in PBS containing 0.02% BSA, 0.05% IGEPAL and secondary antibody (alexa488-conjugated donkey anti-rabbit (Invitrogen) diluted 1:100 in 1% BSA in PBS) applied for 1 hour protected from light in a humidified atmosphere at 37°C. Coverslips or tissue samples were counterstained for 10 minutes with DAPI (Sigma-Aldrich) and Texas red-x-phalloidin (Invitrogen) (diluted 1:100 in 1% BSA in PBS) before a further 3 washes in 0.02% BSA, 0.05% IGEPAL in PBS. Samples were mounted onto glass microscope slides with Hydromount mounting medium (National Diagnostics, Hull, UK) and viewed with fluorescence microscopy (Zeiss) and images captured with a digital camera and AxioVision software (Zeiss). Where required images were quantified using ImageJ 1.45s image analysis software.

2.7 siRNA knockdowns

FHL124 cells were seeded onto 35 mm plastic culture dishes (western blot analysis/cell treatments for alkaline comet assay/LDH measurements) or glass coverslips contained within 35 mm plastic culture dishes (immunocytochemistry) at 17,500 or 10,000 cells, respectively, and grown until approximately 50% confluent. 24 hours prior to siRNA transfection the culture medium was replaced with serum-free EMEM. FHL124 cells were washed with, and subsequently cultured in Opti-MEM (Invitrogen). Cells were either transfected with siRNA targeted against Ku80, PARP-1 or AllStars Negative Control siRNA (scramble siRNA) (Qiagen, Crawley, UK) at a concentration of 5 nM for 48 hours using oligofectamine transfection reagent (Invitrogen). Dilutions were performed in Opti-MEM. Following 48 hours, cells were either treated (for analysis of DNA strand breaks with alkaline comet

assay/LDH release) lysed for protein (western blot) or fixed with 4% formaldehyde (immunocytochemistry).

Table 2.3. Summary of siRNA used in knockdown experiments.

Target gene	Target sequence
XRCC5 (Ku80)	5'-AAGCATAACTATGAGTGTTTA-3'
PARP1 (PARP-1)	5'-ACGGTGATCGGTAGCAACAAA-3'
AllStars Negative Control siNRA (scramble)	Not applicable

2.8 Whole human lens culture

Donor eyes were obtained from the East Anglian Eye Bank with written consent obtained from next of kin. The research was approved by the UK National Ethics Committee (REC 04/Q0102/57) and followed the tenets of the Declaration of Helsinki regarding the use of human material.

Lenses were dissected by anterior approach from donor eyes and placed in serum-free EMEM within 24 hours post-mortem and observed over 24 hours to ensure no damage by physical trauma was inflicted during their removal prior to application of experimental conditions.

2.9 Whole human lens culture treatments

2.9.1 Hydrogen peroxide treatment

H_2O_2 was freshly diluted in serum-free EMEM/Opti-MEM as appropriate and added to culture medium to give a final concentration of 1 mM.

2.9.2 Poly(ADP-ribose) polymerase (PARP)-1 inhibition

PARP-1 was chemically inhibited with AG14361 (Selleck Chemicals, Houston, TX, USA). AG14361 was diluted in DMSO upon delivery to produce a 25 mM stock which was frozen at -80°C. All experiments therefore also contained a DMSO control diluted to the same concentration. AG14361 was freshly diluted in serum-free EMEM and added to cultures to give a final concentration of 10 μM for 1 hour prior to addition of subsequent conditions.

2.10 siRNA knockdown in whole human lenses

Whole human lenses were washed with and then subsequently cultured in Opti-MEM. Lenses were transfected with targeted siRNA at a concentration of 10 nM for 72 hours prior to the addition of experimental treatments, using oligofectamine as a transfection reagent. The paired lens was transfected with AllStars Negative Control siRNA (scramble siRNA) and treated in the same manner.

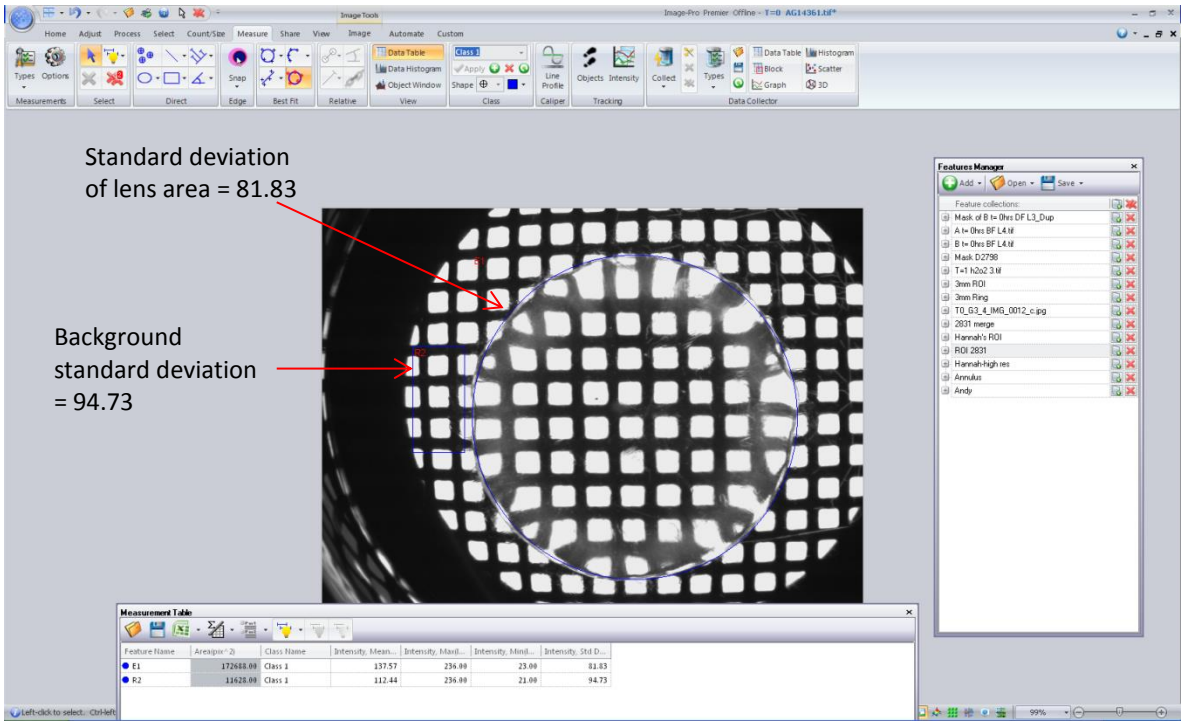
2.11 Verification of siRNA knockdown

72 hours post-transfection whole epithelium was dissected from the lenses. Briefly, lenses were pinned anterior surface down onto 35 mm plastic culture dishes. The posterior capsule and fibre mass were removed leaving the anterior capsule and epithelial cell layer. This was then fixed by addition of 4% formaldehyde and immunocytochemistry performed as described above (Section 2.6). Images were analysed with ImageJ 1.45s analysis software with 100 cells analysed per sample.

2.12 Lens imaging and quantification of opacity

Lenses were imaged using a charge coupled device (CCD) camera (UVP, Cambridge, UK) with Synoptics software (Synoptics, Cambridge, UK) at the experimental start point (day 0) and at 24 hour intervals throughout experiments. Brightfield illumination was used, with a black and white grid placed beneath lenses to give an assessment of visual quality and therefore lens clarity. Visual quality was quantified from these images by measuring standard deviation values of grey scale values obtained from the grid beneath the lens. Values of standard deviation of clear lenses are high whereas when the lens becomes more opaque, the grid becomes less defined/homogenous and the standard deviation values decrease. A background for each image was achieved by selecting a region of the grid adjacent to the lens. This region exhibits the greatest standard deviation and best visual quality and thus a decrease was calculated relative to these values. Image analysis was performed with Image-Pro Premier (MediaCybernetics Inc., MD, USA) analysis software (Figure 2.2).

A



B

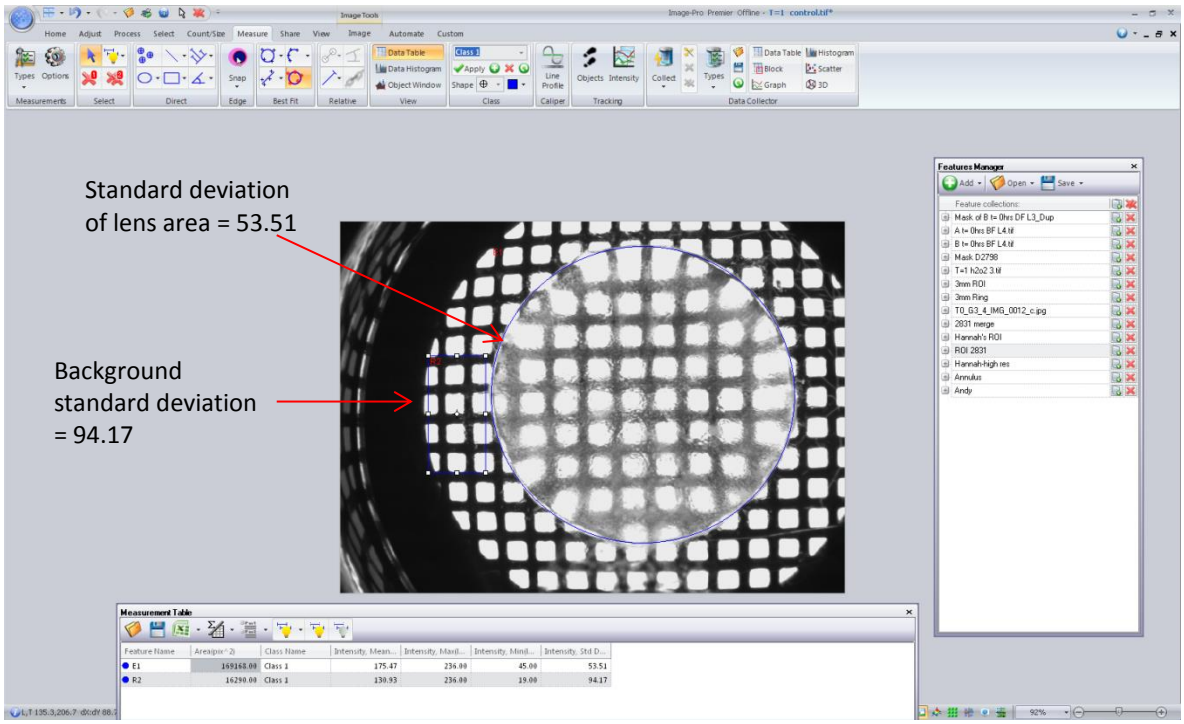


Figure 2.2. Quantification of lens visual quality using Image-Pro Premier image analysis software. Background standard deviation measured from rectangular blue box, lens standard deviation measured from blue circle area. Visual quality of lenses was calculated from lens area standard deviation as a percentage of background standard deviation and plotted as a reduction in visual quality (%). Note standard deviation values of clear lens (A) are higher than those observed for opaque lens (B)

2.13 Statistical analysis

One way analysis of variance (ANOVA) with post hoc Tukey's test was performed to determine statistical differences between multiple experimental groups (SPSS 16.0, SPSS Inc., IL, USA) and Student's t-test to determine statistical differences between two experimental groups (Excel, Microsoft, WA, USA). A p value of ≤ 0.05 was considered significant.

Chapter 3 Hydrogen peroxide induces lens cell death, DNA strand breaks and lens opacification

3.1 Introduction

Oxidative stress has been proposed to play a role in cataract formation (Spector, 1995; Truscott, 2005; Vinson, 2006). The lens possesses numerous anti-oxidant defence systems to protect it from the damaging state of oxidative stress and prevent the onset of cataract. Such defence systems are either enzymatic such as thioredoxin, thioltransferase, superoxide dimutase and catalase or non-enzymatic such as vitamins E and C and reduced glutathione (Michael and Bron, 2011). When antioxidant systems become overwhelmed and unable to cope with the level of oxidants present a state of oxidative stress occurs in which case damage to proteins, lipids and nucleic acids can occur (Evans et al., 2004).

Human cells are constantly exposed to pro-oxidant molecules such as H_2O_2 and the $O_2^{\bullet-}$ and $^{\bullet}OH$ radicals. These can arise from external sources such as exposure to ultraviolet light or ionising radiation that lead to the production of cellular reactive oxygen, or from within the cell itself as they are produced by the mitochondria, peroxisomes and cytochrome p450 (Michael and Bron, 2011). H_2O_2 is a key oxidant in the formation of cataract (Spector, 1995) and is naturally occurring. It produced during the detoxification of $O_2^{\bullet-}$ by superoxide dismutase, which is formed by the incomplete reduction of oxygen in the electron transport chain. H_2O_2 is then converted to water by either catalase or glutathione peroxidase (Michael

and Bron, 2011). In normal, non-cataract human lenses, levels of H_2O_2 have been reported to be 20-30 μM, with levels shown to be significantly increased in approximately one third of cataract patients (Spector and Garner, 1981).

Cataract is an age-related disease and the ability to cope with oxidative stress also declines as humans age. For example, perhaps the most important antioxidant system in the lens is GSH (and glutathione reductase), and its levels are reduced as humans age. A study by Harding (Harding, 1970) reported that levels of GSH in 65 year old lenses were approximately 51% less than levels found in 20 year old lenses. GSH is produced by the lens epithelium and is normally present in millimolar amounts (Giblin, 2000; Michael and Bron, 2011; Truscott, 2005). With age, this decline potentially renders proteins of the lens susceptible to oxidative modification ultimately promoting the formation of cataract.

As mentioned above, oxidative stress causes damage to key cellular constituents. Proteins of the lens are particularly susceptible to oxidative damage as there is no scope to replace damaged proteins. With age, the lens continues to grow by the addition of new layers of fibres – there is no turnover of cells and any oxidative damage to proteins of the lens accumulates and can eventually become so severe as to prevent free passage of light through the lens (Duncan et al., 1997; Spector, 1995).

Another consequence of oxidative stress is damage to DNA. As discussed in Chapter 1 cells are constantly exposed to and damaged by, a variety of DNA damaging stimuli that can arise both exogenously and endogenously. Human cells have evolved numerous DNA repair

mechanisms to cope with various DNA lesions. DNA repair mechanisms have also been shown to decline with age and indeed ageing has been proposed to be a result of DNA damage through accumulation of mutations that result from a decline in DNA repair capability (Freitas and de Magalhaes, 2011).

DNA strand breaks are an early, if not the earliest, event upon a cell's exposure to an oxidant and H_2O_2 is considered prominent in producing DNA strand breaks as it is stable and able to freely diffuse across cell membranes (Ueda and Shah, 1992). DNA damage however is not directly caused by H_2O_2; instead, it reacts with metal ions on, or near to DNA to produce the highly reactive $^{\bullet}OH$ radical which can then attack DNA bases, causing base damage or deoxyribose residues to produce strand breaks. In order to cause DNA damage the $^{\bullet}OH$ radical must be produced within immediate proximity to the DNA itself (Marnett, 2000).

3.2 Aims

H_2O_2 is a pro-oxidant produced during cellular metabolism and found within the aqueous humour of the normal eye at concentrations of approximately 20 to 30 μM, with levels increased within the aqueous humour of cataract patients (Spector, 1995). Oxidative stress and elevated levels of H_2O_2 have been demonstrated in cataract patients (Spector and Garner, 1981). This chapter therefore investigates the use of H_2O_2 as a model for studying the effects of oxidative stress-induced changes in human lens cells, investigating cell death, DNA damage and cataract formation.

3.3 Results

3.3.1 Hydrogen peroxide-induced effects on FHL124 cell death and viability

To test the effects of H_2O_2 on lens cell viability and cell death the ApoTox-Glo Triplex assay was used. FHL124 cells were treated with H_2O_2 and levels of cell viability, cytotoxicity and apoptosis were measured (Figure 3.1). At 4 hours post H_2O_2 treatment no changes in FHL124 cell viability, cytotoxicity or apoptosis were noted at any concentration studied (Figure 3.1 A-C). At 24 hours, a 10 μM treatment remained unable to affect cell viability or cytotoxicity at either time-point studied. However, at this time point, 30 and 100 μM H_2O_2 did significantly promote a loss in cell viability and increased cytotoxicity in a concentration dependent manner (Figure 3.1 D, E). This increase in cell death appeared to largely result from apoptosis (Figure 3.1 F).

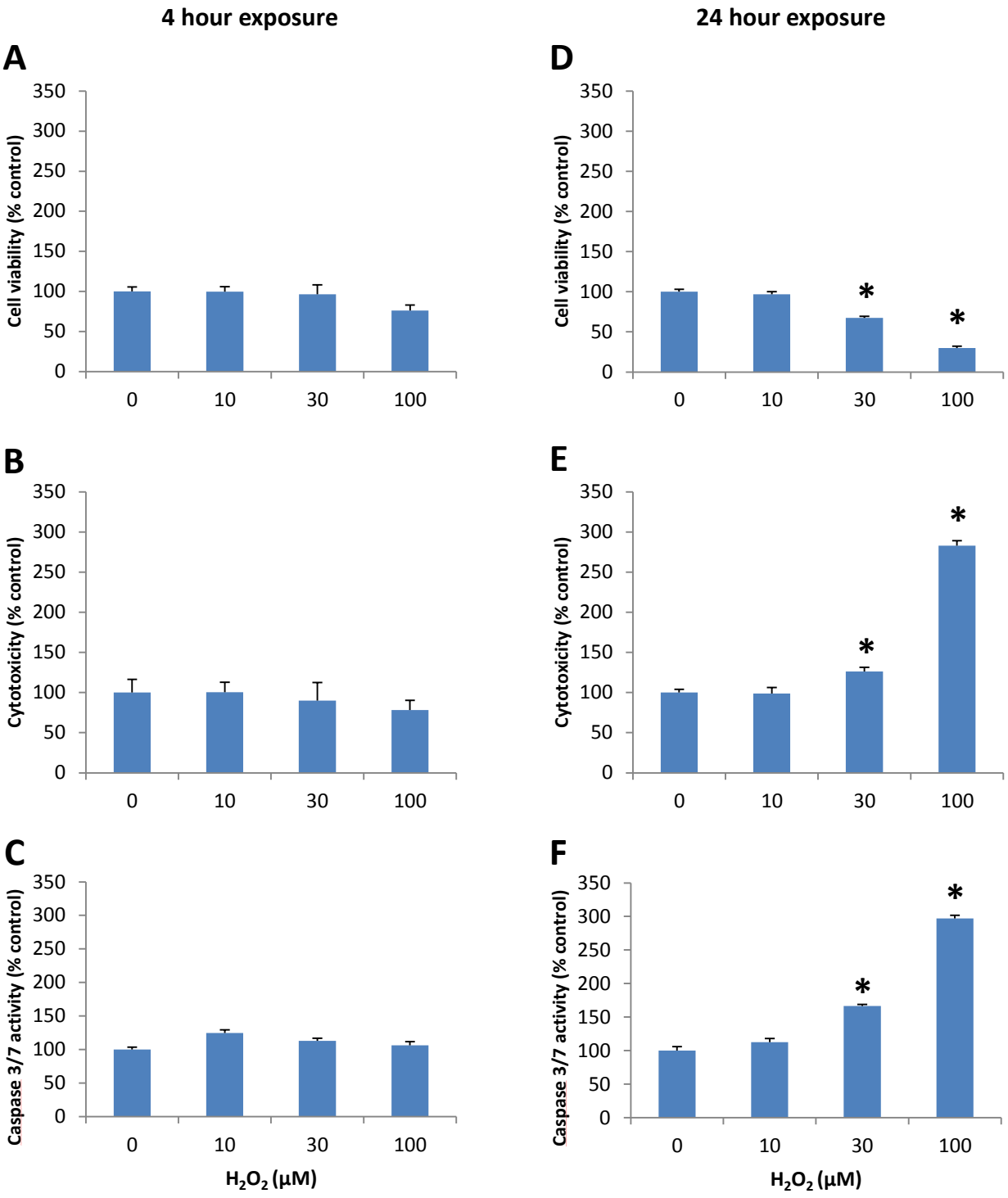


Figure 3.1. Concentration-dependent effect of H_2O_2 on FHL124 cell viability (A,D), cytotoxicity (B,E) and apoptosis (C,F). The ApoTox-Glo Triplex Assay was performed at 4 (A-C) and 24 (D-F) hours. Data representative of 3 independent experiments. * indicates significant differences between H_2O_2 treatments ($p \leq 0.05$;ANOVA with post hoc Tukey test)

3.3.2 Hydrogen peroxide-induced DNA strand breaks

The alkaline comet assay was performed to assess levels of DNA strand breaks in FHL124 cells treated with hydrogen peroxide (Figure 3.2). Treatment with 10 µM H_2O_2 did not produce significantly elevated levels of strand breaks from control treatments at any time point. Increasing the concentration of H_2O_2 to 30µM, dramatically induced DNA strand breaks. Peak levels were observed at 0.5 hours post-treatment and remained significantly elevated relative to untreated controls until 4 hours post-treatment, and remained similar to control levels for the remaining period of study.

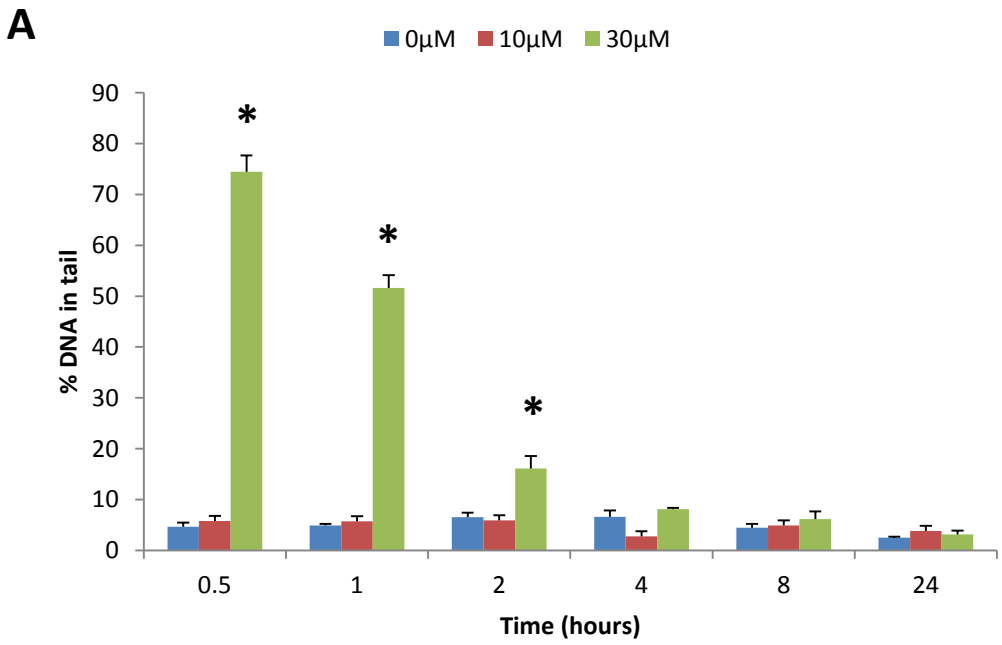


Figure 3.2. H_2O_2-induced DNA strand breaks in FHL124 cells. FHL124 cells were treated with H_2O_2 and strand breaks (% DNA in tail) measured over a 24 hour period with the alkaline comet assay. Data represents 3 independent experiments ± SEM. * indicates significant difference from untreated cells ($p \leq 0.05$; ANOVA with post hoc Tukey test) (A). (B) (see overleaf) shows example fluorescent micrographs illustrating comets in FHL124 cells treated with 30 µM hydrogen peroxide and untreated controls.

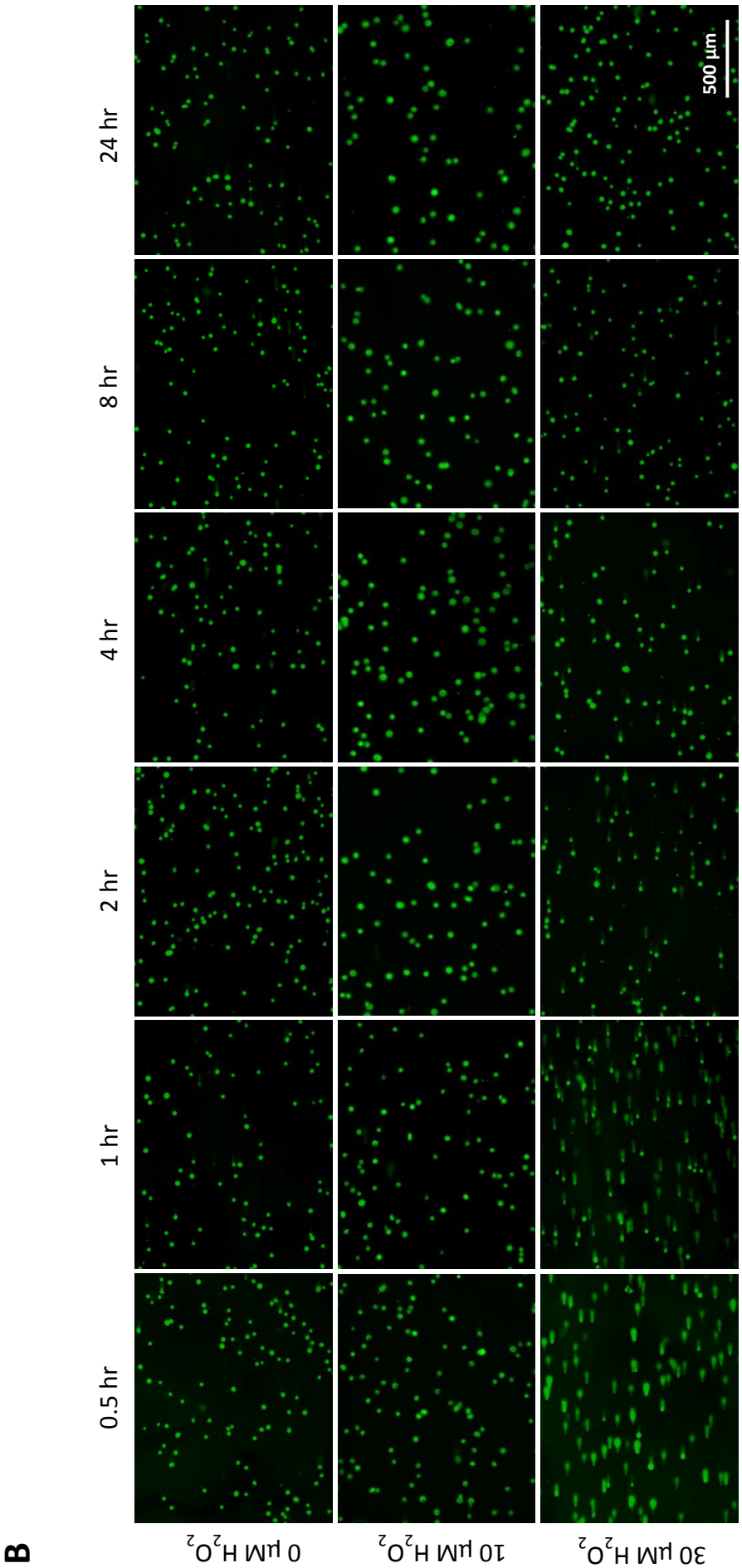


Figure 3.2. Example fluorescent micrographs illustrating comets from FHL124 cells over 24 hours following treatment with 0, 10 and 30 µM H_2O_2.

3.3.3 Hydrogen peroxide induced changes in a whole human lens culture system

To test the effect of H_2O_2 treatment on whole human lenses a whole organ culture system was used. Human lenses were dissected from donor eyes by anterior approach and placed into serum free culture medium and observed over 24 hours to ensure no damage was inflicted during the dissection process. A 1 mM H_2O_2 solution was applied to the lenses with changes in visual quality measured at 24 hours and quantified from brightfield images and compared to untreated match paired control lenses (Figure 3.3). Culture medium was also sampled and assayed for LDH release as a marker of cell death (Figure 3.4).

At the experimental start point (day 0) both lenses were fundamentally transparent and in brightfield images the grid placed below the lens is clearly visible and defined. Exposure to 1 mM H_2O_2 for 24 hours resulted in a significant decrease in visual quality, relative to untreated controls (26.0 ± 2.0% versus 0.3 ± 4.6% reduction in visual quality) (Figure 3.3). These H_2O_2-induced changes also corresponded with a significantly greater release of LDH than observed in untreated lenses with a 4046.8 ± 1025.6% increase noted from untreated lenses (Figure 3.4).

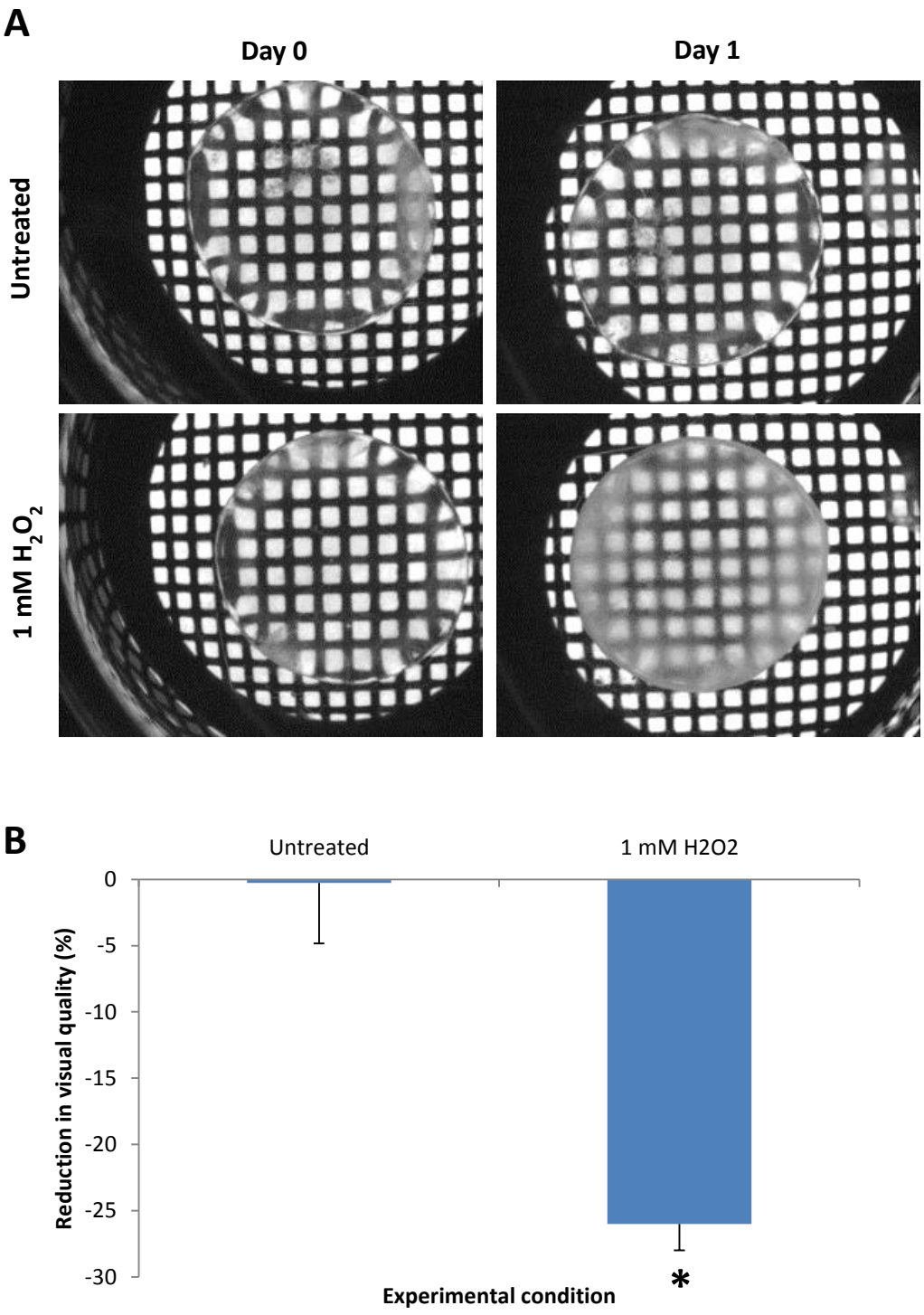


Figure 3.3. H_2O_2-induced reduction in lens visual quality. Representative brightfield images of human lenses over time (A) and quantification of visual quality (B) at day 1. Data pooled from 3 independent experiments ± SEM. * indicates significant differences between experimental conditions ($p \leq 0.05$, Student's t-test).

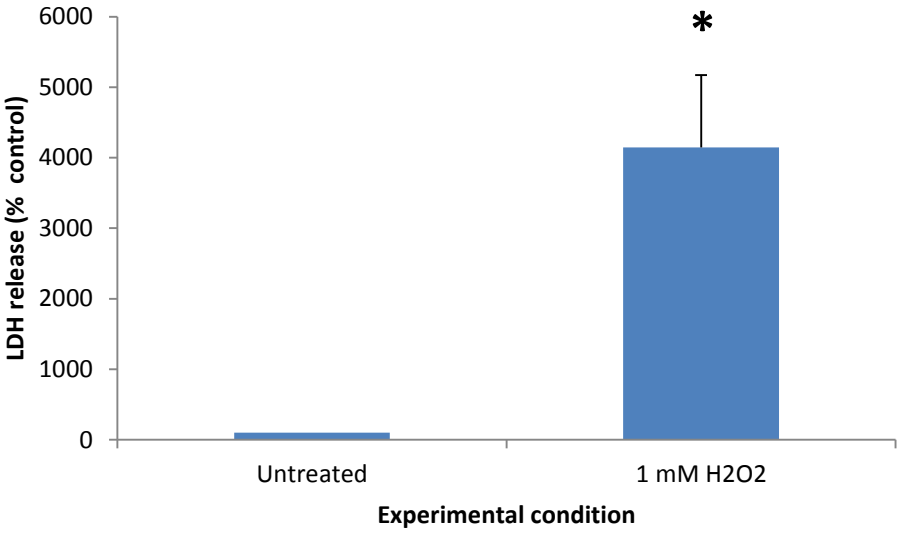


Figure 3.4. H_2O_2-induced cell death in whole human lenses. LDH release was measured at the experimental end point. Data pooled from 3 independent experiments ± SEM. * indicates significant difference between experimental groups ($p \leq 0.05$, Student's t-test).

3.4 Discussion

Oxidation is key to cataract formation (Truscott, 2005) and the ability of the lens to cope with pro-oxidants is greatly diminished in cataract (Harding, 1970). H_2O_2 is a crucial lens oxidant that is present in the aqueous humour of healthy lenses in concentrations between 20 and 30 μM, but this concentration can be significantly elevated in cataract lenses (Spector, 1995). Thus, H_2O_2 was chosen as the experimental model for cataract formation in the current study not only for ease, but also for its significance in the disease.

The FHL124 cell line used in this study is a readily available and useful resource in which to investigate various aspects of lens epithelial cell biology, indeed, it possesses a 99.5% homology in transcript profile to native human lens epithelium (Wormstone et al., 2004) and thus is a good predictive tool indicating likely outcomes within the lens. The cell line does not however allow for observations of organ changes such as cataract. Native human lens tissue is a scarce and precious resource; however, their culture provides an excellent model in which to study whole organ changes. The lens is an avascular organ and thus naturally isolated. It can be removed from the eye and maintained in culture whilst retaining its clarity, therefore allowing a study of opacity formation under various conditions (Wormstone et al., 2006).

Various models have been used to study the effects of H_2O_2 on lens cells and H_2O_2-induced lens opacity. Kleiman and colleagues (Kleiman et al., 1990) investigated H_2O_2-induced DNA damage in a bovine lens cell line derived from primary bovine lens epithelium explants. They found DNA damage to be induced in a H_2O_2 concentration dependent manner, concluding

that DNA lens epithelial cells may be particularly sensitive to oxidative damage. However, no study of the effects of H_2O_2 on whole bovine lens opacity was undertaken. Cui and Lou (Cui and Lou, 1993) and Sanderson et al (Sanderson et al., 1999) utilised whole rat lens culture models to study H_2O_2-induced changes in lens morphology and biochemistry and the protective effects of quercetin against H_2O_2-induced cataractogenesis, respectively.

These animal models allow for the study of H_2O_2 as causative factor of lens opacity, however the current study employed a purely human model for studying a human disease. Recently published work from our laboratory by Liu and colleagues (Liu et al., 2013) has built on the model systems used in the present study. They utilised the FHL124 cell line firstly to establish the protective effects of the dietary component sulforaphane on protecting human lens cells from oxidative stress-induced cell death and DNA damage with H_2O_2 before employing a whole porcine lens culture system to measure effects on lens opacity.

The current body of work firstly established effects of H_2O_2 on DNA strand breaks and measures of viability, cytotoxicity and apoptosis in FHL124 cells; a human lens cell line derived from human caspsule-epithelium explants before investigating changes in whole human lenses. This cell line demonstrates a 99.5% homology to native lens epithelium in transcript profile (Reddan et al., 1999; Wormstone et al., 2004) and so allows greater comparisons to changes noted in the whole human lens culture system adopted.

Initial experiments utilised FHL124 cells to investigate H_2O_2-induced cell viability, cytotoxicity and apoptosis and found a decrease in viability and increase cytotoxicity and

apoptosis in a concentration-dependent fashion. Exposure to greater concentrations of H_2O_2 clearly affected cell survival due to increased oxidative damage. The ApoTox-Glo Triplex assay is an excellent method to assess these changes, allowing for measuring of cell viability, cytotoxicity and apoptosis simultaneously in the same sample. The assay is however limited to cell lines and is not applicable to the whole lens culture system and therefore LDH release was used as an alternative measure of cell death.

Levels of DNA strand breaks induced with H_2O_2 and their repair were assessed with the alkaline comet assay. The comet assay is a simple, quick and sensitive method for determining levels of DNA strand breaks in virtually all eukaryotic cells (the obvious exception being red blood cells) and only requires a very small sample size (100 comets are scored per sample) (Collins, 2004). The alkaline version performed in the current study measures both single and double strand breaks as wells as alkaline labile sites (such abasic sites formed as intermediates of the base excision repair process) and so allows for a broad measure of DNA damage inflicted by an agent (Collins et al., 2008).

The current study found that peak levels of DNA strand breaks were observed at 0.5 hours post-treatment with 30 μM H_2O_2 and that these levels declined over time. This decline of DNA strand breaks allows for a measure of their repair, and so in this instance strand breaks induced were fully repaired at 4 hours post-treatment. H_2O_2 treatments of 10 μM did not produce any elevation in DNA strand breaks from background levels and so FHL124 cells were able to cope readily with this level of oxidant through antioxidant defences. However, data obtained from ApoTox-Glo Triplex assay indicates that at a 30 μM H_2O_2 treatment caused decreases in FHL124 cell viability and increases in cytotoxicity and apoptosis (albeit

to a lesser extent than 100 µM H_2O_2). This could indicate that although some cell death is occurring, the majority of cells are able to cope with, and repair the DNA damage induced.

The whole human lens culture system utilised allows for observations in changes in opacity following a given stress, in this case oxidative stress induced with H_2O_2. At the dose used, H_2O_2 induced both a significant increase in opacity, but also cell death as determined by assaying LDH release from the lens into culture medium. Although not determined during this study, the location of the opacity and therefore the type of cataract produced could also be identified by fixation and sectioning of the lens.

The model adopted does not allow for a measurement of DNA strand breaks in the whole lens and therefore assumptions have to be made that what was observed in the FHL124 cell line is replicated in the lens. Using both a human cell line and human lenses alleviates such concerns. In investigating changes in the whole human lens culture system, a far greater concentration of H_2O_2 was used to induce a response (1 mM in whole lenses versus 0-100 µM in FHL124 cells). The human lens is surrounded by the lens capsule, a thick basement membrane which measures approximately 25-30 µm at the anterior pole in the adult human lens (Barraquer et al., 2006; Danysh and Duncan, 2009), which could potentially act as a barrier to H_2O_2. The effects of H_2O_2 are however also dependent on cell number, with a greater number of cells able to cope with the stress by detoxifying it more efficiently through greater levels of antioxidant present (Ohguro et al., 1999). Cell culture experiments comprised of approximately 100,000 cells per culture dish at the time of treatment, whereas a crude estimate of cell number in the adult human lens epithelium can be calculated as approximately 259,000 (calculated from a mean cell count of 3900 cells per mm^2 as an

average epithelial cell density (Fagerholm and Philipson, 1981) and the average diameter of the adult human lens being 9.25 mm (Augusteyn, 2010)). Under the experimental conditions in the present study therefore FHL124 cells are more susceptible to the effects of H_2O_2 than the whole lens epithelium. 1 mM H_2O_2 was chosen as an appropriate concentration in which to induce oxidative changes in the whole human lens culture system based on previous unpublished work within this laboratory. This work established that a dose of 1 mM H_2O_2 is sufficient to induce opacity with a 24 hour period, thus allowing a timely study of induced changes.

Sensitivity of native lens epithelium to H_2O_2 concentrations applied to FHL124 cells could however be tested without the presence of the lens capsule. By dissecting the epithelial layer from the lens as described in Chapter 2, the epithelial cells could be exposed and H_2O_2 applied directly to the cells.

H_2O_2 is relatively stable in comparison to other reactive oxygen species such as the $O_2^{\bullet-}$ and $^{\bullet}OH$ radicals and is also able to penetrate the lens capsule to cause damage. Coupled with its clinical relevance in cataract formation and the excellent applicability to experimental manipulation, as indicated in this chapter, this makes it an ideal candidate to use in a model system for studying oxidative-stress induced changes in the human lens.

Chapter 4 Elucidation of the role of non-homologous end joining protein Ku80 in protection of the lens

4.1 Introduction

In response to the daily bombardment of insults to the DNA that results in lesions, cells have evolved numerous pathways to repair this damage to DNA. As already highlighted in Chapter 1, these mechanisms range from direct reversal of base damage, BER, NER, MMR to DNA double strand break repair in the form of HR and NHEJ (Hakem, 2008).

The genetic material contained within the human lens, as with other cell types, is constantly damaged by stimuli arising exogenously or endogenously. Failure to efficiently repair this DNA damage could lead to cell death or loss of proper cell function. The central lens epithelium is essentially a non-dividing population of cells present from embryogenesis throughout life (Li et al., 1995; Lovicu and McAvoy, 2005). It is therefore exposed to DNA damaging stimuli over exceptionally long time frames. These cells are essential to maintaining a healthy and transparent lens and are responsible for the production of antioxidants which protect the lens fibres from oxidative damage (Michael and Bron, 2011). Therefore, any damage or death resulting from ineffective DNA repair could impede this important function, rendering lens fibre cells susceptible to oxidative insult and loss of transparency. Indeed, apoptosis in the lens epithelium has been linked to cataract formation (Li et al., 1995).

Furthermore, cataract is an ageing disease. Current theories of the ageing process revolve around the DNA damage response suggesting that ageing results in a decline in the ability to cope with DNA damage and an accumulation of DNA damage over time, resulting in a decline in cellular function (Lombard et al., 2005).

Perhaps the most severe type of DNA damage is the DNA double strand break and failure for these to be repaired efficiently can have drastic consequences for the cell and organism ranging from mutations, genetic instability and cell death (Lees-Miller and Meek, 2003; Ohnishi et al., 2009). As a result of the severe nature and potential consequences of the DNA double strand break the cell has evolved two independent pathways for their repair; HR and NHEJ. It is possible that a DNA double strand break by its very nature could lead to the loss of genetic material because of the lack of an effective template strand from which to reproduce lost information. HR allows the repair of DNA double strand breaks by using a template from the sister chromatid, and, thus can only occur following S phase-G2 of the cell cycle, meaning that it is responsible for a relatively minor proportion of double strand break repair (Mladenov and Iliakis, 2011). The vast majority of DNA double strand breaks are repaired via NHEJ which can occur without the requirement for a template strand and, thus, can occur throughout the cell cycle. NHEJ involves some processing of DNA to create ends that can be ligated without consideration for DNA sequence; as a consequence it is potentially error prone (Costantini et al., 2007; Wyman and Kanaar, 2006). It seems that given the severe potential consequences of such a DNA lesion repair, however error-prone is preferential to no repair at all.

In eukaryotic cells, including those of human origin, NHEJ is initiated by a heterodimer of two proteins, Ku70 and Ku80 which bind to DNA ends with high affinity. In doing so, they act to recruit the DNA-PK catalytic subunit, a protein kinase which phosphorylates and activates itself and other proteins of the pathway such as Artemis, a nuclease that processes DNA ends which cannot be directly re-ligated. Finally, DNA Ligase IV, in complex with XRCC4, is brought in and acts to re-join the break (Jeggo et al., 2011; Mladenov and Iliakis, 2011).

To the best of the author's knowledge there have been no studies investigating the role of NHEJ in the lens. One study by Hawes et al (Hawse et al., 2004) undertook analysis of gene expression profiles of young, old and cataractous lenses. Their data suggest that expression genes involved in DNA double strand break repair decline in the lens with age. Remarkably, this has not been further investigated.

4.2 Aims

DNA double strand breaks are a particularly severe DNA lesion which if unrepaired, or repaired inefficiently can lead to cell death, mutation and genetic instability. NHEJ is an important DNA repair pathway in human cells and is likely to be important in the lens. This chapter investigates the effects of depleting Ku80, a core NHEJ protein, on human lens cells and human lenses in relation to DNA damage and cataract formation.

4.3 Results

4.3.1 Expression of non-homologous end joining proteins in human lens cells

To assess expression of NHEJ repair pathway proteins in FHL124 cells and human lenses immunohistochemistry was performed. In FHL124 cells Ku80, DNA-PK and Ligase IV were detected and were predominantly found in the cell nuclear region (Figure 4.1). A similar pattern of expression and distribution was also observed in the native lens epithelium following its dissection from the lenses, however, it did appear that some cytoplasmic DNA-PK and ligase IV expression was also noted (Figure 4.2).

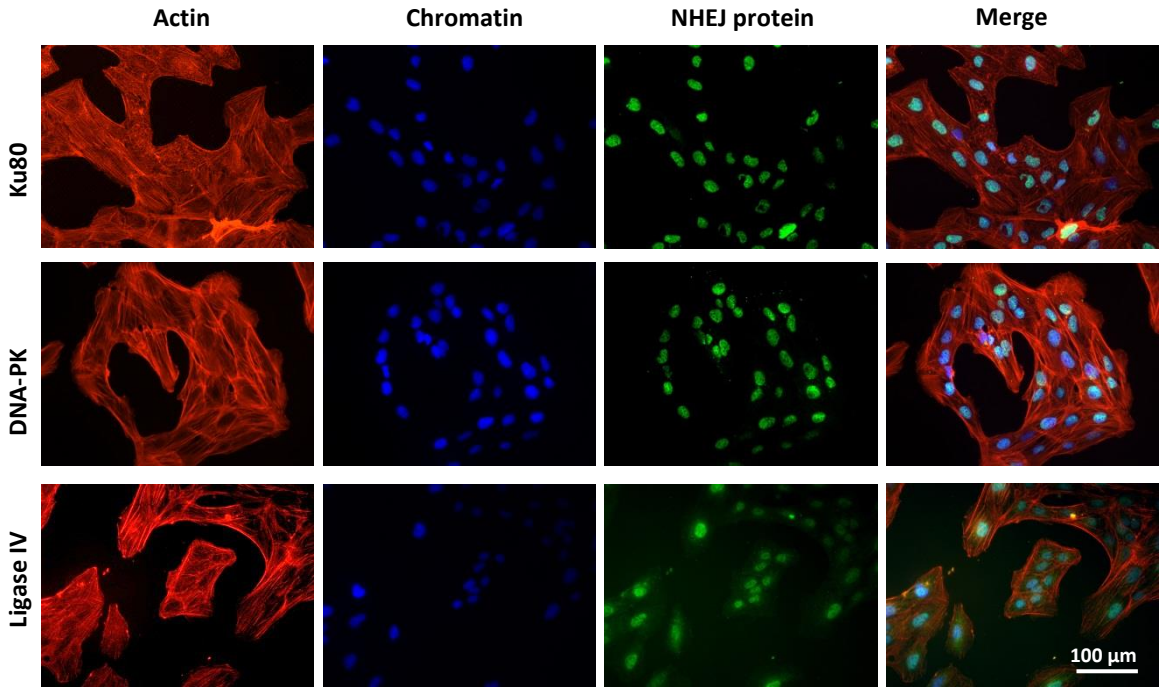


Figure 4.1. Fluorescent micrographs showing NHEJ pathway proteins Ku80, DNA-PK and Ligase IV are expressed in FHL124 cells. Antibodies to target proteins were visualised with an alexa488-conjugated secondary antibody (green) with actin cytoskeleton and chromatin stained with Texas red-x-phalliodin (red) and DAPI (blue) respectively.

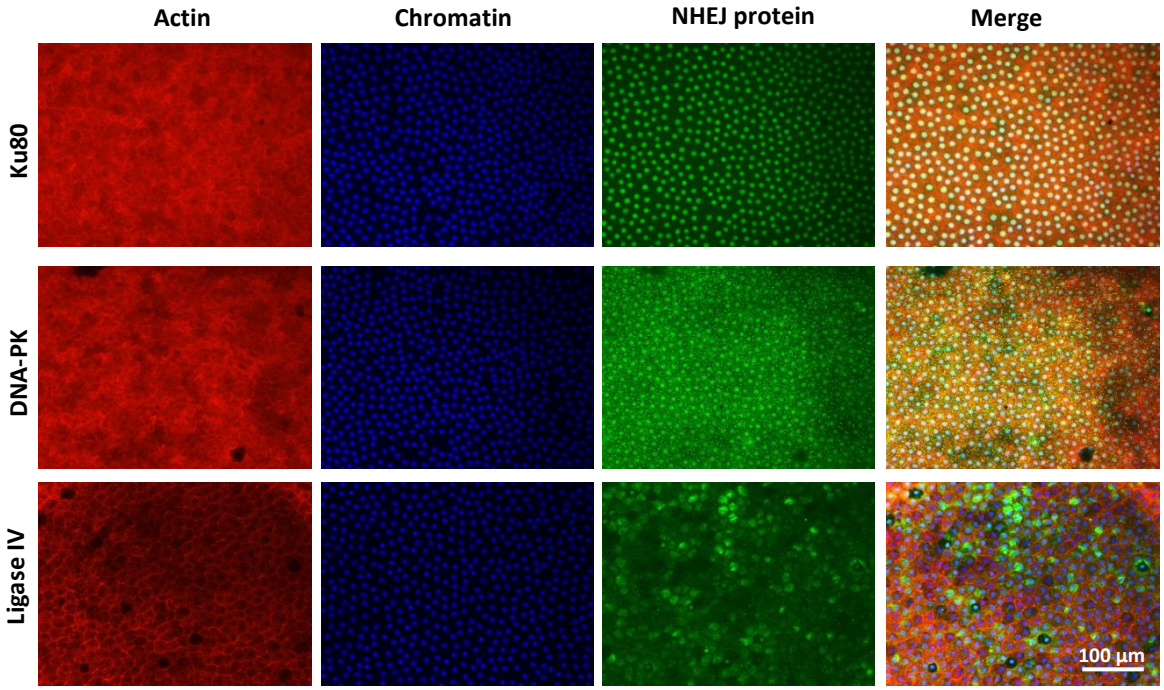


Figure 4.2. Fluorescent micrographs showing NHEJ pathway proteins Ku80, DNA-PK and Ligase IV are expressed in native lens epithelium dissected from donor human lenses. Antibodies to target proteins were visualised with an alexa488-conjugated secondary antibody (green) with actin cytoskeleton and chromatin stained with Texas red-x-phalliodin (red) and DAPI (blue) respectively.

Ku80 as a heterodimer with Ku70 recognises and binds to the site of DNA double strand breaks. This acts to stabilise the DNA and also as a site to which subsequent proteins of the NHEJ are recruited to process the double strand break (Lombard et al., 2005). To ascertain the effects of depleted Ku80 expression on DNA strand breaks, a targeted siRNA approach was adopted using a commercially available siRNA. Ku80 expression was significantly depleted in FHL124 cells, such that a 85.7 ± 7.6 % knockdown was achieved (Figure 4.3) compared to cells treated with non-coding scramble siRNA. No obvious phenotypical changes in cells treated with either Ku80 siRNA or non-coding scramble control siRNA were noted at this stage. Immunocytochemistry was also performed to confirm the knockdown of the protein (Figure 4.4).

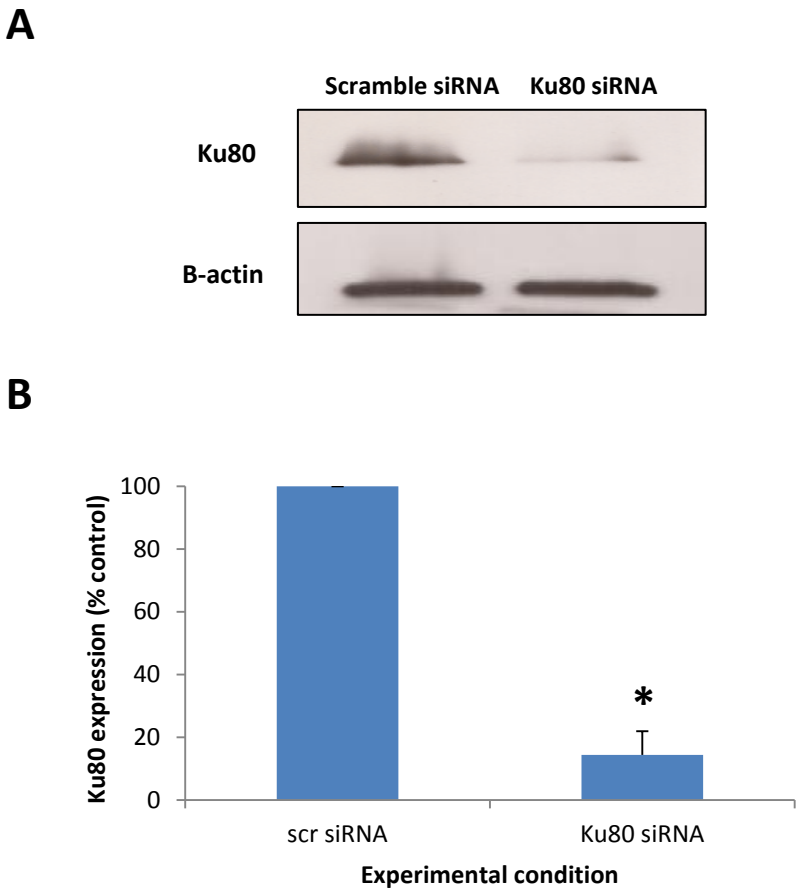


Figure 4.3. Validiation of Ku80 knockdown. FHL124 cells were either transfected with siRNA targeted to Ku80 or a non-coding scramble control siRNA. Knockdown of Ku80 was validated with western blot. (A) shows representative gel and (B) quantification of western blot data pooled from 3 independent experiments adjusted for β-actin loading controls ± standard error of the mean. * indicates significant difference ($p \leq 0.05$; Student's t-test).

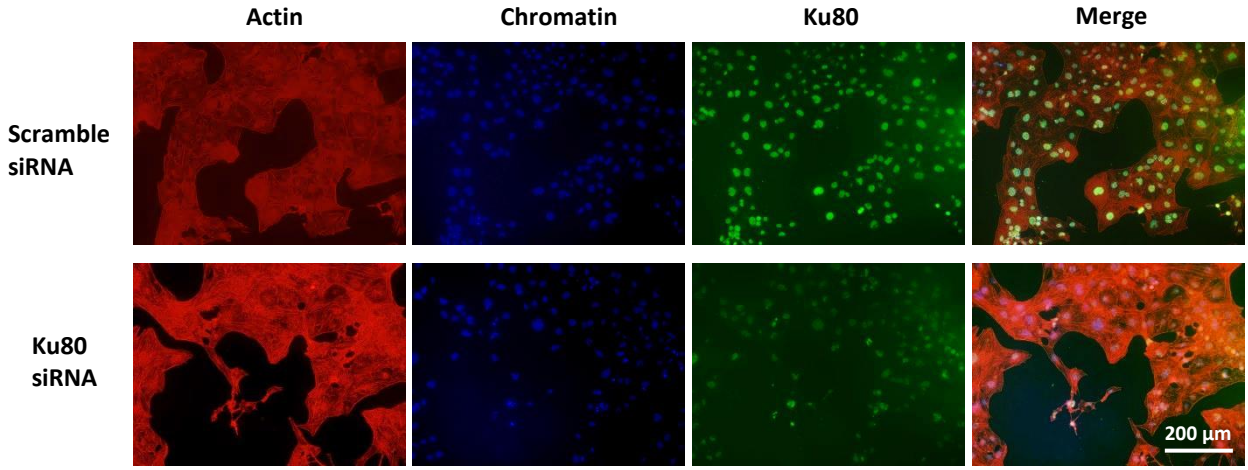


Figure 4.4 Fluorescent micrographs that illustrate successful knockdown of Ku80 in FHL124 cells. FHL124 cells were either treated with siRNA targeted against Ku80 or non-coding scramble control siRNA for 48 hours.

4.3.2 Effects of Ku80 depletion on FHL124 susceptibility to oxidative stress-induced DNA strand breaks

In order to test the effect of depleted Ku80 expression on lens cell susceptibility to oxidative stress-induced DNA strand breaks, FHL124 cells with Ku80 expression depleted with targeted siRNA were treated with H_2O_2. Experiments presented in Chapter 3 demonstrated that a 30 µM H_2O_2 concentration produced peak levels of DNA strand breaks in FHL124 cells at 30 minutes post treatment. Therefore, a 30 µM concentration was used to induce strand breaks in these experiments. The alkaline comet assay was employed to assess levels of DNA strand breaks and compared to levels of strand breaks in cells treated with non-

coding scramble control siRNA (Figure 4.5). Peak levels of DNA strand breaks were observed in Ku80 knockdown cells at 0.5 hours post-treatment (74.8 ± 2.4% DNA in tail). These levels of strand breaks were significantly greater than those observed at 0.5 hours post-treatment in FHL124 cells without depleted Ku80 expression (61.6 ± 2.1% DNA in tail) and remained significantly elevated until 4 hours post-H_2O_2 treatment. In both instances at 4 hours post-treatment levels of DNA strand breaks declined following their initial peaks and returned to baseline levels, consistent with findings presented in Chapter 3.

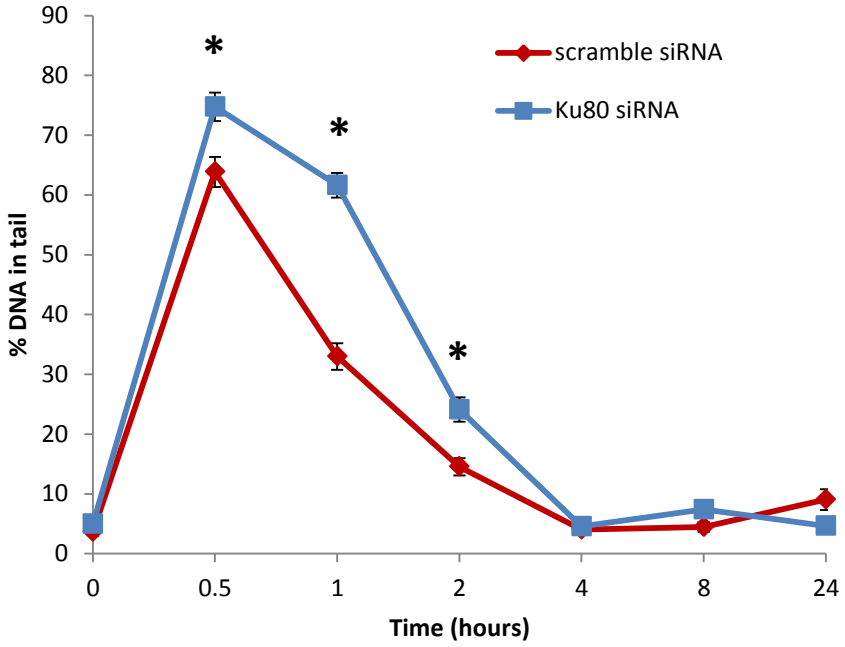


Figure 4.5. Effect of Ku80 depletion on H_2O_2-induced DNA strand breaks. FHL124 cell were treated with siRNA targeted against Ku80 and treated with 30 μM H_2O_2. Levels of strand breaks were measured by alkaline comet assay and compared to levels in FHL124 cells treated with non-coding scramble siRNA. Data presented is representative of 3 independent experiments each with 100 comets scored ± SEM. * indicates significant difference between experimental conditions ($p \leq 0.05$; ANOVA with post hoc Tukey's test)

4.3.3 Targeted siRNA knockdown of Ku80 in whole human lenses

To assess the effect of Ku80 expression on the susceptibility of a human lens to oxidative stress and potential involvement in cataract formation, a whole human lens culture system was employed. siRNA was used to deplete Ku80 expression in whole lenses with expression assessed at 72 hours following transfection and compared to lenses transfected with non-coding scramble control siRNA for the same time period. Ku80 expression levels in Ku80 siRNA treated lenses and scramble controls was assessed by performing immunocytochemistry on the epithelia (Figure 4.6). The overall expression in the Ku80 siRNA group was significantly lower than controls treated with non-coding scramble siRNA, such that a mean knockdown of 32.6 ± 7.3% was achieved.

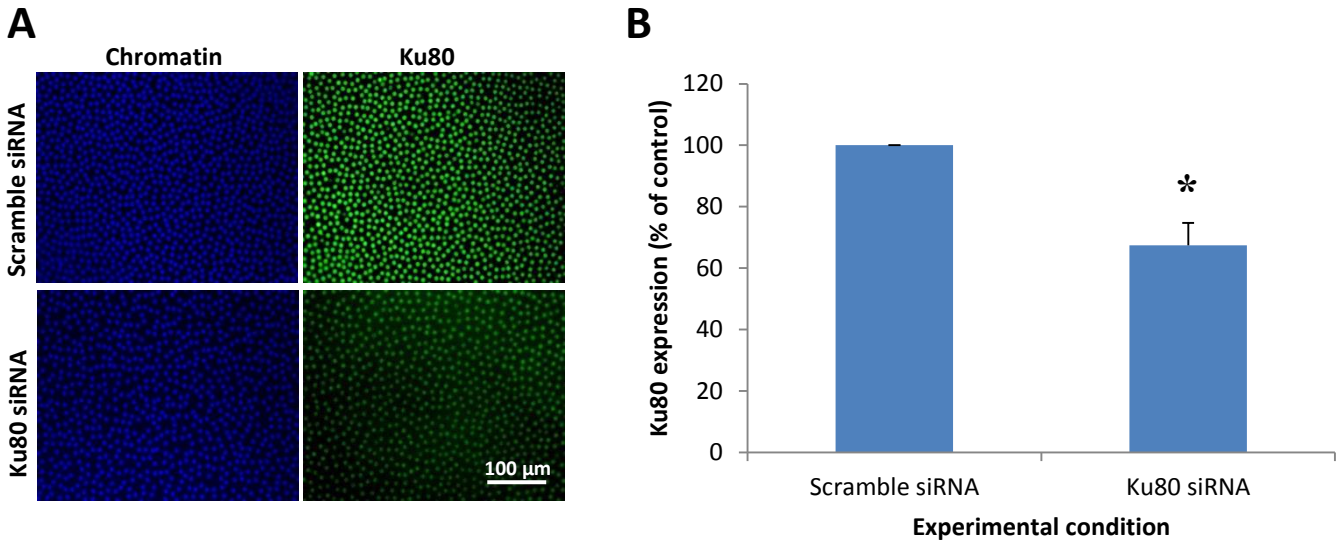


Figure 4.6. Ku80 depletion in whole human lenses using siRNA methods. Immunocytochemistry (A) was performed to assess Ku80 expression in epithelium dissected from whole human lenses following a 72 hour treatment with siRNA targeted against Ku80. Ku80 expression was quantified from fluorescent micrographs (B). Data represent data pooled from 4 independent experiments ± SEM. * indicates significant differences between experimental groups ($p \leq 0.05$; Student's t-test).

4.3.4 Effects of Ku80 depletion on human lens susceptibility to oxidative stress-induced opacity

Oxidative stress is known to induce DNA strand breaks and play a role in the formation of cataract (Marnett, 2000; Truscott, 2005) (see Chapter 3). Therefore, following on from experiments presented in Chapter 3, H_2O_2 was utilised to induce opacity in whole human lens cultures. 24 hours following a treatment with 1 mM H_2O_2 a significant decrease in visual quality was observed in Ku80 knockdown lenses compared to their match-paired controls (transfected with non-coding scramble control siRNA). This is evident from images taken with brightfield illumination whereby a grid is placed beneath the lenses at the time of imaging (Figure 4.7). A marked decrease in visual quality is clearly evident at day 1 compared to day 0 in Ku80 knockdown lenses, with the grid becoming less visible with time. A slight loss in transparency was evident in lenses transfected with non-coding control siRNA at day 1 compared to day 0 following H_2O_2 treatment.

To quantify visual quality, lens images were assessed. A significant reduction in visual quality was induced following hydrogen peroxide treatment in Ku80 knockdown lenses compared to those treated with non-coding scramble control siRNA (Figure 4.7B). In addition, the reduction in visual quality noted in lenses treated with non-coding scramble control siRNA was of similar value to lenses treated with 1 mM H_2O_2 alone as reported in Chapter 3.

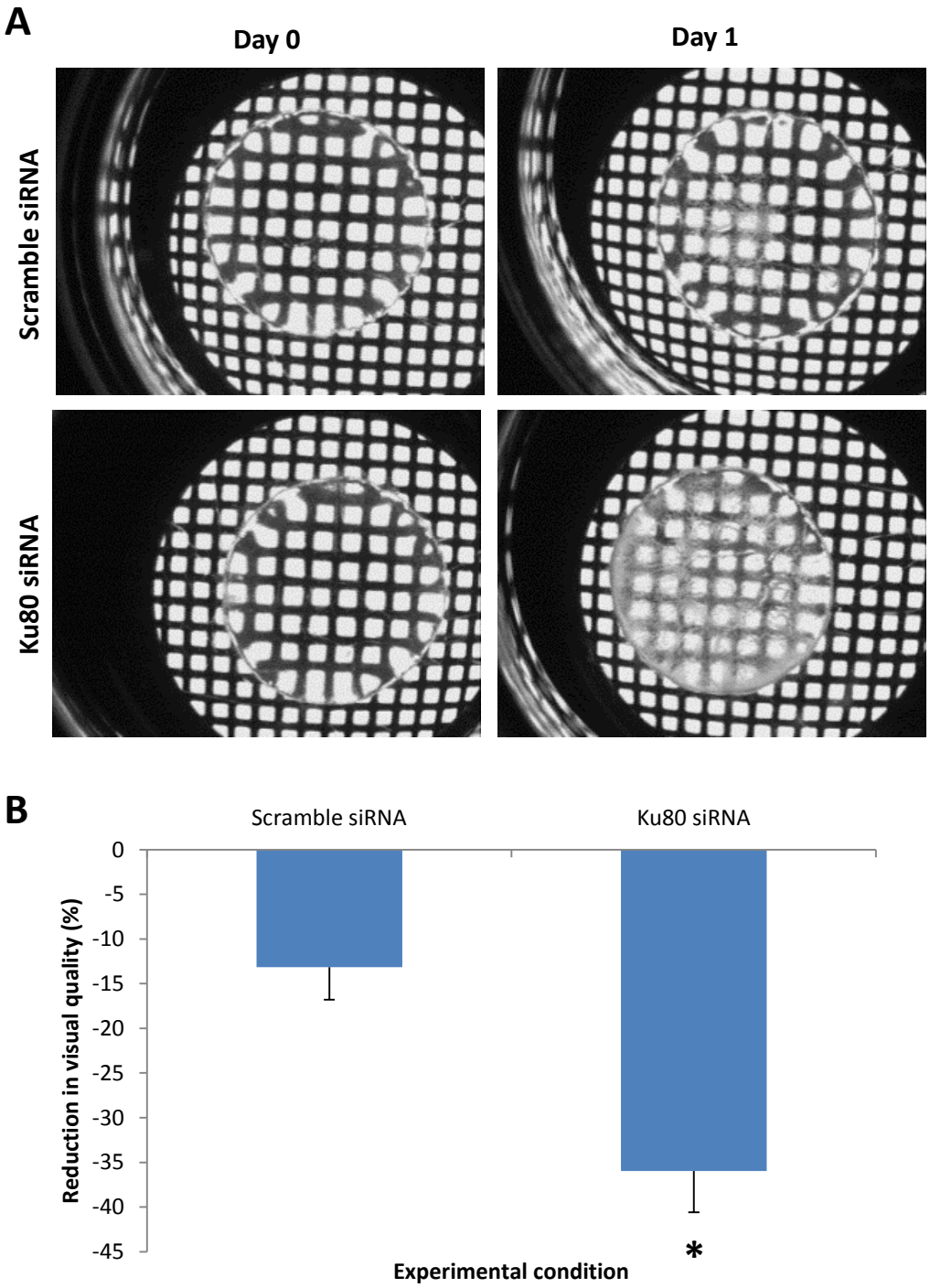


Figure 4.7. Depletion of Ku80 expression renders human lenses more susceptible to oxidative stress. Representative brightfield images of human lenses over time (A) and quantification of visual quality (B) at day 1. Whole human lenses were treated with siRNA targeted against Ku80. 72 hours post treatment 1 mM H_2O_2 was applied (Day 0) and loss of visual quality quantified. Data pooled from 3 independent experiments ± SEM. * indicates significant differences between conditions ($p \leq 0.05$; Student's t-test).

4.4 Discussion

The FHL124 cell line has been previously identified as having a transcript profile with 99.5% homology to native lens epithelium (Wormstone et al., 2004). The current study has demonstrated that three of the core NHEJ repair proteins, Ku80, DNA-PK and Ligase IV are expressed in both the cell line and native human lens epithelium and are considered key (along with Ku70, XRCC4 and Artemis) to the repair of DNA double strand breaks by this repair mechanism (Hakem, 2008).

The current study has demonstrated successfully that targeted siRNA can deplete expression of Ku80 within FHL124 cells and that when such cells are challenged with an oxidative insult of H_2O_2 these cells display significantly greater levels of DNA strand breaks over the first 4 hours post-treatment compared to control cells. This suggests that Ku80 assists in the efficient response to DNA damage induced by hydrogen peroxide within this experimental system. These results could be indicative of a greater susceptibility to DNA damage following Ku80 knockdown or a delayed response to repair of damage inflicted with hydrogen peroxide.

Furthermore, the current study has shown that siRNA can be used to deplete Ku80 expression in whole human lenses, albeit to a lesser extent than observed in FHL124 cells. When these lenses were exposed to H_2O_2 it was found that depletion of Ku80 expression rendered the lenses more susceptible to oxidative stress-induced changes in opacity compared to their match-paired controls, presumably due to increased levels of DNA damage related loss of cell function or death. The fibres of the lens are essentially an inactive mass of cells that rely

on the epithelium for support and maintenance of their transparency, thus damage to the epithelial cell layer which may result in a loss of cell function or death could render lens fibres increasingly susceptible to oxidative damage.

Cataract is an age-related disease and the overwhelming risk factor associated with cataract is ageing and the major contributing factor to the formation of the disease is oxidative stress (Spector, 1995; Truscott, 2005). As we age, the ability of the lens to cope with oxidative stress declines; reduced glutathione, a crucial lens antioxidant is diminished (Giblin, 2000; Harding, 1970) and thus lens fibres become more susceptible to oxidative damage. It is the lens epithelium that produces the anti-oxidants such as reduced glutathione and so damage to the epithelial cells which may diminish cellular function, including DNA damage may have a detrimental effect on the transparency of the fibre mass. With ageing this general decrease in the ability of the lens to cope with oxidative insult renders the lens increasingly vulnerable to oxidative damage and cataract. Should a further failing in DNA repair pathways occur, such as NHEJ for example, this susceptibility to oxidative damage or a loss of epithelial cell function could be further exacerbated.

As we age our ability to cope with DNA damage also declines and ageing has been linked to an accumulation of mutations resulting from a decline in DNA damage repair systems such as NHEJ (Gorbunova and Seluanov, 2005). Ku80, a core protein of NHEJ has been implicated in the ageing process. Seluanov and colleagues (Seluanov et al., 2007) observed that the level of Ku80 (and Ku70) declines during cellular senescence. Vogel et al (Vogel et al., 1999) observed that a deletion of Ku80 in mice causes early onset of age-specific changes such as osteopenia, atrophic skin and hepatocellular degeneration; they were also reported to have a

shorter lifespan. They did not however record any observations of the lenses of the mice. Cataract is a disease closely associated with ageing, and perhaps therefore a measure of lens opacity within Ku80 negative mice would have given an excellent additional measure of ageing within their experimental system.

DNA damage has been suggested to play a role in the formation of cataracts. Ates at al measured 8-OHdG as a marker of DNA damage in leukocytes of 50 cataract patients and found significantly elevated levels than in 50 control group patients, suggesting a greater susceptibility to DNA damage and oxidative insult as a whole, rather than organ specific to the lens (Ates et al., 2010). They also observed greater lipid peroxidation in cataract patients providing further supporting data to an oxidative role in cataract formation (Ates et al., 2010). Kleiman and Spector (Kleiman and Spector, 1993) obtained lens epithelial samples from patients undergoing cataract surgery and found there to be significantly greater levels of DNA strand breaks in approximately 50% of cataract patient samples than in control lenses obtained from eye banks (Kleiman and Spector, 1993). However, of course, this suggests that 50% of cataract patient samples within this study did not have any significant increases in DNA strand breaks compared to control lenses.

The present study depleted Ku80 expression with targeted siRNA and found lens cells to be more susceptible to DNA damage induced with H_2O_2. This study also depleted Ku80 expression in whole human lenses, however to a lesser extent. It could be argued that this is more physiologically relevant to Ku80 (as with other repair processes) declining with age rather than a large depletion of protein expression. These lenses were indeed more susceptible to oxidative stress-induced opacity suggesting an important role for Ku80 and NHEJ in

protecting the lens from cataract formation. Cataract is primarily an age-related disease and so perhaps as we age a declining function in how we cope with oxidative stress and DNA damage may be crucial in the formation of the disease.

Ku80 is a key protein in the NHEJ repair of DNA double strand breaks. Several studies have highlighted its importance. Koike and Koike (Koike and Koike, 2008) demonstrated that Ku80 is necessary for Ku70 accumulation at the site of double strand breaks and that although Ku70 possess DNA binding ability it cannot do so independent of Ku80. Another study by Uematsu and colleagues (Uematsu et al., 2007) also demonstrated the importance of Ku80 in NHEJ showing that it is required for the accumulation of the DNA-PK catalytic subunit and its autophosphorylation at the site of the double strand break. They also determine that un-phosphorylated DNA-PK catalytic subunit blocks DNA ends preventing ligation. Mari et al (Mari et al., 2006) provide further evidence as to the requirement of Ku80 in NHEJ by demonstrating that the Ku70/Ku80 heterodimer is necessary for accumulation of the XRCC4-Ligase IV complex at the site of DNA double strand breaks, required for DNA ligation.

NHEJ accounts for the repair of the majority of DNA double strand breaks which are particularly severe lesions; if they go unrepaired they can result in cell death (Jeggo et al., 2011). Apoptosis in the lens epithelial cell population has been linked to cataract formation (Li et al., 1995). A consequence of unrepaired DNA damage is cell death; any death to the lens epithelium may render the lens fibres susceptible to oxidative damage. The lens fibres are an inactive mass of cells and comprised mostly of crystalline proteins which are required to be kept in a reduced state in order to maintain their transparency (and therefore that of the

lens) and depend on a healthy epithelial cell layer to produce antioxidants such as reduced glutathione to achieve this (Lou, 2003; Michael and Bron, 2011).

Cataract is a disease closely linked to ageing, which has been linked to a general decline in the ability to repair DNA damage and a consequential accumulation of DNA damage and mutation (Gorbunova and Seluanov, 2005). Data from the present study, coupled with current theories of ageing and DNA repair capabilities suggests further study of the link between DNA damage and cataract is worthwhile in elucidating the underlying causes of cataract as an age-related disease.

Chapter 5 The role of poly(ADP-ribose) polymerase in human lens cells

5.1 Introduction

Poly(ADP-ribose) polymerases (PARPs) are a group of approximately 20 proteins involved in protein post-transcriptional modification. They catalyse the production and addition of poly(ADP-ribose) (PAR) polymer chains to target proteins which include PARPs themselves via auto-modification. This modification occurs by covalent attachment predominantly to glutamate or lysine residues using NAD+ as a substrate providing ADP-ribose (Luo and Kraus, 2012; Sousa et al., 2012). Firstly, this reaction produces protein bound mono(ADP-ribose) to which subsequent PAR polymers are added to 2'OH to produce long chained PAR polymer chains reaching hundreds of units in length. This reaction is transient, with PAR polymers degraded by poly(ADP-ribose glycohydrolase) (PARG) (Heeres and Hergenrother, 2007; Krishnakumar and Kraus, 2010). PAR polymers are strongly negatively charged and thus are able to influence protein structure and function (Bouchard et al., 2003). Free PAR, produced in the nucleus has also been implicated in signalling to other proteins within the cell (Wang et al., 2009b).

PARP-1 is the most abundant of the PARP proteins in humans and is present in quantities between 100,000 and 1,000,000 copies per cell (Abd Elmageed et al., 2012; Bouchard et al., 2003). However, as many as 20 PARPs exist which share homology with the catalytic domain of PARP-1. PARPs 1-5 possess PAR activity, with other members possessing or

thought to possess (i.e. not yet fully characterised) either mono(ADP-ribose) activity or to be inactive possessing neither a NAD+-binding site or a glutamate residue required within the catalytic site (Sousa et al., 2012). Classically, PARP-1 has been associated with the DNA damage response, and most proteins that become modified by PARP-1 are those that bind DNA, for example histones, DNA ligases, topoisomerases and DNA and RNA polymerases (Bouchard et al., 2003). Indeed, DNA damage is the primary stimulus of PARP activation and PARPs bind to DNA strand breaks (Sousa et al., 2012). DNA strand breaks (both single and double strand breaks), which may be a consequence of oxidative stress, have been shown to significantly increase levels of PARP-1 activity with PAR levels shown to rise 10-500 times (D'Amours et al., 1999; Virag et al., 2013). In addition to their role in post-translational modification of proteins by PARylation, PAR polymers also act as signalling or targeting molecules. Upon DNA damage PARPs add PAR polymers to themselves, which is thought to act to recruit proteins involved in the DNA damage response (Sousa et al., 2012). Such proteins may possess specific binding domains for PAR (Kraus, 2009).

Observations by Durkacz and colleagues in 1980 (Durkacz et al., 1980) first identified a role for PARP in DNA BER, whereby cells treated with a chemical inhibitor of PARP, 3-aminobenzamide, prevented the re-ligation of DNA strand breaks induced with dimethyl sulphate, thus increasing the dimethyl sulphate cytotoxicity.

PARP-1 has been implicated in both short-patch and long-patch BER and shown to interact with key proteins in both sub-pathways, such as XRCC1, DNA polymerase β and flap endonuclease-1 (Bouchard et al., 2003). However, PARP-1 has been shown to be crucial in long-patch base excision repair by experiments in which cells lacking PARP-1 have

significantly reduced long-patch repair capabilities (Dantzer et al., 2000). The same investigators noted that cells lacking PARP-1 also had reduced short-patch base excision repair capabilities, but not to the same extent.

PARP knockout mice and cells derived from them have been demonstrated to display enhanced sensitivity to DNA damaging agents. De Murcia and colleagues (de Murcia et al., 1997) demonstrated that DNA damage induced by the methylating agent N-methyl-N-nitrosourea (MNU) and ionizing radiation was increased in these experimental systems, which had a knockout of a 113 kDa PARP protein (presumably PARP-1) suggesting a role for PARP-1 in multiple DNA repair pathways.

PARP-1 has also been identified to play a role in the repair of DNA double strand breaks, for example during NHEJ poly(ADP-ribosyl)ation of the DNA-PK catalytic subunit has been shown to stimulate its kinase activity (Ruscetti et al., 1998). PARP-1 has also been implicated in alternative end joining repair of DNA double strand breaks which is thought to act as a back up to classical NHEJ repair which occurs in the absence of functional NHEJ (Wang et al., 2006). Both PARP-1 and Ku proteins bind to DNA double strand breaks, however, Ku with greater affinity; under normal circumstances classical non-homologous end joining prevails. In the absence of functional NHEJ, PARP-1 can readily bind to the DNA double strand break and interact with XRCC1-DNA ligase III complex to re-ligate the strand break (Wang et al., 2006).

Another potential role of PARP-1 in the DNA damage response is in allowing access to the site of DNA damage by repair proteins. Poirier and colleagues observed that poly(ADP-ribosyl)ation of histones allows chromatin remodelling and potentially permitting ease of access to the strand break or damaged base by repair proteins (Althaus, 1992; Poirier et al., 1982). An alternate approach could be to make DNA more available to transcription factors involved in regulating the cellular response to DNA damage and/or genotoxic stress (Malanga and Althaus, 2005).

Not surprisingly, the role of PARPs in DNA damage repair has led to investigation of the potential of chemical inhibitors of PARP as cancer therapies, either to potentiate the effectiveness of chemotherapy or radiotherapy by decreasing repair of DNA lesions repaired by BER for example or by utilising a synthetic lethality approach (De Vos et al., 2012). Synthetic lethality, arises when a mutation in two genes simultaneously causes cell death whereas individual mutations in the same genes has no effect on viability. This principle has led to PARP-inhibitors entering clinical trials for cancers with mutations in BRCA1/2 such as some breast and ovarian cancers. Farmer et al (Farmer et al., 2005) demonstrate that cells with mutations in BRCA1 or BRCA 2 have increased sensitivity to PARP-1 inhibition, leading to chromosomal instability, cell cycle arrest and cell death by apoptosis. The exact mode of action is uncertain but could involve the role of PARP-1 in single strand break repair/base excision repair. By inhibiting PARP-1 an accumulation of unrepaired single strand breaks occur which, when encountered by the replication fork, are converted to double strand breaks that will go unrepaired due to defective double strand break repair by homologous recombination in the absence of BRCA1/2. The ultimate consequence of unrepaired double strand breaks is, of course, cell death (De Vos et al., 2012). Another study by Bryant et al (Bryant et al., 2005) illustrates the necessity for PARP-1 activity in cells with

mutations in BRCA2. The authors suggest that spontaneous strand breaks in the absence of PARP-1 activity by chemical inhibition in BRCA2 mutant cells leads to replication fork collapse and subsequent cell death.

Despite this, the outcomes of clinical trials with PARP inhibitors have been inconclusive. Iniparib, a proposed inhibitor of PARP-1 demonstrated some success in patients with triple negative breast cancer when used in combination with chemotherapeutics gemcitabine and carboplatin during phase 2 clinical trials (Infante and Burris, 2013). However, no overall benefit was noted during phase 3 clinical trials. An inhibitor of both PARP-1 and PARP-2, olaparib, did not proceed to phase 3 clinical trials (Guha, 2011). If PARP inhibitors are to be used as a monotherapy, taking advantage of synthetic lethality, screening of tumours for BRCA1/2 mutants as well as other genes required for effective HR could allow targeting the therapy to patients most likely to respond favourably (Infante and Burris, 2013). PARP inhibition in combination chemotherapy has also shown limited success and fine tuning of dosage to attain the necessary level of PARP inhibition to achieve success is required (Infante and Burris, 2013).

PARP proteins, in contrast to their role in promoting cell survival following genotoxic stress and repair of DNA damage, have also been shown to be involved in cell death. As a result of minor DNA damage, PARP activity is stimulated, resulting in the recruitment of DNA repair proteins, subsequent repair of the damage and cellular survival (Heeres and Hergenrother, 2007). Upon extreme DNA damage however, over-activation of PARP can lead to NAD+ depletion with cells dying by necrosis due to an exhaustion of cellular ATP required during the re-synthesis of NAD+ (Virag et al., 2013).

PARP has also been implicated in apoptotic cell death in response to excessive DNA damage in a process termed parthanatos that involves apoptosis inducing factor (AIF), a protein which resides at the mitochondrial intermembrane in unstressed conditions. Under conditions of stress and DNA damage, PARP-1 activation and subsequent PAR production leads to AIF being released from the mitochondria from where it translocates to the nucleus contributing to caspase-independent cell death, which induces chromatin condensation and DNA fragmentation. It is thought that PAR becomes relocated to the cytoplasm and mitochondria where it induces AIF's release (Wang et al., 2009b).

PARP-1 has also been associated with a role in cell fate decisions in response to cellular stress by controlling intracellular localization of proteins such as p53 and NF-κB by poly(ADP-ribosyl)ation (Kanai et al., 2007; Zerfaoui et al., 2010). Crm1 is a nuclear export protein involved in the export of proteins targeted for removal to the cytoplasm; however, poly(ADP-ribosyl)ation of these proteins prevents their interaction with Crm1, thus preventing their nuclear export, resulting in nuclear accumulation where they can facilitate processes involved in the stress response (Abd Elmageed et al., 2012; Luo and Kraus, 2012).

5.2 Aims

DNA damage and cell death have been proposed to play a role in natural ageing and formation of cataract. Poly(ADP-ribose) polymerases have been identified to regulate DNA repair and control cell survival and death, yet very few studies have investigated the role of PARP or its inhibition in the lens. The objective of the current chapter was therefore to elucidate the roles of PARP in DNA repair and determination of lens cell fate.

5.3 Results

5.3.1 PARP-1 expression in human lens cells.

To assess the expression of PARP-1 in FHL124 cells and native human lens epithelium immunocytochemistry was performed (Figure 5.1). An intense staining pattern for PARP-1 was observed in the nuclear region of both FHL124 cells and the native epithelium.

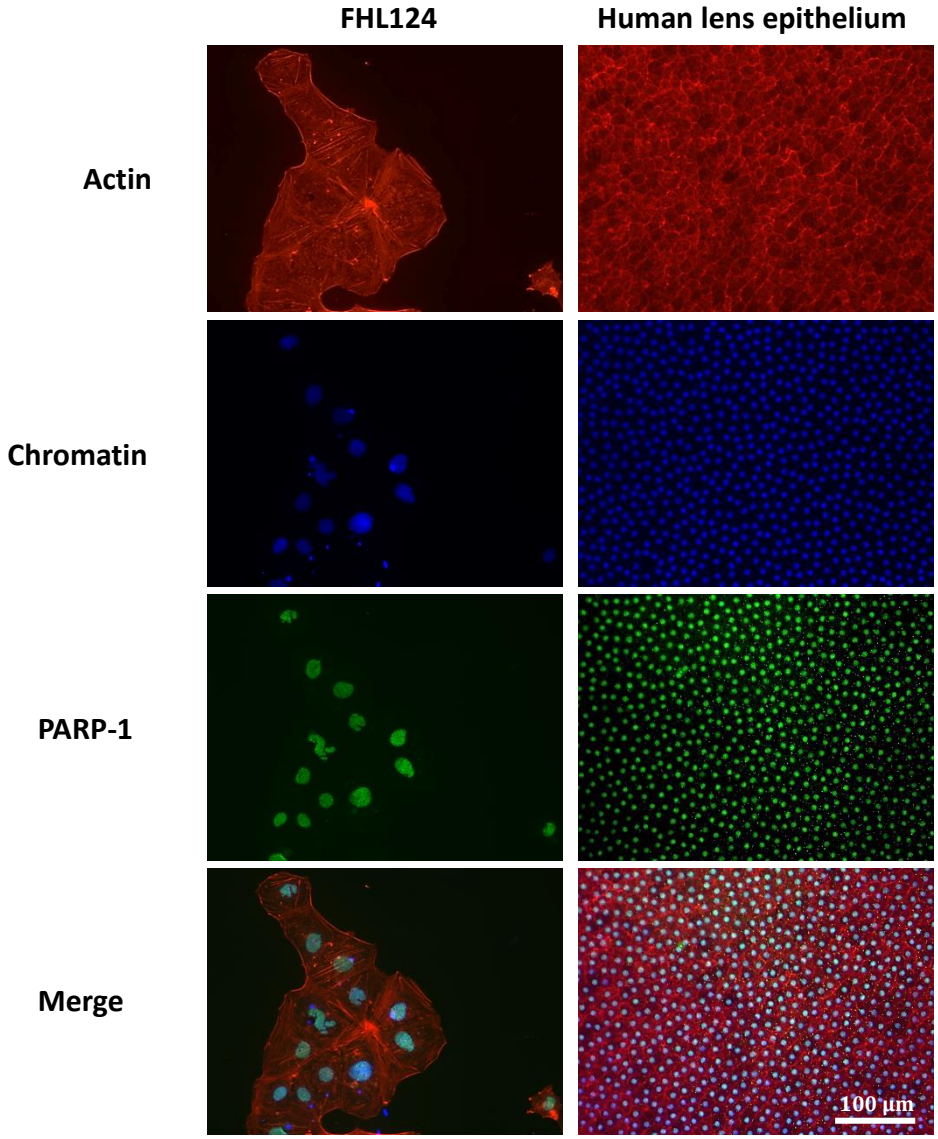


Figure 5.1. Fluorescent micrographs showing PARP-1 distribution in FHL124 cells and the native human lens epithelium. PARP-1 was visualised with an alexa488-conjugated secondary antibody (green), with actin cytoskeleton and chromatin stained with Texas red x-phalliodin (red) and DAPI (blue) respectively.

5.3.2 Effects of PARP-1 chemical inhibition on oxidative stress-induced DNA strand breaks in FHL124 cells

PARP-1 has been described as playing a role in the repair of oxidative DNA damage and single strand break repair via the BER pathway (Bouchard et al., 2003; Dantzer et al., 2000). As shown in Chapter 3 and 4, H_2O_2 is a pro-oxidant able to cause oxidative stress and subsequent damage to DNA. To investigate the role of PARP-1 in oxidative stress-induced DNA strand breaks and their repair in human lens cells a chemical inhibitor of PARP-1, AG14361, was applied at a concentration of 1 μM to FHL124 cells for 1 hour prior to treatment with 30 μM H_2O_2. DNA strand breaks were measured over 24 hours following treatment (Figure 5.2). A 1 μM AG14361 concentration has been shown previously to inhibit PARP-1 activity by greater than 90% (Calabrese et al., 2004).

H_2O_2 treatment in FHL124 cells without PARP-1 inhibition produced a peak level of DNA strand breaks at 0.5 hours (76.8 ± 2.0% DNA in tail) following treatment, after which levels of DNA strand breaks were shown to steadily decline over the 24 hours studied. These findings are consistent with data presented in Chapter 3 and 4. In FHL124 cells pre-treated with PARP-1 inhibitor these levels of strand breaks also peaked at 0.5 hours post H_2O_2 treatment (97.0 ± 0.5% DNA in tail), but this peak was significantly greater than control cells without PARP-1 inhibition. In fact, levels of DNA strand breaks generally remained elevated over the 24 hour study period.

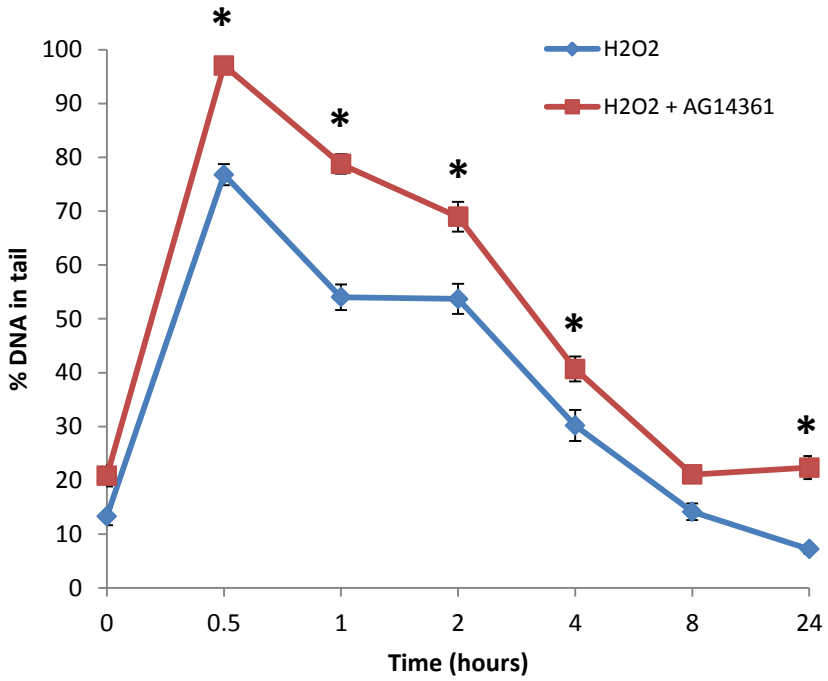


Figure 5.2. Effect of PARP-1 inhibition on H_2O_2-induced DNA strand breaks. FHL124 cells were pre-treated with 1 μM AG14361 for one hour prior to treatment with 30 μM H_2O_2. Levels of DNA strand breaks (% DNA in tail) were measured by the alkaline comet assay and compared to H_2O_2 treated cells without AG14361 pre-treatment. Data presented is representative of 3 independent experiments each with 100 comets scored ± SEM. * indicates significant difference between experimental conditions ($p \leq 0.05$; ANOVA with post hoc Tukey's test).

5.3.3 Effects of PARP-1 inhibition on oxidative stress-induced FHL124 cell death

To investigate the effects of PARP-1 inhibition on oxidative stress-induced cell death and survival, FHL124 cells were pre-treated PARP-1 chemical inhibitor, AG14361, followed by H_2O_2 treatment. On this occasion FHL124 cells were treated with 0 or 100 μM H_2O_2. A concentration of 100 μM was shown in Figure 3.1 to induce the highest increase in cytotoxicity of the dose range studied. Thus, it was considered an appropriate concentration

in which to induce FHL124 cell death in these experiments. At 24 hours cells were washed free of treatment medium and dead cells before being fixed and stained with coomassie blue protein stain to measure cell survival. Culture dishes were then imaged (Figure 5.3) and coomassie blue staining measured (Figure 5.4). A greater level of cell survival is indicated by increased levels of coomassie blue protein stain owing to a greater level of cells present following treatment. Coomassie blue protein staining revealed greater cell numbers remaining following hydrogen peroxide treatment in FHL124 cells pre-treated with AG14361. FHL124 cells treated with H_2O_2 alone demonstrated a 69.6% decrease in cell survival compared to untreated cells at the experimental end point. However, cells pre-treated with AG14361 prior to application of H_2O_2 demonstrated only a 44.8% decrease in cell survival. No significant changes in cell survival were noted with pre-treatment alone.

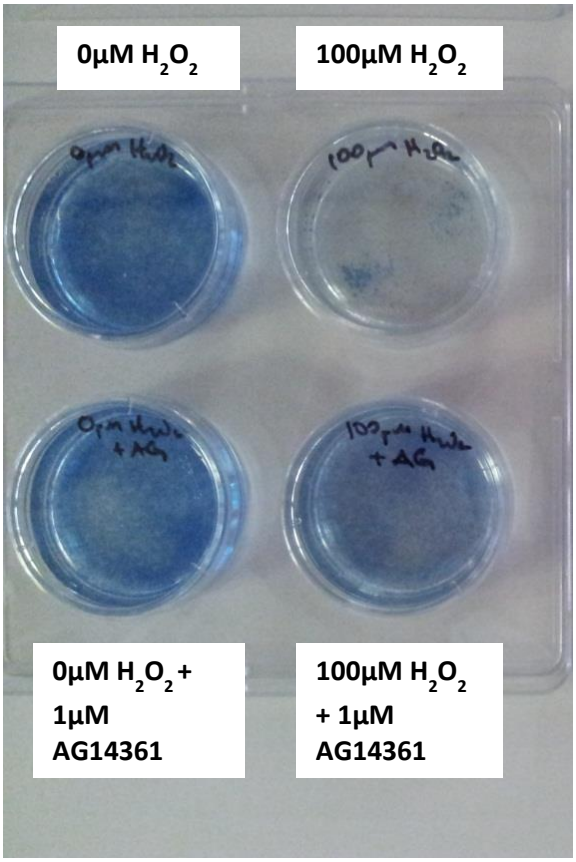


Figure 5.3. Coomassie blue protein stain of FHL124 cells. PARP-1 inhibition reduces hydrogen peroxide-induced cell death in FHL124 cells. FHL124 cells were pre-treated with 1 µM AG14361 before application of 100 µM H_2O_2. Detached/dead cells were washed free at 24 hours, cells fixed and stained with coomassie blue.

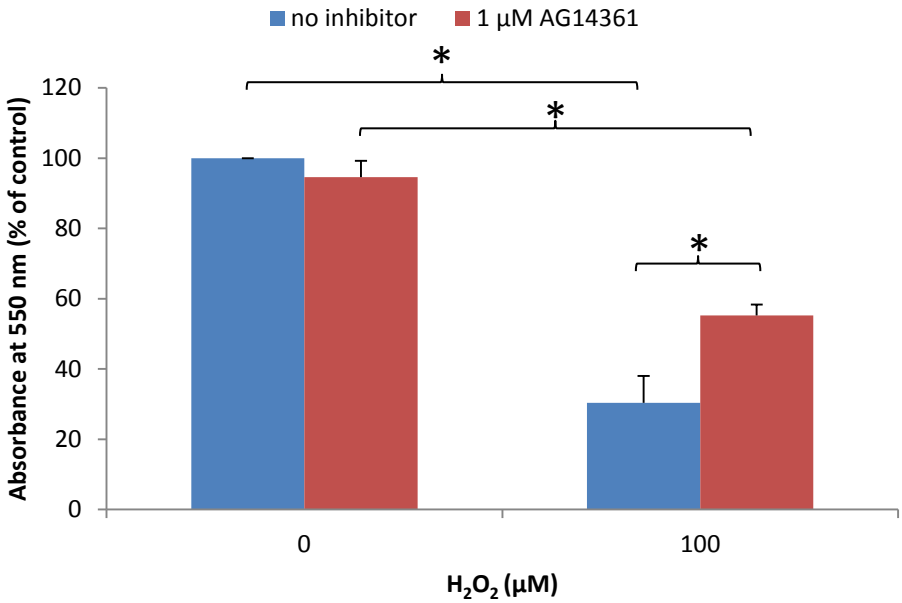


Figure 5.4. PARP-1 inhibition reduces H_2O_2-induced FHL124 cell death. FHL124 cells were pre-treated with 1 µM AG14361 before application of 100 µM H_2O_2. Detached/dead cells were washed free at 24 hours, cells fixed and stained with coomassie blue protein stain. Absorbance was measured at 24 hours. Data represents 3 independent experiments ± SEM. * indicates significant difference between experimental groups ($p \leq 0.05$; ANOVA with post hoc Tukey's test).

Levels of LDH released into the cell culture medium at the experimental end point were also assayed as a measure of cell death (Figure 5.5). At 24 hours post H_2O_2 treatment, levels of LDH were significantly lower in FHL124 cells pre-treated with AG14361 compared to those without. Levels of LDH were significantly elevated in culture medium of FHL124 cells treated with H_2O_2 alone, with a 109.6% increased noted compared to untreated cells. FHL124 cells first pre-treated with AG14361 prior to addition of H_2O_2 demonstrated a 32.9% increase in LDH release following H_2O_2 treatment compared to untreated cells. Thus, a suppression of H_2O_2-induced LDH release/cell death was noted in cells pre-treated with AG14361. No

changes in LDH release were noted with inhibitor pre-treatment alone, consistent with data presented in Figure 5.4.

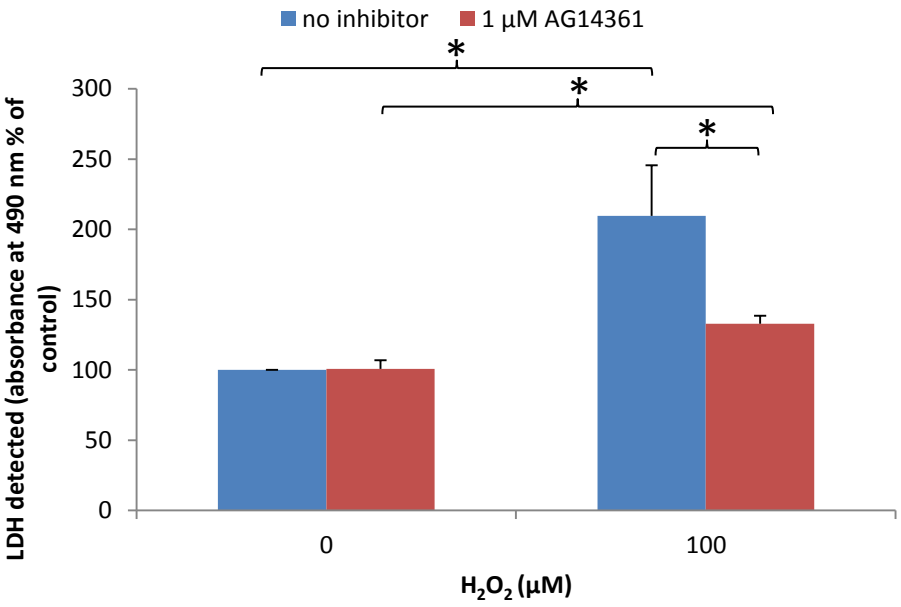


Figure 5.5. PARP-1 inhibition reduces H_2O_2 -induced FHL124 cell death. FHL124 cells were pre-treated with 1 µM AG14361 before application of 100 µM H_2O_2 peroxide. LDH release (as a marker of cell death) was measured at 24 hours (absorbance at 490 nm) and compared to cells without pre-treatment with AG14361. Data represents 3 independent experiments ± SEM. * indicates significant difference between experimental groups ($p \leq 0.05$; ANOVA with post hoc Tukey's test).

To reduce concerns that AG14361 could be directly acting as an anti-oxidant against H_2O_2, experiments were undertaken whereby AG14361 and H_2O_2 were added to FHL124 cells simultaneously; following 24 hours LDH release was again measured (Figure 5.6). No

statistically significant difference in LDH release was observed between cells treated with AG14361 and H_2O_2 simultaneously and those treated with H_2O_2 alone.

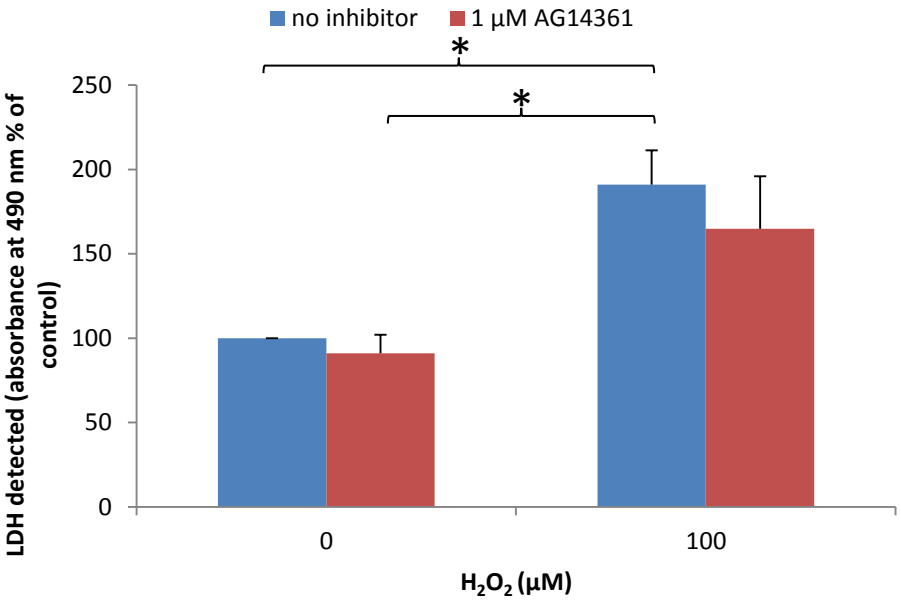


Figure 5.6. PARP-1 inhibitor (AG14361) is not a direct antioxidant. AG14361 and H_2O_2 were added to FHL124 cells simultaneously. LDH release (absorbance at 490 nm) was measured 24 hours post treatment and compared to cells treated with H_2O_2 alone. Data represents the mean of 3 independent experiments ± SEM. * indicates significant difference between experimental groups ($p \leq 0.05$; ANOVA with post hoc Tukey's test).

5.3.4 Effects of targeted siRNA mediated PARP-1 knockdown on oxidative stress-induced DNA strand breaks in FHL124 cells.

To investigate the effect of PARP-1 expression on FHL124 cell responses to oxidative stress, PARP-1 was depleted with targeted siRNA. Knockdown of PARP-1 was confirmed by immunocytochemistry (Figure 5.7) and western blotting (Figure 5.8). Quantification of PARP-1 western blotting demonstrated that PARP-1 was significantly depleted (77.7 ± 3.3%) compared to cells transfected with non-coding scramble control siRNA. This depletion in PARP-1 expression was in line with expectations provided by the manufacturer.

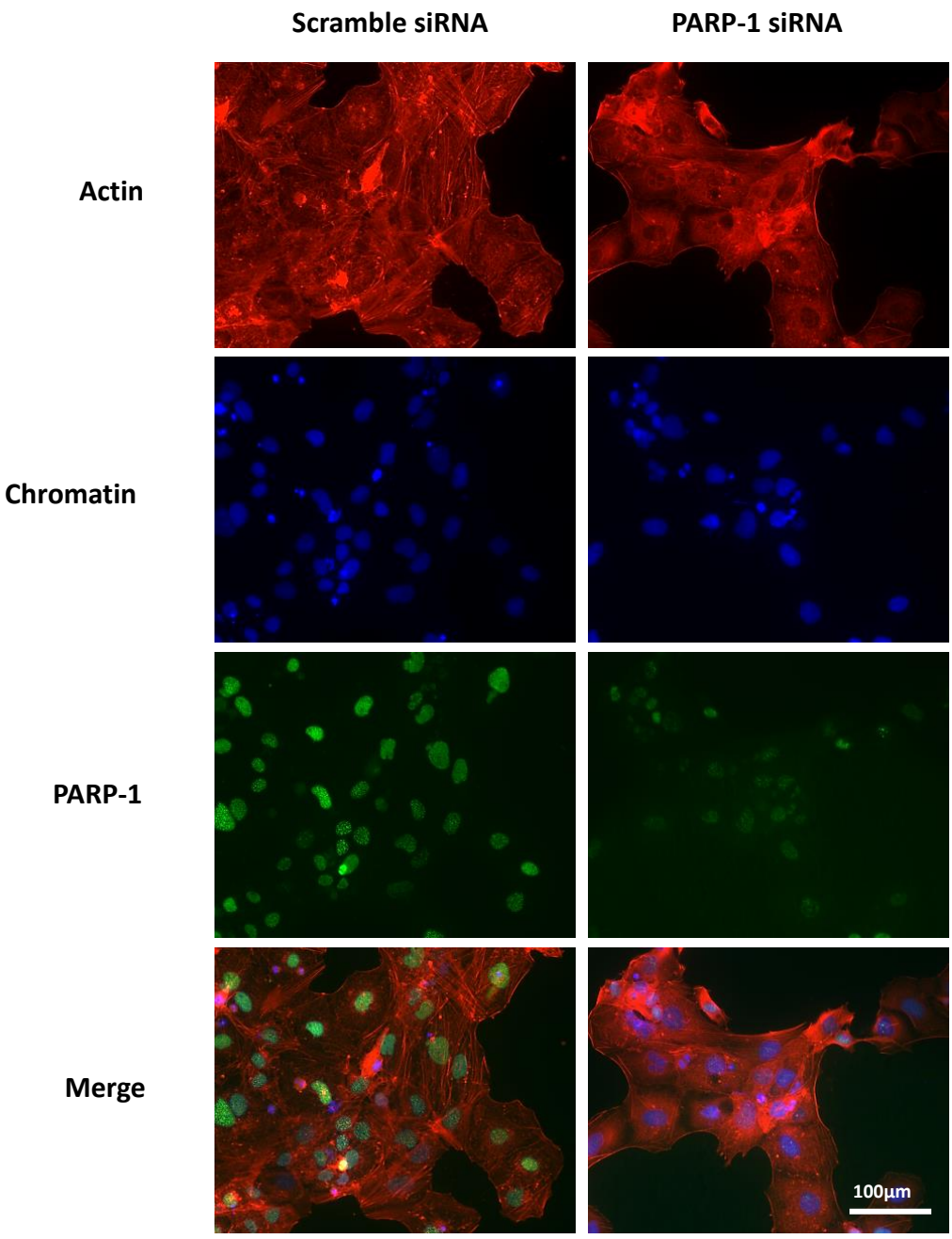


Figure 5.7. Fluorescent micrographs illustrating successful PARP-1 knockdown. FHL124 cells were either treated with siRNA targeted against PARP-1 or non-coding scramble control siRNA for 48 hours and immunocytochemistry performed.

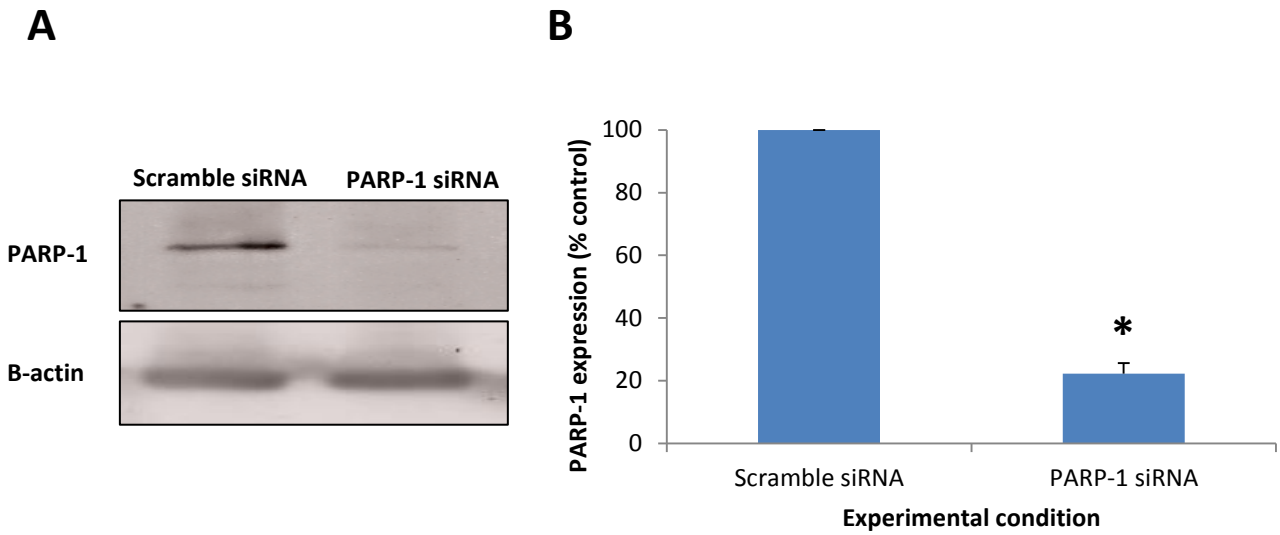


Figure 5.8. Targeted siRNA significantly depletes PARP-1 expression in FHL124 cells. Representative gel from western blot (A) and quantification of western blot data pooled from 3 independent experiments adjusted for β-actin loading controls ± standard error of the mean (B). * indicates significant difference ($p \leq 0.05$; Student's t-test).

To investigate the effect of PARP-1 depletion on oxidative stress-induced DNA strand breaks, FHL124 cells were either transfected with PARP-1 siRNA or non-coding scramble control siRNA and treated with 30 μM H_2O_2. The alkaline comet assay was performed and levels of DNA strand breaks measured over 24 hours (Figure 5.9). FHL124 cells treated with non-coding scramble siRNA demonstrated a peak level of DNA strand breaks at 0.5 hours post H_2O_2 treatment (23.4 ± 2.1% DNA in tail) with levels steadily decreasing thereafter. Levels of DNA strand breaks in PARP-1 knockdown cells also peaked at 0.5 hours post H_2O_2 treatment demonstrating an increase in strand breaks observed in cells without PARP-1 knockdown at the same time point (42.0 ± 2.7% DNA in tail). However, these levels of strand

breaks were significantly elevated compared to cells treated with scramble control siRNA cells at 0.5 and 1 hours post H_2O_2 treatment.

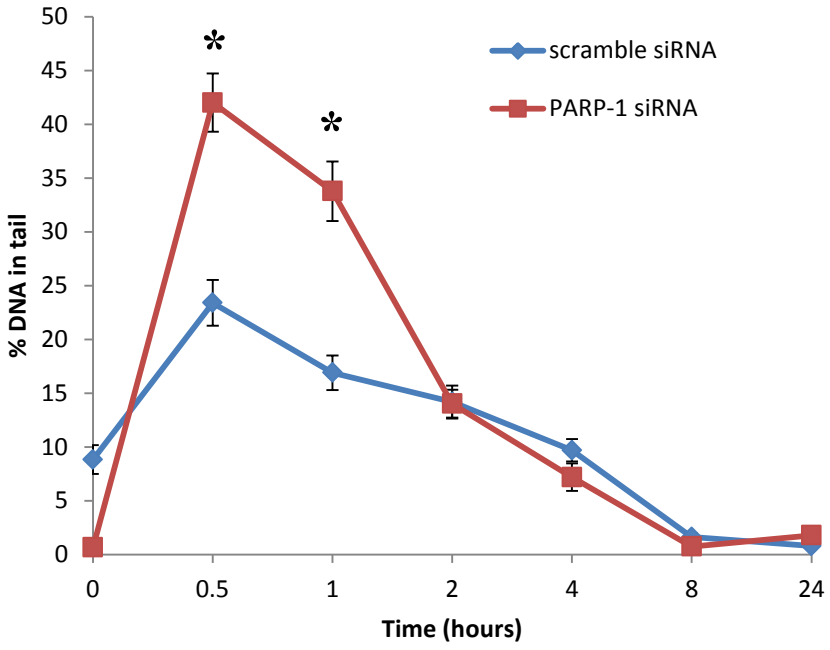


Figure 5.9. Effect of depleting PARP-1 on H_2O_2-induced DNA strand breaks. FHL124 cells were treated with siRNA targeted against PARP-1 and treated with 30 μM H_2O_2. Levels of DNA strand breaks were measured by alkaline comet assay and compared to levels in FHL124 cells treated with non-coding scramble control siRNA. Data presented is representative of 3 independent experiments each with 100 comets scored ± SEM. * indicates significant difference between experimental conditions ($p \leq 0.05$; ANOVA with post hoc Tukey's test).

5.3.5 Effects of PARP-1 inhibition on oxidative stress induced changes in a whole human lens culture system

To test the effect of PARP-1 inhibition on oxidative stress induced changes associated with cataract formation, a whole human lens culture system was once again adopted. A 10 μM solution of AG14361 was applied to the lenses for 1 hour prior to treatment with 1 mM H_2O_2. A greater concentration of AG14361 was used to inhibit PARP-1 activity in these experiments compared to those using FHL124 cells for reasons alluded to in Chapter 3, including increased cell number and potential of the lens capsule to act as a barrier. At 24 hours following treatment, lenses were imaged and changes in visual quality were quantified from brightfield images and compared to match paired controls treated with H_2O_2 but without pre-treatment with AG14361 (Figure 5.10). Culture medium was also sampled and assayed for LDH release at 24 hours as a marker of cell death (Figure 5.11).

At the experimental start point (day 0) lenses in both conditions (1 mM H_2O_2 and 1 mM H_2O_2 + 10 μM AG14361) were fundamentally clear. This is demonstrated by the grid beneath the lenses being clearly visible and defined in each instance.

At day 1, control lenses treated with H_2O_2 only demonstrated a marked decrease in visual quality, with the grid beneath the lens being less visible and defined; however lenses pre-treated with AG14361 were protected from the effects of H_2O_2 with the grid remaining visible and only a slight central opacity was noted. Quantification demonstrated a significant protection in visual quality compared to match paired controls without pre-treatment with the

PARP-1 inhibitor AG14361 (18.4 ± 3.2% versus 3.7 ± 1.4% reduction in visual quality respectively).

Pre-treatment with AG14361 also protected lenses from H_2O_2-induced cell death. LDH release measured at the experimental end point (day 1) was significantly lower in culture medium sampled from lenses pre-treated with AG14361 compared to lenses without pre-treatment (Figure 5.11) with a 78.9% suppression in LDH release noted.

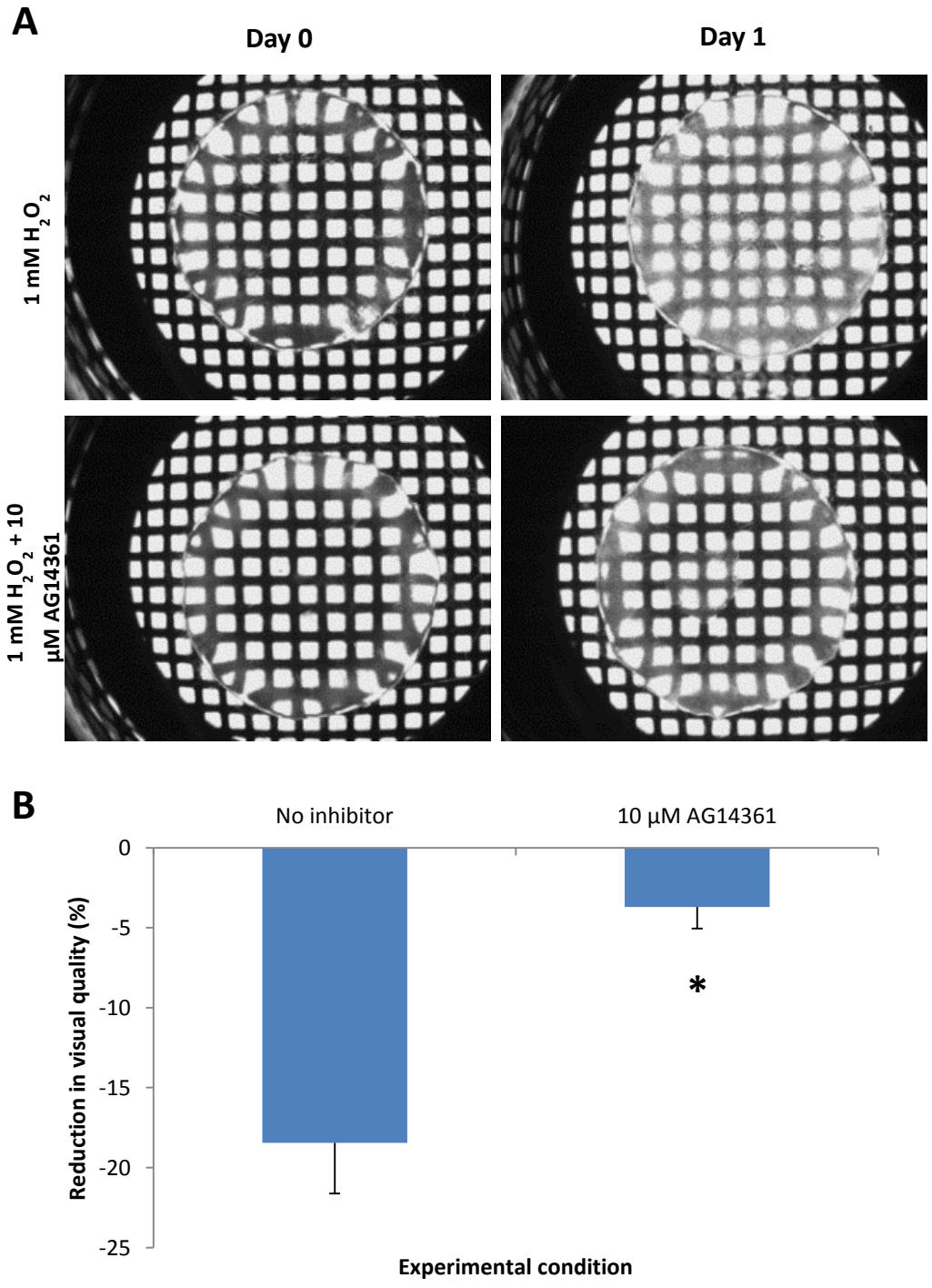


Figure 5.10. PARP-1 inhibition protects human lenses from oxidative stress. Representative brightfield images of human lenses over time (A) and quantification of visual quality (B) at day 1. Whole human lenses were treated for 1 hour with 10 µM AG14361 followed by the application of 1 mM H_2O_2 (Day 0) and of visual quality quantified at day 1. Data pooled from 3 independent experiments ± SEM. * indicates significant differences between conditions ($p \leq 0.05$; Student's t-test).

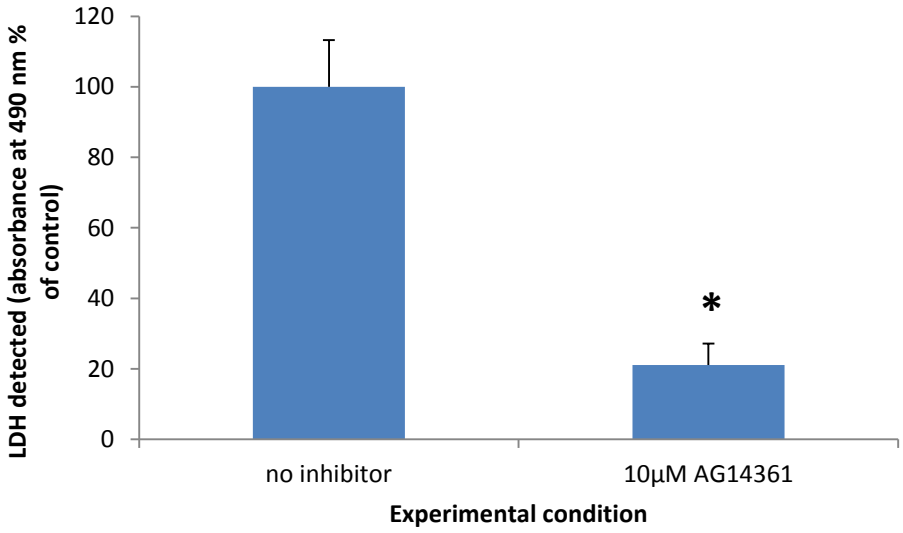


Figure 5.11. PARP-1 inhibition protects human lenses from oxidative stress-induced cell death. LDH release was measured at the experimental end point (day 1). Data pooled from 3 independent experiments ± SEM. * indicates significant difference between experimental groups ($p \leq 0.05$, Student's t-test).

5.4 Discussion

PARP-1, and poly(ADP-ribosyl)ation has long been reported to play a role in DNA damage repair (Durkacz et al., 1980; Virag and Szabo, 2002), particularly BER via interactions with XRCC1, DNA polymerase β and flap endonuclease-1 (Bouchard et al., 2003). It has also been implicated in the repair of DNA double strand breaks by NHEJ (Wang et al., 2006). A further role of PARP-1 in the DNA damage response is to act as a nick sensor allowing for recruitment of DNA repair machinery, but also to allow the DNA repair machinery access to act up on DNA damage. Histones are target proteins of PARP-1 and it is thought that poly(ADP-ribosyl)ation by PARP-1 and subsequent negative charge provides repulsion between DNA and histones allowing the DNA to be accessed by the repair machinery (Virag and Szabo, 2002).

The present study demonstrated PARP-1 expression in the human lens cell line FHL124 and in the native human epithelium, and in each instance this expression was predominantly nuclear. PARP-1 activity was inhibited in FHL124 lens cells with a specific and potent chemical inhibitor, AG14361 (Calabrese et al., 2004; Smith et al., 2005). FHL124 cells pre-treated with AG14361 displayed increased sensitivity to H_2O_2-induced DNA strand breaks with levels shown to be increased from those treated with H_2O_2 alone. However, in both instances repair of strand breaks was observed. Therefore, it is clearly evident from these inhibition studies that functional PARP-1 is important in the response to DNA damage in human lens cells. It can be concluded that PARP-1 inhibition by AG14361 renders FHL124 cells more susceptible to oxidative stress-induced DNA strand breaks.

Several investigators have produced work highlighting the importance of PARPs in the DNA damage response. Studies by de Murcia et al and Trucco et al (de Murcia et al., 1997; Trucco et al., 1998) investigated the requirement of PARP in recovery from DNA damage and sensitivity to DNA damage in PARP $^{-/-}$ mice and cells. De Murica and colleagues found that PARP $^{-/-}$ mice displayed increased sensitivity to MNU and γ-irradiation. The study by Trucco and colleagues noted similar results adding that PARP $^{-/-}$ mice embryonic fibroblasts displayed delayed re-ligation of methylmethane sulfonate (MMS) induced strand breaks, resulting in cell growth retardation, cell cycle arrest and chromosome instability; importantly, this loss of cell viability was restored if PARP expression was also restored in these cells. Dantzer et al (Dantzer et al., 2000) note that PARP-1 interacts with several key proteins in BER such as XRCC1, DNA polymerase β and DNA ligase III. These investigators concluded a role for PARP in the BER pathway.

PARPs have also been implicated in the repair of DNA double strand breaks via the alternative end joining pathway and classical NHEJ. Alternative end joining acts to re-join double strand breaks in the absence of functional NHEJ and PARP-1 is thought to bind DNA ends in this instance with ligation performed by XRCC1 and DNA ligase III (De Vos et al., 2012). In classical NHEJ, PARylation of DNA-PKcs by PARP-1 has been shown, in vitro, to stimulate its kinase activity (Ruscetti et al., 1998). In addition to these findings, it has been proposed that PARP-1 may be involved in double strand break signalling via interaction with ATM, contributing to its localisation at the site of double strand breaks and may result in ATM activation (Haince et al., 2007).

The present study has also investigated the effect of PARP-1 inhibition on lens cell survival and death following oxidative insult. Survival of FHL124 cells following H_2O_2 treatment was found to be increased when PARP-1 was chemically inhibited, and conversely, this inhibition of PARP-1 afforded protection from oxidative stress-induced cell death. On first observation, this result is in conflict with data suggesting PARP-1 inhibition renders lens cells increasingly sensitive to DNA damage induced by H_2O_2. However, PARP enzymes appear to have a dual role, acting both in cell survival and death; cell survival in their role in the DNA damage response, aiding in the repair of DNA strand breaks and oxidative base damage in the BER pathway and subsequent cell survival but also increasingly implicated in cell death pathways (Sousa et al., 2012; Yu et al., 2002).

Poly (ADP-ribsoyl)ation by PARP was hypothesised by Nagele in 1995 (Nagele, 1995) to mediate a cell suicide mechanism in response to high levels of DNA damage. PARP is activated in response to DNA strand breaks and should the insult be severe or long lasting then the resulting excessive PARP activation leads to depletion of NAD+ and subsequently that of ATP. This cellular energy depletion causes cell necrosis (Virag et al., 1998), thus limiting the possibility of cells with potentially high mutation frequency surviving. Previous investigations (Schraufstatter et al., 1986) have concurred with the present study, whereby inhibiting PARP activity chemically with 3-aminobenzamide protected stimulated leukocytes from hydrogen peroxide-induced cell death.

PARP-1 may also mediate cell death in response to DNA damage in an alternative pathway to cellular energy depletion, termed parthanatos. AIF is a death signalling molecule which becomes released from the mitochondria in response to DNA damage from where it

translocates to the nucleus to affect chromatin condensation and recruitment of nucleases (Heeres and Hergenrother, 2007). PARP-1 is essential for AIF release from the mitochondria. One study ulitised PARP-1 knockout fibroblasts in which there was no AIF translocation to the nucleus upon MNNG treatment (Yu et al., 2002). It is therefore the activation of PARP-1 upon DNA damage that acts as the signal for AIF release from the mitochondria. Indeed, the PAR polymer itself maybe the death signal in response to DNA damage, and is produced in the nucleus in response to DNA strand breaks, from where free PAR translocates to the mitochondria where it causes the release of AIF (Hong et al., 2004; Wang et al., 2009b). Future work investigating AIF distribution patterns with immunocytochemistry within human lens cells following PARP-1 inhibition and H_2O_2 treatment would be worthwhile. This would allow for elucidation of the role that PARP-1 and parthanatos play in the lens cells survival following oxidative stress, a key causative factor in the formation of cataract.

Alternative avenues of future investigation of the role of PARP-1 in cell death and survival in human lens cells could also involve study of other proteins implicated in cell fate decisions, such as p53 and NF-κB. Both are target proteins of PARP-1 and their subcellular location is controlled by the nuclear export protein Crm1; however, their poly(ADP-ribosyl)ation by PARP-1 prevents interaction with Crm1 and their nuclear export. Thus, nuclear accumulation is thought to occur, promoting transcription of genes involved in the stress response and apoptosis (Luo and Kraus, 2012). Localisation of p53 and NF-κB could be studied under conditions of cellular stress, and effects of inhibition of PARP-1 elucidated.

In the present study it became apparent that oxidative stress-induced cell death could be suppressed by the inhibition of PARP-1. Thus, experiments were undertaken to ensure that

AG14361 was not acting as a direct anti-oxidant. It was found that pre-treatment of cells was required for suppression of oxidative stress-induced cell death. When AG14361 and H_2O_2 were added simultaneously to cell culture no difference in cell death was noted compared to H_2O_2 treatment alone, suggesting pre-incubation with AG14361 was necessary to produce its inhibitory effect. Crystallographic analysis of AG14361 binding to PARP-1 and found it to bind to specific sites within its catalytic domain producing a specific and potent inhibitory effect (Calabrese et al., 2004). This concurs with findings by other investigators that PARP inhibitors do not act as direct antioxidants (Schraufstatter et al., 1986).

A targeted siRNA approach was also undertaken to deplete PARP-1 expression in FHL124 cells and this knockdown was found to render the cells more susceptible to oxidative stress-induced DNA strand breaks. This result confirmed that effects observed with chemical inhibition of PARP-1 are likely to not be due to a direct anti-oxidant effect, but due to depleted PARP-1 function. Future experiments should assess the effects of oxidative stress-induced cell death in cells with siRNA mediated knockdown of PARP-1 expression. Indeed, investigations could be extended to study the effects of other PARP protein family members to ascertain their role, if any, on lens cell fate following oxidative damage.

Work presented in Chapter 4 of this thesis has demonstrated that siRNA can be used to suppress gene expression within the whole human lens. However, on this occasion it was decided to use AG14361 to chemically inhibit PARP-1 in the whole lens culture system because of similar responses noted when using AG14361 or PARP-1 targeted siRNA to study effects on hydrogen peroxide-induced DNA strand breaks. A one hour pre-incubation with

AG14361 was considered preferable to transfecting with siRNA for 72 hours, as performed in Chapter 4.

The current study investigated the role of PARP-1 inhibition on oxidative stress-induced cataract and found that PARP-1 inhibition supressed lens opacity and decline in visual quality as a result of oxidative stress. This inhibition also afforded protection against cell death in line with experiments within FHL124 cells.

To date, there have been two investigations as to the role of PARP inhibition in the lens. There is conflicting evidence for the role of PARP proteins in cataractogenesis, with both studies to date utilising rat models. The first of these concluded that PARP inhibitors nicotinamide and 3-aminobenzamide accelerated MNU-induced cataractogenesis (Miki et al., 2007). Drel et al (Drel et al., 2009) evaluated effects of PARP inhibition by 1,5 iso-quinolinediol and 10-(4-methyl-piperazin-1-ylmethyl)-2H-7-oxa-1,2-diaza-benzo-[de]anthracen-3-1 in diabetic-induced cataracts; they concluded that PARP inhibition by these compounds counteracted cataract formation. The current body of work determines for the first time the effects of PARP-1 inhibition on cataract formation in a human model.

The present study clearly demonstrates that PARP-1 inhibition in this experimental model protects human lenses from oxidative-stress induced changes in opacity and cell death and that this protection is afforded from an inhibition of cell death pathways mediated by PARP-1. These, as discussed, could involve necrotic cell death as a consequence of cellular energy depletion or via inhibition of PARP-1 mediated parthanatos, or indeed through disruption of

interactions between p53 and/or NF-κB with the nuclear export protein Crm1. The data as presented cannot however establish by which means; indeed it could involve a combination of PARP-1 related pathways. Although not investigated here, the PAR polymer has also been shown to interact with key apoptotic proteins such as p53, p21 and caspases (Hong et al., 2004). Interestingly, during caspase dependent apoptosis, PARP-1 becomes cleaved by caspase-7 and caspase-3, supressing PARP-1 activity suggesting that this suppression is essential for caspase dependent apoptotic cell death. This, therefore, presumably prevents energy depletion resulting from excessive PARP-1 activity stimulated by DNA cleavage during apoptotic process (Virag and Szabo, 2002).

Indeed, Luo and Kraus (Luo and Kraus, 2012) propose that PARP-1 is able to promote different cellular outcomes in response to varying levels of cellular stress, suggesting that under conditions of mild stress PARP-1 promotes DNA repair and cell survival. However, when cells are severely stressed PARP-1 promotes cell death via apoptosis, necrosis or parthanatos (Figure 5.12). Differences in the outcomes of previous studies (Drel et al., 2009; Miki et al., 2007) and the present study could indeed result from differing levels of stress being applied in the experimental systems used.

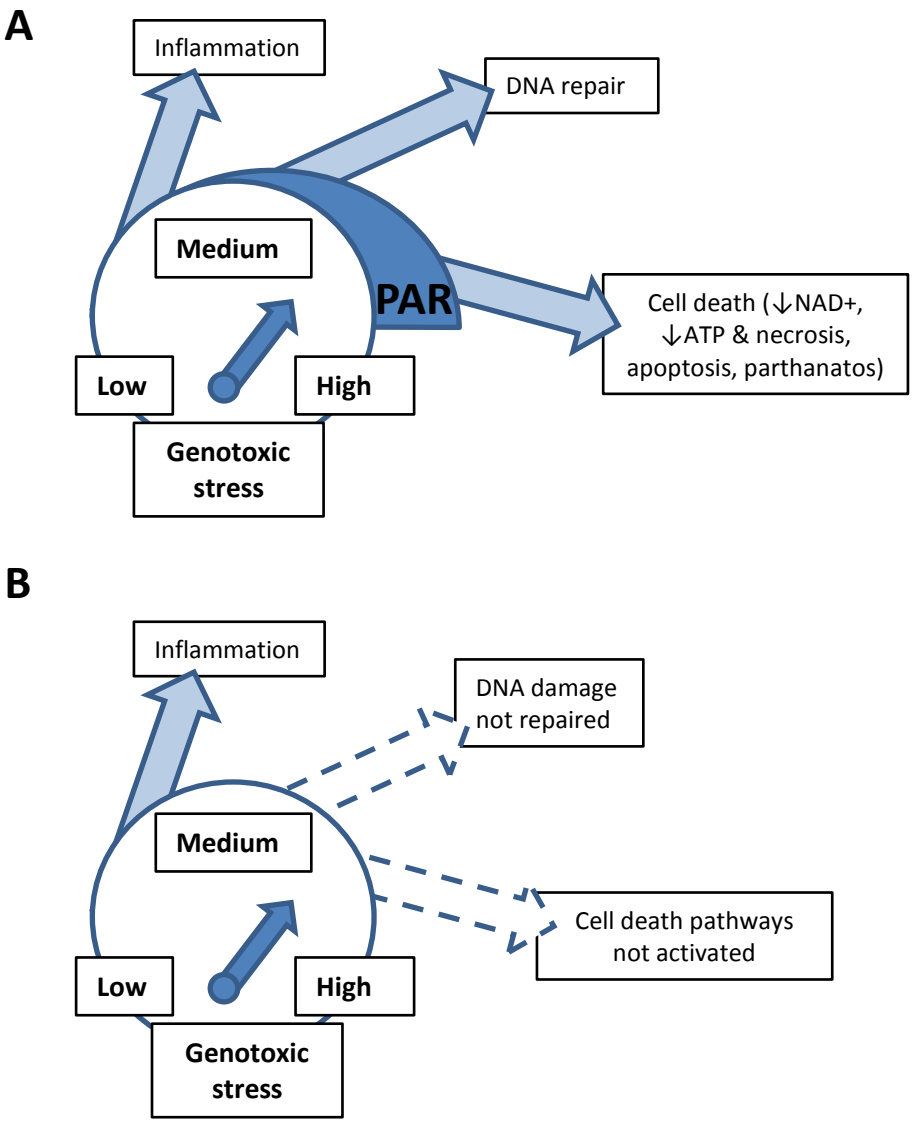


Figure 5.12. PARP-1 as a regulator of cell fate. (A) As levels of genotoxic stress are increased, PARP-1 activity and PAR production increases giving rise to varying cellular outcomes. Under low to medium levels of genotoxic stress cell survival prevails, however, high levels genotoxic stress cause activation of cell death pathways. (B) In the absence of PARP activity, possibly through PARP inhibition, DNA damage is not repaired and PARP-associated cell death pathways are not activated. Adapted by R. Bowater from Luo and Kraus, 2012.

Despite the well documented dual role of PARP-1 on both cell survival and cell death in response to DNA damaging stimuli and the current study's findings of relating to cell death and DNA damage, much of PARP's role within the cell remains elusive. Clearly, PARP inhibition has the ability to dramatically protect the cell from oxidative insult and future studies should focus further on clarifying the role of PARP proteins in the lens.

Chapter 6 General discussion

Cataract is a disease of great significance affecting millions worldwide (WHO, 2014). Currently, the only means of treating of cataract is by surgical intervention, which has a high success rate in restoring visual quality; however, with time a secondary loss of vision known as posterior capsule opacification occurs in many patients, which then requires further costly treatment (Wormstone et al., 2006). Cataract impinges on the wellbeing of individuals and places a huge strain on healthcare budgets. In 2010, cataract surgery in the USA alone cost $2498 million, with a further $222 million spent on treating posterior capsule opacification (Spalton et al., 2014). Therefore, investigations into the underlying causes of this disease could lead to elucidation of potential preventative therapies.

The involvement of DNA damage and DNA damage repair pathways are a much neglected area of cataract research; the current study therefore aims to investigate links between DNA damage, its repair and cataract formation. The lens faces a constant exposure to DNA damaging stimuli over long time frames. Cataract is a disease closely associated with ageing, one consequence of ageing is an accumulation of DNA damage throughout life which is associated with a decreased DNA repair capability. The present study has identified potentially important roles of DNA damage in the formation of cataract and has implicated the specific DNA repair process of NHEJ as well as a role for PARP-1 as potential factors in this disease.

Oxidative stress is a key factor in cataract formation (Truscott, 2005; Vinson, 2006) and one consequence of oxidative stress is damage to DNA. If this is unrepaired it may lead to cell death; indeed, apoptosis in the lens epithelium has been linked to the formation of cataract (Li et al., 1995). There have been few studies investigating the role of DNA damage to the lens in the formation of cataract. Kleiman and Spector (Kleiman and Spector, 1993) measured DNA strand breaks in lens epithelial samples obtained from cataract patients following surgery and compared these to DNA strand breaks in healthy, eye bank samples of similar age. They found levels of strand breaks to be significantly elevated in approximately 50% of cataract patients. Ates and colleagues (Ates et al., 2010) measured levels of oxidative DNA damage in cataract patients by assessing 8-OHdG levels in isolated blood cells and compared these to age- and gender-matched control subjects, finding a significant increase in cataract patients. However, this allows only for a measure of an individual's overall susceptibility to oxidative DNA damage rather than any measurement of DNA damage in the lens itself. A further study assessed DNA damage directly in lens epithelial cells from cataract patients following surgery and compared these to samples from non-cataractous lenses (Sorte et al., 2011). Significantly elevated levels of strand breaks were observed in samples obtained from cataract patients compared to control subjects.

These studies certainly provide suggestions of a role for DNA damage in the formation of cataract. The present study builds on these investigations utilising both a human lens cell line and native lenses obtained from human donor eyes. Thus, this project allowed for proof of principle investigations in the lens cell line before continuing investigations on precious donor material. The experimental model employed also utilises match-paired controls from the same individual donor, allowing for direct and robust comparisons between experimental conditions. The aforementioned studies (Kleiman and Spector, 1993; Sorte et al., 2011)

obtained donor material from cataract patients and compared it to tissue obtained from healthy eye bank lenses and, thus, those comparisons are less robust. They have however measured levels of DNA strand breaks in the lens epithelium from these samples, something the present study has not undertaken.

The current study has established a human cell line to human native tissue model for studying the effects of oxidative stress on human lens cells and whole human lenses, thus utilising a purely human model for a human disease. Previous studies have used animal lens models for investigating aspects of lens biology using various species including mouse (Spector et al., 2001), pig (Liu et al., 2013), bovine (Manna et al., 1996) and rat (Sanderson et al., 1999). However, in utilising a purely human model, the current study removes any possibility of variation between species. One such example of variation is provided by differences identified between human and rat whole lens culture models (Wormstone et al., 2006). This study demonstrated differing responses in calcium signalling profiles in response to various agonists of calcium signalling including histamine, ATP, EGF and adrenaline, however similarities were noted between species in response to acetylcholine.

Another example in inter-species variation can be given in relation to NHEJ, although it is not lens specific example; mice lacking Ku80 are viable, but show symptoms of premature ageing (Vogel et al., 1999) whereas no human with mutations in Ku80 has been described (Seluanov et al., 2004; Wang et al., 2009a), suggesting any such absence is lethal in humans. It may be that different species utilise these proteins in different ways, or that the cellular roles of this protein differs between species. DNA repair mechanisms appear to be largely conserved between species; nevertheless, it is clear that species respond to DNA damage

different ways (Freitas and de Magalhaes, 2011). At the very least, there are clear differences in lens gene expression profiles between species. The experimental model employed in the current study utilised both a human lens cell line and a human whole lens culture system and is thus preferential to any system utilising varying species.

The present study established a model using H_2O_2 to induce oxidative damage in the human lens cell line FHL124 and that it induced DNA strand breaks in these cells in a concentration-dependent manner. H_2O_2 was found to also induce concentration-dependent changes in FHL124 cell viability/cytotoxicity and apoptosis. The effects were also studied on whole human lens cultures and it was found that in this experimental system H_2O_2 could induce cataract; this decline in visual quality was found to correspond to increased cell death. Thus, there is good correspondence between the different systems used. This model makes assumptions that, for example, responses to DNA damage observed in the cell line are also occurring within the lens epithelium on the whole lens culture. A difficulty encountered was that whilst DNA strand breaks in the lens cell line could be readily detected with the alkaline comet assay, no such method was developed to measure their presence in the native epithelium. Future work could be directed to establishing methods whereby this could be achieved and could involve removal of lens epithelial cells for comet assay analysis. This in itself will provide some difficulties, however, since processing of the tissue could well induce further DNA damage. The dissection of lens epithelium from the whole lens, although a delicate and precise procedure, by its very nature would more than likely stress the epithelium. A procedure to minimise any mechanical or oxidative damage would require significant optimisation and future work could be directed to this.

Cataract is a disease primarily associated with ageing (Wormstone et al., 2006) and indeed ageing itself is considered a risk factor, with incidence of the disease increasing exponentially above 50 years (Duncan et al., 1997). One theory suggests that ageing results from a decline in the ability to cope with DNA damage, which in turn leads to an accumulation of damage over time with the consequence of declining cellular function (Lombard et al., 2005). ROS are considered a significant source of age-related DNA damage (Lombard et al., 2005) and, indeed, GSH is a key antioxidant defence in the lens that becomes diminished with age (Harding, 1970; Lou, 2003). Hawse et al compared gene expression profiles of young and old lenses and those with cataract to clear lenses (Hawse et al., 2004). They found that genes involved in maintenance of lens homeostasis and protection from oxidative stress, such as glutathione peroxidase, are decreased in older lenses. Interestingly, gene expression of proteins involved in DNA double strand break repair were noted to decrease in aged lenses compared to those from younger lenses. Building on these observations, the current body of work investigated links between oxidative stress induced-DNA damage, DNA damage repair and cataract formation.

The present study demonstrated that depletion of Ku80 expression, a core protein in the repair of DNA double strand breaks by non-homologous end joining, rendered lens cells more susceptible to oxidative stress-induced DNA strand breaks. Furthermore, whole human lenses with depleted Ku80 expression were rendered more susceptible to oxidative stress-induced loss of visual quality and, therefore, cataract.

NHEJ is the major pathway involved in the DNA damage response to double strand breaks, and Ku80 one of the pathway's key proteins (Jeggo et al., 2011). Ageing has been linked to a

decline in the ability to repair DNA damage and the level of expression of Ku proteins implicated in the ageing process (Seluanov et al., 2007; Vogel et al., 1999). Studies investigating levels of DNA strand breaks in cataract patients have also found increased levels of DNA strand breaks in cataract patients compared to controls in terms of overall susceptibility of individuals to DNA damage (Ates et al., 2010) and in the lens epithelium (Kleiman and Spector, 1993; Sorte et al., 2011).

DNA damage to the lens epithelium could have a detrimental effect on the transparency of the lens. The bulk of the lens is comprised of fibres, which are a passive mass of cells reliant on the epithelial layer for protection from oxidative damage. The epithelium is the site of antioxidant production, from where they are transported to the fibres (Michael and Bron, 2011). Damage to the epithelium, therefore, may lead to a decrease in the ability to protect the lens from oxidative stress-induced changes and such impairment may result from a DNA damage-induced loss of cellular function or cell death.

PARP-1 is a protein that has been heavily implicated in the DNA damage response (Bouchard et al., 2003; Dantzer et al., 2000; Durkacz et al., 1980). The present study utilised chemical inhibition and targeted siRNA to decrease the level of PARP-1 activity in human lens cells. FHL124 cells demonstrated increased susceptibility to oxidative stress-induced DNA strand breaks with PARP-1 inhibition and depleted expression with siRNA. However, in contrast to these findings, chemical inhibition of PARP-1 protected these cells from oxidative stress-induced cell death and this protection was not a result of direct antioxidant effects. Co-treatment with PARP-1 inhibitor and H_2O_2 offered no protection and thus, pre-incubation was required to produce the protective effects observed. Chemical inhibition of

PARP-1 in whole human lenses was also found to protect from an oxidative stress-induced loss of visual quality and cataract and associated oxidative stress-induced cell death

Throughout the present study it became increasingly clear that PARP-1 possesses many roles in the response to cellular stress. Clearly, PARP-1 is involved in the DNA damage response in human lens cells, but also in cell death following oxidative insult. Future studies should further investigate the role of PARP-1, and other PARP proteins, in cell death pathways within the lens.

Under conditions producing extreme DNA damage, PARP over-activation causes a depletion of NAD+ and ATP resulting in cell death, in a pathway whereby cells with large amounts of DNA damage are removed (Virag et al., 2013). PARP is also implicated in the release of AIF from the mitochondria from where it translocates to the nucleus where it contributes to caspase-independent cell death (Yu et al., 2002). However, the exact mechanism and PARP's involvement is yet to be elucidated and, therefore, future work could aim to clarify this.

PARP-1 has also been shown to control the intracellular localization of key proteins involved in decisions of cell fate, such as p53 (Kanai et al., 2007) and NF-κB (Zerfaoui et al., 2010). Evidently, PARP-1 is an important protein with multiple cellular roles and the current study has determined the effects of inhibiting its function on cataract formation in a human model, but the exact mechanisms by which this is achieved remain unresolved. Certainly, future work should investigate intracellular location of AIF, p53 and NF-κB with immunocytochemistry and western blotting in response to oxidative stress. This could be

performed with or without PARP-1 inhibition and/or siRNA mediated PARP-1 depletion to clarify the involvement of PARP-1 in these processes within the experimental model.

The PARP family contains as many as 20 proteins, each sharing homology to the catalytic domain observed in PARP-1, but much of their function within the human cell remains unknown (Sousa et al., 2012). Future work could certainly investigate roles of these proteins within the human lens cell model developed within the present study. Specific siRNAs could be used to deplete their expression to ascertain their roles within the response to oxidative stress.

PARP-1, like other proteins involved in the DNA damage response, has been implicated in the ageing process. Species with longer lifespans have been shown to display greater poly(ADP-ribose) formation than shorter-lived species (Burkle et al., 2005), suggesting a tentative link between the catalytic function of PARP-1 and the rate of ageing. PARP-1 has also been shown to be implicated in ageing process indicated by the progeroid disease, Werner's syndrome, a disease characterised by premature ageing and caused by a loss of function in the WRN gene (Burkle et al., 2005). WRN is a protein with both helicase and exonuclease activity and is therefore implicated in the DNA damage response (Li et al., 2004). Interestingly, a potential interaction between PARP-1 and WRN was identified (Lebel et al., 2003) and one of the age-related symptoms associated with Werner's syndrome is cataract (Lee et al., 2005).

Future investigations could explore DNA repair protein expression, including that of PARP-1 as a function of age within the lens. Donor lenses could be characterised by age, sectioned and stained with specific antibodies with expression profiles noted. Another advantage of the current model is the broad age range of donor eyes received. This allows a study of age-related changes over a variety of age groups, something that animal models do not easily account for.

The current study has investigated the roles of the NHEJ protein Ku80 and PARP-1 with respect to lens cell responses to oxidative stress-induced DNA damage and cataract. The DNA damage response is comprised of many pathways to respond to differing types of damage, which must act in a collective manner to deal with the daily onslaught of damage to the genome. PARP-1 has been shown to interact with DNA and many proteins involved in the DNA damage response, for example those involved with base excision repair such as APE1, DNA polymerase β, DNA ligase IIIα and FEN1 (Sousa et al., 2012). PARP-1 is also implicated in the repair of DNA double strand breaks by non-homologous end joining, interacting with DNA-PKcs stimulating its activity (Ruscetti et al., 1998) and the alternative end joining pathway, binding to DNA strand breaks in the absence functional NHEJ (Wang et al., 2006). PARP-1 clearly has multiple roles in the DNA damage response and indeed in the cellular stress response overall and the current study has highlighted some of these. Future avenues of investigation could investigate interplay between Ku80 and PARP-1 within the experimental system developed and explore links to ageing and cataract.

In conclusion, the present study has found DNA damage and DNA repair processes to be implicated in the formation of cataract in the experimental systems employed. Surprisingly,

few investigations to date have centred on studying DNA damage as a causative factor in cataract formation considering its role in the ageing process; therefore further investigation is considered both important and exciting.

The eukaryotic cell, which includes cells of the lens epithelium, possesses mechanisms which allow for the sensing of various types of DNA damage. The sensing of DNA damage is followed by the induction of a response that will either result in repair of damage via one or more of the repair mechanisms previously discussed, or, if the damage is particularly severe, will lead to the induction of cell death (Surova and Zhivotovsky, 2013).

DNA damage is recognised by sensors of DNA damage, such as the MRN complex. In Turn the MRN complex recruits and activates kinases of the PIKK (phosphatidylinositol 3-kinase-related kinase) family, such as ATM, which is recruited to DNA double strand breaks, and ATR, which recognises regions of single stranded DNA and stalled replication forks (Ciccia and Elledge, 2010). In response to mild, repairable DNA damage, this leads to the recruitment of proteins involved in DNA repair and, if required, cell cycle arrest, which ultimately, promotes cell survival. However, should DNA damage be particularly severe, there is an induction of cell death that can be initiated via phosphorylation of p53 by ATM and/or ATR promoting p53 stabilisation and thus expression of pro-apoptotic proteins under its transcriptional control, such as p53-upregulated modulator of apoptosis (PUMA), Bax and apoptotic protease activating factor-1 (Apaf-1). This promotes the intrinsic apoptotic pathway and, activates the caspase cascade to ultimately remove the damaged cell (Nowsheen and Yang, 2012; Surova and Zhivotovsky, 2013).

It is proposed that by such a mechanism, cell death in the lens epithelium may occur in response to oxidative stress-induced DNA damage, in the current study induced with H_2O_2. Since lens epithelial cell apoptosis has been implicated in cataract (Li et al., 1995), it is therefore feasible that if sufficient DNA damage in the lens epithelium occurs, this could induce cell death and in turn accelerate cataract formation.

The experimental model developed allowed for the investigation of the roles of Ku80 and PARP-1 within the lens with respect to cataract formation, Many investigators have used animal models to investigate various aspects of lens biology, however, the cell line and whole lens complementary approach adopted here allows for the assessment of the role of DNA repair from the cell to organ, providing an ideal model with a defined clinical outcome, which has relevance to the lens and ageing process as well as that of DNA repair.

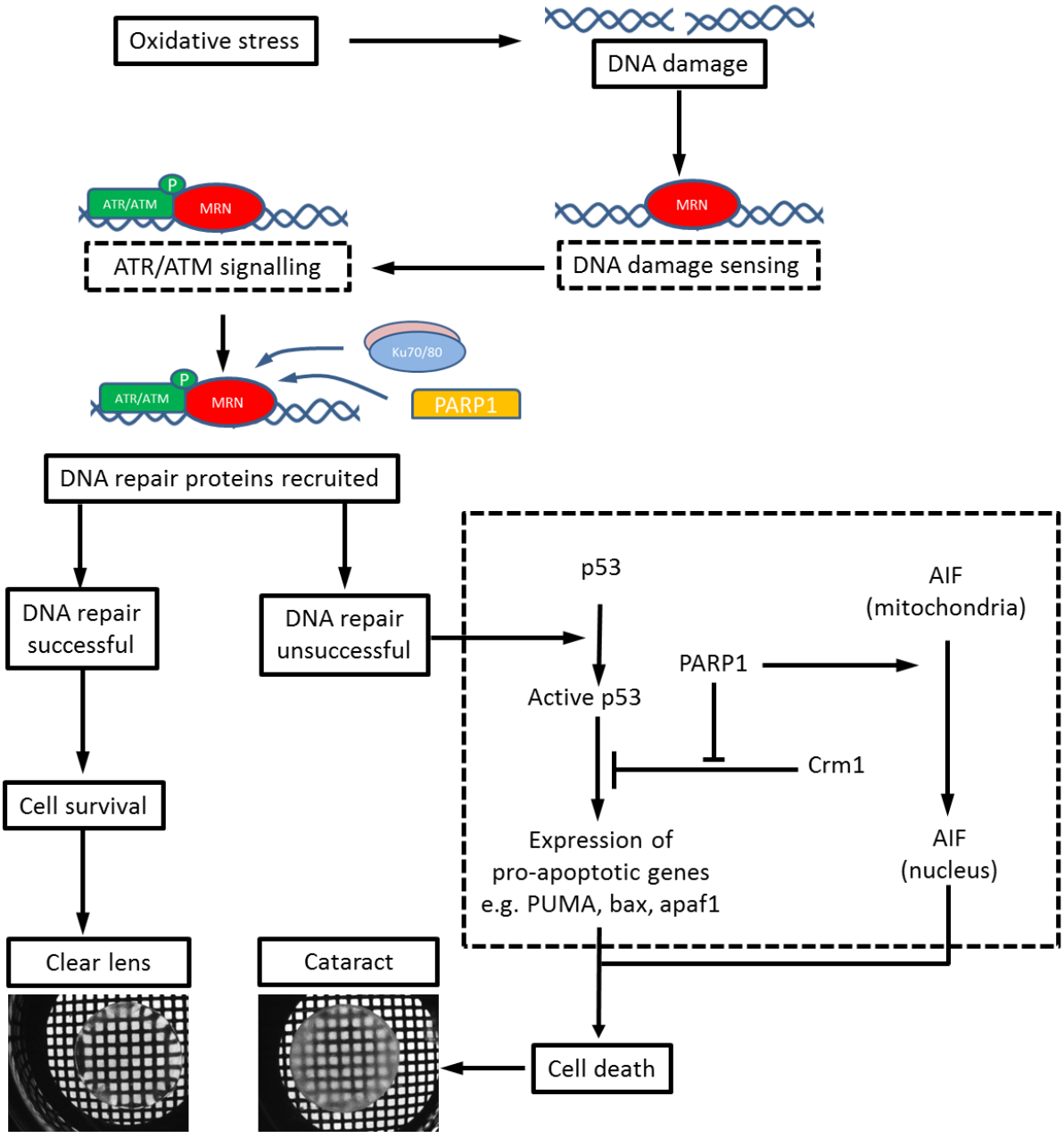


Figure 6.1. A proposed sequence of events following oxidative stress-induced DNA damage in the lens epithelium and its putative role in cataract formation. Oxidative stress, for example induced by H_2O_2, can cause DNA damage, which is identified by DNA damage sensors, such as the MRN complex. This in turn activates signalling proteins such as ATM/ATR that initiate the DNA damage response through recruitment of selected DNA repair proteins such as Ku70/80 and PARP-1. If NHEJ is compromised DNA repair is impaired with the consequence of increased cell death resulting in cataract. PARP-1 also plays a role in DNA repair and cell survival. If DNA damage is severe and repair unsuccessful, cell death pathways are activated, predominately resulting in apoptosis. PARP-1 is also involved in cell death pathways. Information based on the findings of the thesis are presented with a solid line; elements based on the findings of other studies (reviewed by Surova and Zhivotovsky, 2013; Luo and Kraus, 2012) are indicated by dashed lines.

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