The Application of a Statistical Model Investigating Reactive Oxygen Species in Premature Ageing Syndromes

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Abstract

The premature ageing syndromes Hutchison-Gilford Progeria Syndrome (HGPS) and Restrictive Dermopathy (RD) are rare genetic disorders that result in greatly accelerated ageing. RD is neonatal fatal, whereas children suffering from HGPS age approximately 8 times faster than normal individuals and die during their second decade of life -usually from either stroke or heart disease.

The underlying genetic causes of these diseases affect the post-translational processing of the protein lamin A. Lamin A is a Class V intermediate filament protein which resides in the cell nucleus, where it forms a cage-like structure against mechanical strain and is involved in transcriptional regulation, protecting the cell's genome from damage.

HGPS is caused by a mutation in LMNA, the gene encoding lamin A, which results in a truncated mutant protein termed progerin. RD, on the other hand, has genetic roots in the mutation of the metalloprotease Zmpste24, which has a vital role in the processing of prelamin A. The mutation results in the accumulation of immature prelamin A at the nuclear membrane.0

As a result of increased understanding of the defects in HGPS arising from LMNA mutations a number of therapeutic routes are currently being trialled, but with little success. Work in my supervisor's laboratory has suggested that defective forms of lamin A may lead to the production of reactive oxygen species (ROS) in cells. ROS can cause a range of damage in cells including DNA damage. This type of damage eventually leads to cellular senescence which is thought to be a precursor to normal ageing.

The results put forth here suggest that primary dermal fibroblasts from these premature ageing syndromes show defects in both DNA damage repair and ROS buffering ability. This is shown from my statistical modelling of immunofluorescence data showing DNA double strand break repair over time. RD and HGPS fibroblasts showed persistent γ-H2AX foci after DNA damage induction with oxidative stress, yet showed improved repair after induction with a direct DNA break inducing agent, etoposide.

Proliferative markers indicated a reduced proliferative capacity in premature ageing fibroblasts, which was heightened after treatment with oxidative stress. Taken together these results suggest that RD and HGPS fibroblasts more readily enter a state of cellular senescence when exposed to oxidising conditions than control fibroblasts from both and young and old donors.

Phenotypical aberrant cellular morphology associated with these diseases was shown to be independent of the defect in ROS buffering ability, supporting evidence showing improvements in abnormal nuclear shape by treatment with farnesyltransferase inhibitors (FTIs) have little impact on the DNA damage repair defects associated with these diseases.

From the results here, N-acetyl cysteine (NAC), a powerful antioxidant, has shown to be effective in reducing the proportion of irreparable DNA damage in HGPS and RD fibroblasts. It has also been shown to increase repair rates by actively reducing ROS levels as detected by flow cytometry. NAC treatment therefore shows beneficial properties in treating certain phenotypes associated with progeria and may require consideration as a cooperative form of treatment.

The implications of these results are that HGPS is a useful model for normal ageing as fibroblasts from an elderly donor showed signs not dissimilar to those of HGPS, albeit in milder form. These included an increase in the number of persistent γ-H2AX foci and increased ROS levels. This suggests that activation of the HGPS cryptic splice site in normal ageing may account for increased age-associated genomic instability Thus it is tempting to speculate that lamin A is a modulator of both premature and normal ageing through its regulation of cellular oxidative damage.

Acknowledgments

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The Nuclear Envelope and the Nuclear Lamina

1.1.1 The Nuclear Envelope

The nuclear envelope (NE) is a double phospholipid bilayer encapsulating the nucleus in eukaryotic cells. It serves as a physical barrier, separating chromosomes from the cytoplasm, and transcription from translation, allowing complexity and regulation within the cell (Pennisi, 2004). This specialised structure is composed of two lipid membranes, the outer nuclear membrane (ONM), which is continuous with the endoplasmic reticulum (ER), and the inner nuclear membrane (INM) which associates with the nuclear chromatin and the nuclear lamina (Chi, Chen and Jeang, 2009).

The two membranes are separated by the perinuclear space (30-50nm), but are also fused together at multiple points at the nuclear pore complexes (NPC), which regulate transport and communication across the nuclear envelope (Capelson, Hetzer, 2009).

One major role of the nuclear envelope is to act as a scaffold for internal nuclear structures; this is achieved by the proteins associated with the NE. As the ONM is continuous with the rough ER, they share a similar protein composition, whilst the INM has a more unique protein content, responsible for a wide variety of cellular functions, including cell signalling, gene regulation and enzymatic activity (Ye and Worman, 1996; Markiewicz et al., 2006).

A recent convergence of clinical observations and proteomic research has highlighted the association between many human diseases and NE protein defects. It revealed over 60 integral membrane proteins, most of which reside within the INM. Strikingly this revealed significant distinctions of NE composition between different tissue types, helping to explain tissue specific diseases arising from mutations in NE proteins (Schirmer et al., 2003).

Once thought of as a merely passive structure, evidence now shows that minor changes in NE organisation results in altered cellular mechanics; the NE acts as a dynamic connection between the nucleus and cytoskeleton to integrate both the nuclear and cytoplasmic architecture (Crisp and Burke, 2008).

1.1.2 The Nuclear Lamina

Underlying the NE is the nuclear lamina. This is a thin (20-50nm), protein network than lines the INM, composed of type V intermediate filaments, classified as either A- or B-type lamins and associated proteins. It is thought to act as a tensegrity element; a lightweight but elastic structure, providing structural integrity to the NE (Burke and Stewart, 2002; Hutchison and Worman, 2004) (Figure).The increasing number of interactions between the nuclear lamina and other nuclear and cellular structures, alongside the vast number of diseases caused by lamin mutations, makes the nuclear lamina a highly complex structure, involved in a vast array of nuclear activities including DNA replication, RNA transcription, nuclear and chromatin organisation, cell differentiation and apoptosis (Gruenbaum et al., 2003).

1.1.3 Lamin Proteins and Filament Assembly

Lamins are part of the intermediate filament (IF) supergene family and central components of the nuclear lamina (Hutchison and Worman, 2004). As IFs they share a common tripartite domain structure, with common globular N- and C-terminal domains organised around a central α-helical rod domain, split into four α-helical segments (1A, 1B, 2A and 2B) which are separated by flexible, hinge-like linker regions. Uncommon to IFs, they contain a nuclear localisation sequence (NLS) in the tail domain, which interacts with importins allowing transport across the NPC, permitting correct localisation at the INM (Loewinger and McKeon, 1988).

Crucially lamins (apart from lamin C) are the only IF to contain a C-terminal CaaX motif, the critical site for multiple post-translational modifications (Broers et al., 2004). Additionally they contain a characteristic additional 42 amino acids (six heptad repeats) within coil 1B (Hutchison, Alvarez-Reyes and Vaughan, 2000; Hutchison and Worman, 2004). (Figure 1)

Lamins are classified into two categories, A and B-type lamins, dependent upon their structure, behaviour during mitosis and their tissue expression patterns and specificity (Broers et al., 1997; Hutchison, Alvarez-Reyes and Vaughan, 2000; Broers et al., 2006).

B-type lamins are constitutively expressed in all cell types, both embryonic and somatic, and are required for cell survival, nuclear integrity and normal development (Broers et al., 2006). There are two types of B-type lamins, and although they are very similar in amino acid sequence, they are the product of two separate genes. B type lamins, B1 and B2, are expressed from the LMNB1

and LMNB2 genes on 5q23 and 19q13 respectively (OMIM 150340; OMIM 150341). A further B-type lamin has also been identified, lamin B3, a male germline-specific LMNB2 splice variant, important for spermatogenesis (Furukawa and Hotta, 1993).

Conversely, A-type lamins are all alternative splice variants of a single gene, LMNA, a 12 exon gene on 1q21.1-21.3. Four A-type lamins have been identified (A, AΔ10, C and C2), however lamin A and lamin C make up the major products of LMNA. Lamin AΔ10 is expressed primarily in cancer cell lines (Broers et al., 1997), as well as several normal cell types, whilst the other minor splice variant C2 is expressed only in male gametes (Furukawa et al., 1994). Lamins A and C are identical except for their C-terminal, with lamin C containing five unique amino acids, and lamin A containing an additional 98 amino acids (Burke and Stewart, 2006).

In contrast to B type lamins, A-type lamins are expressed only in differentiated cells, regulated by developmental control (Nigg, 1989).

Lamins A and C exist in ratios, which are variable depending on the tissue type, whilst overall levels of A type lamins are dependent upon the differentiation status of the tissue (Worman et al., 1988; Broers et al., 1997; Lattanzi et al., 2003). Due to their differentiated state dependent expression it has been hypothesized that A type lamins may hold a role in the maintenance of tissues in a differentiated state (Constatinescu et al., 2006).

It has been reported that A-type lamins are dispensable for embryonic development, as LMNA-null mice develop normally, but do not survive for more than 8 weeks and exhibit growth retardation, muscular dystrophy and cardiomyopathy phenotypes (Sullivan et al., 1999). This shows that A-type lamins are not required for early embryonic development yet are essential for postnatal survival (Broers, ref 360. In humans, on the contrary, haploinsufficiency of LMNA results in either *in utero* death, or death very shortly after birth (Bonne et al., 1999).

Lamins must form polymers to assemble into a network of filaments that act to line the INM. Through various studies it has been elucidated that lamins form lateral interactions between two lamin chains to create parallel unstaggered homodimers of 50nm, via coiled-coil association of the α-helical domains (Hutchison, Alvarez-Reyes and Vaughan, 2000). These lamin dimers assemble into head-to tail polymers, via associations between the N and C-terminus. These 10nnm filaments can then form a higher order network by associating laterally, creating the nuclear lamina (Herrmann, Kreplak and Aebi, 2004, Hutchison and Worman, 2004, Goldman et al., 2005)

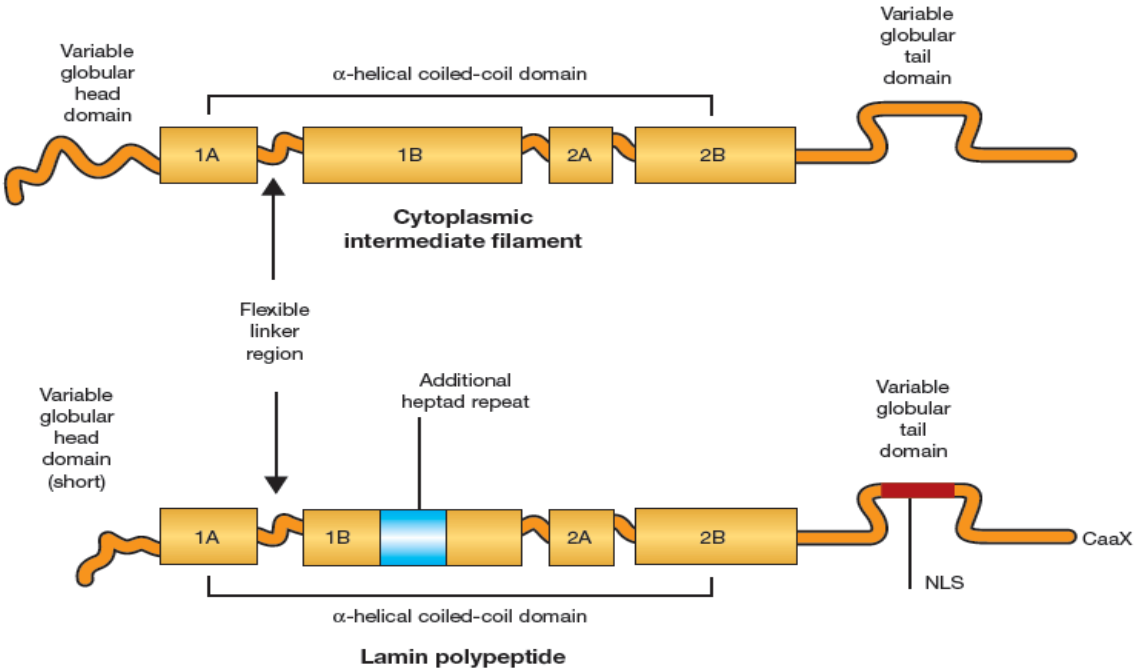


Figure 1: Structure of lamin peptides, indicating the additional heptad repeat in α-helical coil 1B from cytoplasmic Ifs. (Taken from Hutchison and Worman, 2004)

1.1.4 Post-translational regulation of lamin proteins

Studies have shown that for correct localisation of lamins, post-translational modifications of the C-terminal CaaX motif are essential (Holtz et al., 1989; Hutchison, Alvarez-Reyes and Vaughan, 2000). The CaaX (C, cysteine; a, any aliphatic amino acid; X, any amino acid) motif is site for these post-translational modifications.

Lamins are synthesised as precursor molecules before they are inserted into the nuclear lamina. The first stage of the processing is the farnesylation of the thiol group of the cysteine residue located in the CaaX motif. This involves the addition of a 15-carbon farnesyl isoprenoid via a thioether bond by protein farnesyl transferase (FTase) and is thought to occur at discrete sites within the nucleoplasm (Moir et al., 1995; Broers et al., 2006). Farnesylation is thought to be crucial for the assembly of lamin A into the nuclear lamina and assists in the correct targeting of the lamins to the NE (Holtz et al., 1989). This has been demonstrated by the use of a farnesyl transferase inhibitor, preventing the addition of a farnesyl moiety to the cysteine residue. When this tactic is employed, nonfarnesylated prelamin A accumulates in nucleoplasmic aggregates (Lutz et al., 1992).

After this farnesylation step, the final three amino acids (aaX) are cleaved by either one of two proteins, Zmpste24 (alternatively called FACE1), a zinc metalloproteinase, or Rce (Ras-converting enzyme 1) (Corrigan et al., 2005).

Following this first cleavage event, the cysteine residue is further modified by methylation by prenyl cysteine carboxyl methyltransferase (pcCMT), which is able to localise to the nuclear membrane (Sinensky, 2000). This additional hydrophobic chain aids localisation to the INM (Hennekes and Nigg, 1994). Once at the nuclear membrane, the differences between lamin A and B-type lamins diverge. B-type lamins require no further processing and remain isoprenylated, allowing them to be tightly associated with the INM. In A type lamins, however, the 15 terminal amino acids are cleaved, including the farnesylated, carboxymethylated cysteine by the endonuclease Zmpste24 to produce mature lamin A (Bergo et al., 2002)(Figure 2). In contrast, lamin C (an A-type lamin isoform) lacks the CaaX motif and it is thought is inserted into the nuclear lamina through associations with prelamin A (Hutchison, Alvarez-Reyes and Vaughan 2001).

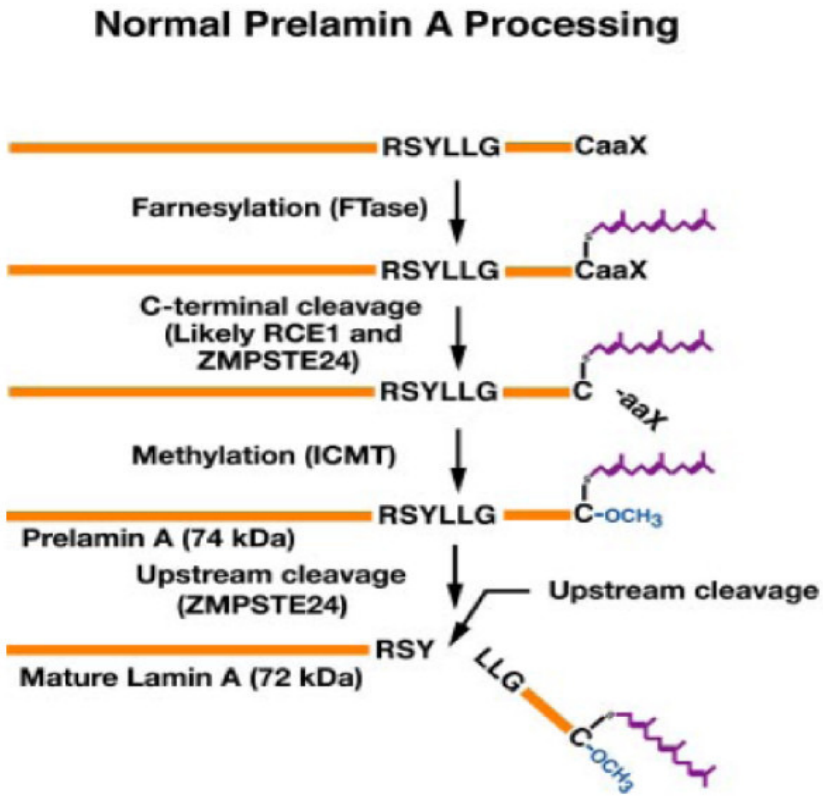


Figure 2: Post-translational modification of wild-type prelamin A. See text for details. (Adapted from *Coutinho* et al., 2008)

1.1.5 Roles of the Nuclear Lamina

The roles of the nuclear lamins are largely based on their various interactions in the nucleus and can be classified into broadly three categories. Although these will be discussed sequentially, it is important to note that these functions are not mutually exclusive as the nuclear lamina is a dynamic and complex structure (Zastrow et al., 2004).

Tensegrity Element

Several lines of evidence have suggested that the lamina acts as a 'tensegrity element' by which it acts to resist deformation and protect the genetic material inside the nucleus, and thus acts to preserve the structural integrity of the nucleus (Hutchison and Worman, 2004). The contributions of lamins A, C and B1 to nuclear morphology have been analysed. Lmna-/- mice show gross defects in nuclear shape, structure and stability, whilst transgenic mice overexpressing lamin C but not lamin A show mild alterations in nuclear structure and mechanics. Fibroblasts from a genetically engineered mouse with a severely truncated lamin B1 also show severe nuclear herniations (Dahl et al., 2008). It is thought that lamin A and C are the main contributors to nuclear stiffness, whilst lamin B1 may be more involved in mechanics (Lammerding et al., 2004; Lammerding et al., 2005). Nuclei from lamin-depleted *Xenopus* egg extracts are also highly fragile and vulnerable to stress (Newport, Wilson and Dunphy, 1990). Nuclear structural abnormalities are also prominent in cells containing transiently expressed disease causing lamin A/C mutations (Eriksson et al., 2003; Navarro et al., 2004).

A major structural role can also be attributed to lamins, through their formation of a link between the lamina and the cytoskeleton via the LINC complex of Sun proteins and nesprins (Zhen et al., 2002; Crisp et al., 2006; Haque et al., 2006; Padmakumar et al., 2005). Nesprins, located at the ONM are a family of cytoskeletal linker proteins and have been shown to provide a link between lamin A (and emerin) and the actin cytoskeleton (Crisp et al., 2006). Nesprins 1 and 2 act as an actin bundling proteins (Broers, ref 292), whilst nesprin-3 binds to the cytolinker protein plectin, which in turn interacts with actin (Wilhelmsen et al., 2005). The INM protein SUN1 has also been shown to interact with both nuclear lamin A and nesprin proteins and provides a mechanical link between the nuclear lamina and the cytoskeleton (Haque et al., 2006). Dynamic interactions between the cytoskeleton and lamina act to preserve structural and mechanical integrity throughout the cell, as well as supporting a signalling capability. Mutations affecting lamina proteins have been shown to impair these interactions as demonstrated by the

detachment of desmin filaments from the nuclear surface in LMNA knockout cardiomyocytes (Nikolova et al., 2004).

Lamins have also been implicated in nuclear size. Depletion of lamins in cell-free extracts of *Xenopus* oocytes leads to the assembly of very small nuclei (Meier et al., 1991), whereas overexpression of CaaX-motif containing lamins results in increased membrane growth and larger nuclei (Ralle et al., 2004).

As such, lamins are able to determine, size, shape and strength of the NE and therefore possess all the roles required to be described as a tensegrity element (Hutchison and Worman, 2004).

Chromatin Scaffold

Evidence exists to show that the nuclear lamina may act as a chromatin scaffold, providing binding sites for the attachment of inner nuclear membrane proteins and chromatin. Lamins have the ability to bind DNA, chromatin and histones via the lamin A/C C-terminal domain (Luderus et al., 1992; Luderus et al., 1994) and thus provide a scaffold for chromosomal architecture.

BAF (barrier-to-autointegration factor) is a highly conserved chromatin protein that is able to bind double stranded DNA without any sequence preference, and its presence is essential for cell survival (Lee and Craigie, 1998; Zheng et al., 2000). LEM (LAP2α, emerin, MAN-1) domain proteins are able to bind to both BAF and lamin A (Furukawa 1999; Shumaker et al., 2001), mediating a chromatin association, whereby lamin A may act as a scaffold for chromatin binding factors which are critical in DNA compaction, chromatin decondensation and post-mitotic nuclear assembly (Vleck and Foisner, 2007). A loss of INM proteins such as MAN1 and emerin, resulting in the loss of BAF interactions, leads to aberrant chromatin structure, nuclear morphology and defects in cell division (Liu et al., 2003).

Internal nucleoplasmic sites of A-type lamins may also provide a platform for transcriptional machinery and gene interactions required to engage gene expression. This idea may gain support as it is interesting to note that disruption of nuclear lamins is accompanied by a dramatic reduction in the transcriptional activity of RNA polymerase II (Spann et al., 2002).

Transcriptional Regulators

Other transcriptional regulators have been shown to interact with lamins. One of the most vital transcriptional regulators, the retinoblastoma (Rb) protein is known to interact with lamin A

(Ozaki et al., 1994). Hypophosphorylated pRb forms a complex with the E2F family of transcription factors and prevents E2F from activating cell cycle inhibitor genes, resulting in continued cell proliferation. Whereas when pRb is hyperphosphorylated, it is unable to bind E2F. Cell cycle inhibiting genes are therefore induced, resulting in exit from the cell cycle. A-type amins and LAP2α are able to bind to hypophosphorylated Rb and tether it to the nucleus, ensuring E2F repression by a range of mechanisms including recruitment of the SWI/SNF chromatin remodelling complex and histone and DNA methyltransferases (Markiewicz et al., 2002; Dorner et al., 2006).

Prelamin A has been found to be a binding partner for the adipocyte transcription factor sterol regulatory element binding protein1 (SREBP1), which acts to regulate genes involved in adipocyte differentiation including PPARγ (Lloyd et al., 2002).

Other transcription factors linked to lamins involve MOK2, a DNA binding transcriptional repressor which has been shown to bind to lamins A/C, (Harper et al., 2009), Oct-1, shown to bind to lamin B1 acts to repress the collagenase gene associated with cellular ageing. (Imai et al., 1997; Zastrow et al., 2004) and c-Fos and lamin A/C interactions suppress AP-1 transcription factor activity. (Ivorra et al., 2006).

Due to mutations in LMNA causing a diverse range of diseases, lamin A must therefore interact with multiple chromatin organisation proteins, transcription factors and transcriptional regulators, to be able to cause such a wide spectrum of syndromes.

1.1.6 Proteins that Interact with Lamins

A large number of NE proteins have been shown to interact with lamin proteins. The vast majority of these are INM proteins and bind to lamins with high affinity. A few ONM proteins also bind to lamins and play important roles. All these are shown in the table below. (Figure 3)

	Protein	Isoforms	m.w. (kDa)	Motifs	Interactions	Functions	Identifier
Outer nuclear membrane proteins with interactions with lamins	Nesprin 1	B myne-1 α	380 131 112	Actin-binding domain (1β) KASH domain, bipartite NLS, spectrin repeats	Actin Nesprin 1α interacts with lamin A/C and emerin	Muscle specific anchoring of sunsynaptic nuclei, nuclear differentiation, organisation of nucleoskeleton	Uni:Hs.19102
	Nesprin 2	NUANCE γ β β2 α α2 NUANCE-N33	796 377 87 76 62 48 30	Actin binding domain, KASH-domain, bipartite NLS, leucine zipper, spectrin repeats	Actin, SUN proteins	Nuclear anchoring, nuclear migrartion	PID:29839588 Uni:Hs.57749
	Nesprin 3		110	KASH-domain, spectrin repeats	Plectrin, SUN proteins	Nuclear anchoring	Uni:Hs161176
Inner Nuclear Membrane Proteins with interactions with lamins	Emerin	-	29	LEM	BAF, lamin A	Scaffold protein, involved in chromatin structure and gene transcription. Linked to Emery-Dreifuss muscular dystrophy	Uni:Hs.2985
	Lamin B receptor (LBR)	-	71	RS-domain TUDOR domain	HP1, histone H3, lamin B, LBR-kinases, p34/32, heterochromatin	Heterochromatin anchoring, targeting membranes to the NE, linked to Pelger-Huet anomaly	Uni: Hs.152931
	LAP-1	A B C	75 68 57		Lamins A/C, lamin B		Uni:Rn.11373
	LAP-2	A β γ δ ε ζ	 50 38 43 46	β, γ, δ, and ε long N-terminal nucleoplasmic domain, single TM domain, α and ζ no TM domain. All have LEM domain. LEM-like domain	BAF, DNA, GCL, lamin B, lamin A/C, HA95	Regulation of gene expression, nucear architecture, both structural and functional	Uni:Mm.124
	MAN-1	-	100	LEM domain	BAF, DNA, lamins, SMADS	TGFβ/BMP signalling,	Uni:Hs.7256
	SUN-1	-	92	SUN domain	Prelamin A, telomeres, nesprin 2	Nuclear anchoring, nuclear migration, NPC distribution	PID:3882341
	SUN-2	-	83	SUN domain	Telomeres, nesprin 2	Nuclear anchoring, nuclear migration	PID:3327150

Table 3: Table showing proteins of the NE known to interact with lamins. (Adapted from Evans, Hutchison and Bryant, 2004

1.1.7 Nuclear Envelope, Nuclear Lamina and the Cell Cycle

As lamins perform the wide variety of functions described above, it is believed that they are dynamic structures, as opposed to part of a rigid and inert structure. The most striking of these dynamics occurs during mitosis. At the switch from prophase to prometaphase both the nuclear membrane and the nuclear lamina disassemble. Microtubules bind to fragments of the nuclear membrane via dynein, disrupting the nuclear envelope. This permits access to the nucleus for kinases to phosphorylate the nuclear lamins. Lamin proteins are phosphorylated by $p32^{cdc}$ kinase at domains either side of the rod domain (Peter et al., 1990), and as a result of this phosphorylation event depolymerise from higher order lamin polymers to form smaller monomers and dimmers and become distributed throughout the cytoplasm (Moir et al., 2000). Disassembly of the nuclear lamina is a gradual process with timings differing between the lamin isotypes. Whilst lamin A/C are quickly disassembled during early prophase, lamin B remains polymerised at the nuclear periphery until prometaphase (Georgatos et al., 1997), thus providing a rate-limiting step in nuclear disassembly. Other factors including expression of LAPs and other lamin associated proteins may also affect the rate of depolymerisation. During the process of disassembly, chromosomes remains attached, implying a role for the lamin in chromosomal organisation during mitosis (Moir et al., 2000).

It is not until the full completion of cytokinesis that A-type lamins reassemble. GFP tagged lamin A imaging shows A-type lamins surrounding the chromatin very rapidly at the end of cytokinesis where the cytoplasm is distributed between the two daughter cells (Broers et al., 2005). LAP2α, BAF, emerin and A-type lamins have been shown to localise to 'core' regions of chromatin during telophase.

In consideration of B type lamins, recent GFP imaging studies have shown that lamin B1 begin to associate with membrane particles (Broers et al., 2005). It can then associate with peripheral regions of chromosomes at mid-late-telophase along with INM proteins LBR and LAP2β.. Some researchers suggest that lamin B1 is required for chromosome decondensation, whilst others propose that lamin reassembly occurs after cytokinesis (Daigle et al., 2001) and is not required for the early reassemble of the nuclear envelope. It is assumed that although there is some association of nuclear lamins to chromatin during the early stages of reassembly, the majority of lamins are reassembled to form a functional nuclear lamina are imported across the nucleus after NE assembly (Guttinger, Laurell and Kutay, 2009).

1.2 Lamina Proteins and Disease

1.2.1 Laminopathies

Nuclear envelopathies are a group of diseases associated with mutations encoding nuclear envelope proteins. Laminopathies, fall into this category of diseases, and are caused by mutations in either the lamin A/C gene (LMNA) known as primary laminopathies or mutations in the FACE-1 gene which affect the processing of prelamin A (Broers et al., 2006). LMNA gives rise to over twenty different genetic disorders caused by over two hundred different mutations in the LMNA gene.

Laminopathies affect tissues that are mesenchymal in origin, however the diseases can be categorised into four subclasses depending on the type of tissue they affect. Group one affects striated muscle, group two affects the peripheral nerves, group three affects adipose tissue and those in group four are classified as “premature ageing syndromes” or “systemic laminopathies” (Broers et al., 2006)

What is not understood fully is the relationship between genotype and phenotype and how mutations in *LMNA* are able to cause such diverse and yet tissue restricted diseases. This results in one of the most intriguing mysteries in medical genetics. The correlation between the location of the mutation and the laminopathy is not fully understood, with many disease causing mutations spread throughout the whole gene.

1.2.2 Hutchison Guilford Progeria Syndrome

One of the most devastating laminopathies is Hutchison Guilford Progeria Syndrome (HGPS). It is an extremely rare disorder affecting approximately one in eight million births. It was first described in 1886 by Jonathan Hutchinson and also described independently in 1897 by Hastings Gilford and thus named Hutchison Guilford Progeria Syndrome.

The genetic disease is characterised by symptoms recalling normal aging, but at a much accelerated rate. It is thought that these individuals age approximately eight times faster than normal individuals and have a mean lifespan of thirteen years.

The premature aging observed is described as segmental, defined with respect to normal ageing as only certain phenotypes are manifested, whilst others are noticed by their absence such as cataracts and the incidence of cancer (Cohen et al., 2008; Ramirez et al., 2007) Children suffering from this disorder generally have no visible signs of such a devastating disorder at birth

and birth weight is usually normal. However, during the first year of life failure to thrive is observed as the child generally fails to put on much weight. The average age of diagnosis is 2.9 years (Pereira et al., 2008) and is recognised by a low weight, sclerodermatous skin, lipodistrophy and the appearance of characteristic facial dysmorphia (Mazereeuw-Hautier, Wilson et al., 2007). Throughout the lifespan symptoms include facial abnormalities including micrognathia (small jaw), prominent scalp veins, alopecia, prominent eyes and frontal bossing. Skin is thin, atrophic and scaly with hyperpigmented lesions and scars. (Jimbow et al., 1988) Bone abnormalities are also present with clavicular hypoplasia and generalised osteopenia. (Pereira et al., 2008)

Despite these physical abnormalities, mental development progresses as normal. Strokes are frequent, however the most common cause of death is myocardial infarction as the left ventricle is larger and heart valves are frequently calcified.

In 2003, a surprising breakthrough was made when the discovery of the underlying genetic mutation was identified and was achieved by a genome wide scan of several patients. This resulted in the identification of a 6-megabase paternal interstitial deletion in the 1q21 region, allowing directed gene sequencing. (Eriksson et al., 2003), which then permitted the identification of a mutation in LMNA, the gene encoding for lamin A/C.

Classical HGPS is a mutation in LMNA exon 11, caused by a heterozygous substitution (c.1842C>T; pGly608Gly). Yet, whilst this is a silent mutation on the level of amino acids, in reality is causes an aberrant splicing event resulting in a heavily truncated protein. The classical form of HGPS is due to a dominant de novo germline mutation, which, more often than not occurs in the paternal allele and has been shown to be linked to paternal age. (D'Apice et al., 2004)

Other mutations have also been shown to cause HGPS, but these differ from the classical form in both symptoms and severity (Scaffidi and Misteli, 2006). Other HGPS mutations to be documented include E145K, R471C, R527C, G608S, T623S and 1824C>T (Erriksson et al., 2003; Csoka et al., 2004; Cao and Hegele, 2003; Fukuchi et al., 2004)

The c.1842C>T classical mutation activates a cryptic splice site in exon 11 of the gene and results in the expression of a truncated pre-lamin A protein with a deletion of an internal (pVal607-Gln656del) 50 amino acids from the C-terminus. (Eriksson et al., 2003) This abberantly truncated protein, named Progerin, retains the CAAX farnesylation motif, but crucially lacks the second endoproteolytic cleavage site RSY↓LLG, required for Zmpste24 dependent processing. This results in a mutant protein termed progerin which remains permanently farnesylated and bound to the inner nuclear membrane. (Figure 4)

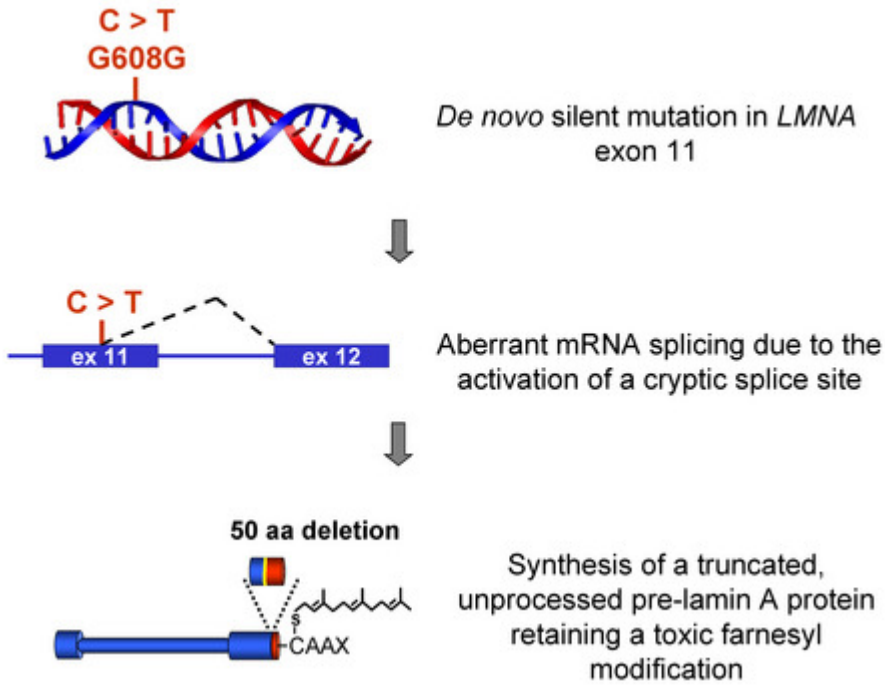


Figure 4: Schematic diagram showing aberrant processing steps found in classical HGPS. The active splice site results in the removal of the second endoproteolytic cleavage site, resulting in a permanently farnesylated form of pre-lamin A termed progerin. (Adapted from Scafidi, Gordon and Mistelli, 2005)

HGPS is characterised by the accumulation of progerin with age, as observed by a serial passage assay where patient cell lines are grown in culture. The cell line showed an increase in the progerin levels with increasing population doublings (McClintock et al., 2006).

Progerin levels make up approximately 40% of total lamin A transcripts, suggesting the use of the cryptic splice site is not exclusive, but also demonstrates that the progerin mutant protein has a dominant negative effect over normal lamin A transcripts (Reddel and Weiss, 2004). This is supported by evidence which shows that deletion of Lmna in a mouse model results in a muscular dystrophy, whereas introduction of progerin results in a progeria like phenotype (Sullivan et al., 1999; Varga et al., 2006).

1.2.2.1 HGPS as a Model for Ageing

There are obvious similarities between normal ageing and HGPS, however particular aspects of ageing are noticeably absent from HGPS patients. It is therefore essential to investigate and understand how such a syndrome relates to normal ageing.

Several recent studies have demonstrated that Lamin A is involved in normal physiological ageing (Scaffidi and Mistelli, 2006; McClinktock et al., 2007). There is now evidence that progerin is produced in healthy individuals without mutations in LMNA by occasional use of the cryptic splice site in exon 11. Fibroblasts were taken from elderly individuals and were shown to exhibit nuclear phenotypes similar to laminopathic fibroblasts such as herniations and after several population doublings in culture were shown to be progerin positive. (McClintock et al., 2007)

These accumulated abnormalities associated with progerin expression with age may account for part of the ageing process, hence cells expressing higher levels of progerin or other lamin A mutant proteins will age prematurely in addition to abnormal nuclear morphology (Huang et al., 2005).

Similarities are shown to exist between cells from HGPS patients and cells from aged donors, including nuclear deformations, re-localisation of heterochromatin as well as defective DNA damage responses. (Scaffidi and Mistelli, 2006)

The presence of progerin positive cells were also investigated in skin biopsies to determine if progerin was present *in vivo*. It was noted that progerin accumulates with age in terminally differentiated keratinocytes towards the epidermis and showed nuclear rim staining consistent with expression in HGPS keratinocytes (McClinktock et al., 2007).

The use of HGPS as a model for ageing is further supported by a study showing that the cellular abnormalities associated with ageing can be reversed by the introduction of morpholinos designed to reduce progerin levels (Scaffidi and Mistelli, 2006). This vital discovery indicates that some of the nuclear changes associated with ageing require a certain level of progerin to be present, which is obviously much higher in progeria (Kudlow and Kennedy, 2006).

A slight increase in the expression of wild-type prelamin in healthy fibroblasts has shown to be sufficient to induce phenotypes that are seen in cells from progeric patients, including changes in nuclear morphology, growth rate reductions and ultimately premature cellular senescence or cell death (Candelario et al., 2008). These effects can be ameliorated by overexpression of ZMPSTE24, suggesting that the defective processing of wild-type prelamin A is responsible for these changes. This in turn could plausibly lead to the implication that a decrease in the activity of ZMPSTE24 in ageing individuals could at least in part contribute to the normal ageing process (Li, Jog and Comai, 2009).

It has also been reported that a decrease in the levels of normal lamin A/C proteins may be associated with normal ageing, with reduced expression in ageing bone marrow and osteoblasts (Duque and Rivas, 2006). This suggests that the loss of normal lamin A/C function and increased

levels of mutant progerin may cause nuclear and cellular fragility which may ultimately lead to cellular senescence and contribute to ageing.

1.2.2.2 Cellular Defects

To gain a greater understanding of the pathogenesis of laminopathies, researchers began looking at the cells of affected patients. The most obvious and striking abnormality is misshapen nuclei, otherwise known as herniations or "blebs", suggesting that the presence of an altered lamin A protein has a drastic effect on cellular morphology. (Worman et al., 2009)

The presence of herniations within the nuclear envelope has often been used as a measure of severity of the disease, and the success of putative therapies.

Other observed cellular defects include "honeycombing" of the nuclear lamina, increased nuclear surface area, loss of peripheral heterochromatin, abnormal clustering of nuclear pore complexes, and atypical intranuclear foci of lamins. (Worman et al., 2009)

Previously, it has been suggested that muscle and heart defects associated with mutations within LMNA could be caused by motion-related fragility of the nuclear envelope. Lammerding et al., (2004) tested this assumption by subjecting nuclei from Lmna-/- and Lmna+/+ control fibroblast to biaxial strain. This experiment demonstrated that the nuclear envelope of Lmna-/- fibroblasts was explicitly more mechanically fragile than control counterparts, with some A-type lamin mutants more susceptible to heat shock.

Fibroblasts from progeria patients typically are characterised by a short period of hyperproliferation and then consequently a large increase in both apoptosis and senescence, contributing to a much decreased growth rate over increasing passage (Bridger and Kill, 2004). In addition there is also a decrease in cell viability as a result of both increased necrotic and apoptotic cells, alongside a decrease in the expression levels of mechanosensitive genes in response to mechanical strain. (Lammerding et al., 2004).

Key questions to raise therefore, are how and why do the misshapen nuclei occur, and how do the misshapen nuclei correlate with altered gene expression?

An interesting finding has demonstrated that emerin, a nuclear envelope binding partner of lamin A/C is also mislocalised in LMNA null fibroblasts, thus indicating that lamin A/C is required for its correct targeting to the nuclear envelope (Sullivan et al., 1999), which may further impact on nuclear structure.

Navarro et al., (2004) has suggested that aberrations in nuclear morphology could be correlated with the quantity of Prelamin A accumulation, which in turn could correlate with the severity of

the symptoms observed in different cases. However, the presence of nuclear abnormalities are unclear in relation to the pathogenesis of the disease; only a subset of fibroblasts from patients exhibit misshapen and abnormal nuclei with cell culture conditions and passage numbers greatly affecting the proportion of nuclear abnormalities.

1.2.2.3 Genomic Instability

Loss of peripheral heterochromatin

As well as a clearly defined structural role in the nuclear lamina, lamins are also found throughout the nuclear interior, where it is thought they play a major role in genome organisation and DNA replication (Stuurman et al., 1998; Bridger et al., 2007).

Cells from HGPS patients and mouse models of the syndrome have shown alterations in epigenetic control. It has been proposed that the nuclear lamina acts as a molecular scaffold for chromatin, and alterations within the nuclear lamina as a result of mutations lead to increased genomic instability (Shumaker et al., 2006; Bridger et al., 2007). One of the most evident losses is that of peripheral heterochromatin (Goldman et al., 2004). Heterochromatin has recently been re-defined in the context of epigenetics, as inactive or silenced chromatin based upon histone and DNA modifications (Schirmer, 2008). The accumulation of progerin causes a reduction of the levels of heterochromatin markers including H3K27me3 (marker of facultative heterochromatin), HP1α and H3K9me3, as well as heterochromatin associated with Xi Barr body in a female patient's fibroblasts as the cells progressed through passage (Shumaker et al., 2006). The methyltransferase (EzH2) responsible for the methylation patterns of the histones was markedly down-regulated in HGPS cells at both the protein and the mRNA transcript level, indicating major alterations in the peripheral heterochromatin (Li, Jong and Comai, 2009).

This loss of epigenetic control through the inactivation of heterochromatin results in transcriptional activation in regions of DNA that are usually transcriptional silenced. A significant increase in the levels of usually inactive pericentric Satellite III DNA repeats were detected in HGPS fibroblasts (Shumaker et al., 2006). The presence of progerin is sufficient to alter epigenetic control and chromatin organisation in early passages, even before the observable changes in nuclear shape associated with the cumulative accumulation of progerin in later passages. These changes in epigenetic control subsequently lead to massive changes in gene expression patterns, contributing to the observed phenotypes.

Interestingly, the changes in heterochromatin organisation in HGPS reflect features of those changes observed in normal ageing, including the loss of H3K27me3, supporting the notion of a "premature ageing syndrome" and the relevance of HGPS in understanding physiological ageing.

DNA Damage response

DNA damage accumulation and the consequences of DNA repair deficits can contribute to genomic instability and are thought to have roles in normal ageing (Lombard et al., 2005). DNA damage accumulation along with defects in DNA damage repair have been shown to be a large contributing factor to the genomic instability in HGPS fibroblasts (Liu et al., 2005). As well as playing a largely structural and scaffolding role at the nuclear lamina, lamins are also found located throughout the nuclear interior as indicted by FRAP studies (Broers et al., 1999), where they interact with major components of the nucleoplasm and are believed to play additional roles in further genome organisation, DNA replication and DNA damage repair (Bridger et al., 2007)

Recent studies have shown that double strand breaks (DSBs) characteristically accumulate in both HGPS and RD cells, contributing to genomic instability which is linked to cellular senescence, and to the premature ageing phenotype (Liu et al., 2005).

DNA damage in cells induces a checkpoint response, arresting cell cycle progression allowing the damage to be repaired, preventing the damage being replicated into the daughter cells and being converted into inheritable mutations (Li and Zou, 2005). HGPS fibroblasts and Zmpste24 null mouse embryonic fibroblasts show increased DNA damage as indicated by the presence of γ-H2AX foci, a marker of DNA damage (Liu et al., 2005). This increase in DNA damage correlated with an up-regulation of p53 target genes (Varela et al., 2005) which leads to cell cycle arrest, cellular senescence and apoptosis, contributing to the progeria phenotype. Furthermore, double knockout of Zmpste24-/- and p53-/- partially rescued the phenotype (Varela et al., 2005).

Due to the accumulated DNA damage in HGPS and RD cells, the DNA checkpoint response is persistently activated (Cortez et al., 2001; Liu et al., 2006) with continuing activation of ATM and ATR, two protein kinases, regulating downstream targets involved in cell cycle progression and DNA synthesis (Liu et al., 2006). Lamin mutant proteins induce defects in these signalling pathways, causing mislocalisation of ATM and ATR to the cytoplasm and leading to delayed recruitment of DNA damage repair proteins such as 53BP1 (Manju et al., 2006).

More recently, greater detail has been revealed as to how defective lamin A maturation is correlated with increased DNA damage. The repair proteins Rad51 and Rad50 do not localise to

damage sites where they are required; instead there is a mislocalisation of the nucleotide excision proteins xeroderma pigmentosum group A (XPA) to DSBs formed in HGPS and RD cells (Liu et al., 2008). XPA is a specific and essential factor for NER, but is not required or involved in the repair of DSBs. This therefore indicates that the incorrect localisation of XPA prevents the correct localisation of Rad50 and Rad51 thereby preventing DNA damage repair.

XPA was shown to bind to γ-H2AX (a sensitive target for DSBs), and this association, absent in control cells, is shown to be mediated by chromatin (Liu et al., 2008). This is intriguing, as HGPS and RD show a massive disorganisation of heterochromatin. Heterochromatin is thought to act as a barrier to DSB repair, which must be overcome, at least in part by ATM (Goodarzi, Noon and Jeggo, 2009). Yet, if ATM is incorrectly localised, the heterochromatin barrier cannot be permeated, causing the incorrect localisation of XPA, hence causing a severe defect in DNA damage repair.

Farnesyltransferase inhibitors as discussed below have been shown to improve the aberrant nuclear morphology of HGPS and RD fibroblasts, however they are unable to reduce the accumulation of DNA DSBs in both HGPS and RD cells, (Liu et al., 2006), nor the levels of γ-H2AX protein, suggesting DNA damage accumulation and misshapen nuclei are two independent phenotypes in premature ageing laminopathies (Liu et al., 2008). Potential therapeutic treatments would therefore have to target both of these phenotypes in order to be an effective option in the treatment of these premature ageing laminopathies.

Telomere shortening

It has been observed that compared to age-matched controls, telomere length in HGPS fibroblasts are consistently shorter (Allsopp et al., 1992). Telomeres are repetitive regions of G rich DNA sequences and associated proteins located at the ends of linear chromosomes, protecting the coding DNA from damage and are a major defence in protecting against genomic instability.

Telomeres shorten with each cellular division, acting as a buffer for the ends of chromosome and are thought to be actively involved in ageing, with cells from older individuals having a shorter telomere length, than those from younger individuals. When a telomere reaches a critical length, the cell either undergoes apoptosis or enters cellular senescence, thus contributing to the loss of cellular and tissue function with increasing age (Decker et al., 2009).

In HGPS, quantified fluorescence in situ hybridisation (Q-FISH) is used to quantify the length and rate of attrition of telomeres and found that HGPS fibroblasts showed significantly shorter

telomeres and a faster rate of loss than age matches controls (Decker et al., 2009; Gonzalez-Suarez et al., 2009). This is unsurprising as evidence linking A-type lamins with ageing and cancer, with regard to telomeres has been forthcoming, along with evidence of impaired genomic stability in progeria. Interestingly, overexpression of progerin or wild type lamin A in control fibroblasts resulted in increased telomere attrition, suggesting a major role for lamin A in telomere length.

The loss of peripheral heterochromatin as discussed previously may cause these defects at the telomeres as the aberrant chromosomal organisation could affect telomere structure.

The localisation of telomeres changes throughout the cell cycle, whilst in G0/G1/S phases they are distributed throughout the nucleus, at G2 they form a telomere disc at the centre of the nucleus. It is thought that A-type lamins may have a role in regulating their distribution. This has been shown, as the loss of A-type lamins there is an observable shift of the telomeres towards the nuclear periphery, correlating with the loss of peripheral heterochromatin.

Lamins may act to tether telomeres to a lamin scaffold or attach to the nuclear matrix, and loss or mutation of A-type lamins may perturb this interaction (Gonzalez-Suarez et al., 2009; Decker et al., 2009).

One alternative explanation is that loss or mutation of A-type lamins might perturb the recognition and processing of damaged telomeres through the non-homologous end joining (NHEJ) pathway. By inducing telomere damage through a dominant negative form of the transcription factor TRF2, it was found that loss of A-type lamins does not prevent the recognition of dysfunctional telomeres, but the NHEJ pathway is affected. 53BP1, a vital member of the DNA damage response pathway, as discussed previously, shows a significant decrease upon A-type lamin loss, this results in restricted motility of telomeres and defects in their repair (Gonzalez-Suarez et al., 2009).

This would lead to premature cellular senescence or apoptosis leaving the remaining cells to divide a greater number of times, thereby reducing the length of their telomeres (Decker et al., 2009).

Others have suggested aberrant interactions with lamin A binding proteins could contribute to the reduced telomere length. LAP2α resides in the nucleoplasm where it binds to and forms stable complexes with lamin A/C. It also forms dynamic interactions with telomeric chromosome regions during the cell cycle (Dechat et al., 1998). Lamin B also binds to chromatin, in domains of high and low binding stability (Guelen et al., 2008). It is thought that either loss or mutation of

lamin A may interfere with these interactions, resulting in destabilisation of telomeric tethering (Huang et al., 2005), and defects in dysfunctional telomere repair (Huang et al., 2008).

Although the regulatory mechanisms of telomere length such as telomerase, and the shelterin complex are not affected in HGPS or upon loss of lamin A, recently identified components known as TERRAs (non-coding telomeric RNAs) show markedly decreased levels in Lmna -/- cells. TERRAs are transcribed by RNA polymerase II, which is known to be perturbed by lamin A mutation (Azzalin et al., 2007). TERRAs are thought to inhibit the action of telomerase, which logically would lead to an increased telomere length phenotype. Therefore it is likely that a more global defect in chromatin structure and DNA metabolism due to mutation of A-type lamins is responsible for this phenotype (Gonzalez-Suarez et al., 2009).

1.2.2.4 Farnesylation

The processing of the immature pre-lamin A has been described previously. Within this post-translational processing stages, HGPS and RD mutations become apparent. The farnesyl lipid meoity is added to the thiol group of the cysteine residue in the Caax motif at the C-terminus of prelamin A by farnesyltransferase enzyme (FTase).

Progerin, the abnormal protein found in HGPS patients, undergoes farnesylation at the Caax motif, yet lacks the cleavage site for the endoprotease ZMPSTE24, preventing further processing. This produces progerin; a permanently farnesylated protein which forms stable associations with the nuclear membrane (Scafiffi and Misteli, 2005), resulting in a compromised nuclear lamina causing the characteristic misshapen nuclei.

A different mechanism causes restrictive dermopathy. Here the mutation resides in the gene encoding the endoprotease ZMPSTE24 itself, resulting in accumulation o f high levels of permanently farnesylated prelamin A.

It has been hypothesised that the severity of alterations in nuclear structure as well as function could correlate with the amount of accumulated farnesylated progerin or prelamin-A. Therefore, restrictive dermopathy lies at the extreme end of the severity scale, as no mature lamin A is produced, and is therefore neonatal fatal. Whilst with HGPS, the patients have a longer life expectancy due to the presence of some mature lamin A, and lower levels are farnesylated progerin (Navarro et al., 2004).

The farnesyl group attached to pre-lamin A and progerin targets the protein to the nuclear rim, where it exerts its dominant negative effect, (Yang et al., 2006) with this working hypothesis, it

was suggested that an FTI (Farnesyltransferase inhibitor) would prevent this mislocalisation away from the nuclear envelope, and therefore improve the misshapen nuclei.

Lonafarnib (an FTI) is already used clinically as an anti-cancer drug, inhibiting the farnesylation of the oncoprotein Ras, hence it is known to be well tolerated and safe for use in patients (Adjei et al., 2000) (Kim et al., 2005) (Doll et al., 2004).

Initial trials were carried out on fibroblasts from HGPS patients and demonstrated a dramatic, dose depending improvement in nuclear shape upon treated with FTI (Capell et al., 2005). Due to these encouraging results, it was thought that FTIs may be a potential therapy for patients with HGPS, and began with gene targeted mouse models. Yang et al., 2006 created a mouse model harbouring a HGPS mutation producing progerin exclusively either as a heterozygous (LmnaHG/+) or homozygous (LmnaHG/HG). Treatment with FTIs partially mislocalised Prelamin A away from the nuclear envelope and reduced the incidence of misshapen nuclei. This was corroborated in an additional Zmpste24-/- mouse model. (Toth et al., 2005) The FTI improved body weight, prevented loss of adipose tissue, reduced rib fractures and improved bone density (Yang et al., 2006).

However, although the FTI treatment began human clinical trials in HGPS patients, the beneficial effects fell far short of a cure. The mouse models used still showed signs of premature aging including reduced weight curves, rib fractures, and died prematurely. (Yang et al., 2008)

Questions have been raised over the effectiveness of FTIs as a therapeutic treatment for laminopathic patients. One reason for trepidation is the status of other farnesylated proteins, such as B-type lamins and Ras in cells treated with FTIs. Additionally, evidence suggests that FTIs may lead to cell cycle arrest by targeting the proteasome (Efuet and Keyomarsi, 2006), thereby counteracting its beneficiary effects.

Furthermore, Lui et al., (2006) have evidence demonstrating that many of the functional defects associated with HGPS are not improved upon FTI treatment. Most prominently, DNA damage checkpoints are consistently activated due to genomic instability leading to large numbers of DNA double strand breaks, even in the presence of FTIs thereby questioning their functionality. Although the FTIs improved nuclear shape, DNA damage repair remained unchanged, leading to the conclusion that these manifestations of the syndrome are independent from one another (Lui et al., 2006).

Yet, most importantly, prelamin A may be alternatively prenylated through geranyl-geranylation, (Basso et al., 2006) in the presence of FTIs, producing a geranylated protein that is perhaps even more toxic than its farnesylated counterparts.

The combined use of statins (pravastatin) and aminobisphosphonates (zoledronate) to block prelamin A prenylation by inhibiting the mevalonate pathway (Varela et al., 2008) led to a substantial increase in non-prenylated pre-lamin A in a Zmpste24-/- fibroblast. This was associated with a percentage reduction in misshapen nuclei in HGPS patient fibroblasts (Varela et al., 2008).

This treatment has gone on to be used as a clinical trial in Europe on children suffering progeria with results yet unknown at the date of publication.

Other researchers have questioned the basis on which FTIs are used, suggesting permanent farnesylation may not be the exclusive factor leading to the development of progeria. This is based on the discovery of two cases of atypical progeria caused by amino acid substitutions. Although the patients presented with classical HGPS phenotypes, this compound heterozygous T528M and M540T mutation showed no accumulation of either progerin or pre-lamin A in cells, indicating another mechanism of HGPS (Smallwood and Shackleton, 2010).

Further evidence however, has suggested that merely the presence of progerin, farnesylated or not is intrinsically toxic to cells. As the cysteine residue is the target for prenylation, it was suggested that by replacing the cysteine residue with that of a serine, which is unable to be prenylated would prevent the build up of progerin (Yang et al., 2008).

In HGPS mouse models with this targeted cysteine to serine mutation ($Lmna^{nHG/+}$), the same disease phenotypes appear, yet with a lesser severity of those with the original cysteine ($Lmna^{HG/+}$). Noticeably the transcript levels of progerin in were lower than that $Lmna^{nHG/+}$ were lower than that of $Lmna^{HG/+}$, thereby suggesting that the steady state levels of progerin could explain the differing severities of the phenotypes observed (Yang et al., 2008).

As progerin acts in a dominant-negative fashion, reversal of the aberrant cellular morphology requires the complete elimination of the mutant protein. This was achieved by the introduction of a 25-mer morpholino oligonucleotide designed specifically to block the cryptic splice in progeria fibroblasts. Upon treatment with this oligonucleotide, the cellular phenotype was reversed, including loss of herniations and severe wrinkles and adopted a normal fibroblast shape. Genes, typically altered by the presence of progerin were also restored to wild-type levels including lamin B, LAP2 proteins and heterochromatin markers including H3K9me3 (Scaffidi and Misteli, 2005). Such a method may be used therapeutically as cells can be rescued independently of mitosis, but is yet to be tested on mouse models.

1.2.3 Restrictive Dermopathy

Restrictive dermopathy (RD) is a lethal human genetic disorder caused by a mutation in the gene ZMPSTE24 and is characterized by taught, slightly translucent skin with erosions at joints. In most patients death results from respiratory failure due to the tightness of the skin. (Moulson et al., 2005).

Clinically at birth, several features of the RD phenotype are recognisable as a premature ageing disorder, including bone density reductions, joint contractures and hypoplastic clavicles, all of which are present in HGPS. Only 60 cases have been reported worldwide and is generally classed as rarer than HGPS, although many missed diagnosis may have been made at birth.

Signs are often present during pregnancy and include growth retardation, reduced fetal movements and premature rupture of membranes. (Moulson et al., 2005)

Other clinical features at birth include a fixed facial expression with typical “O-shaped” mouth, absent eyelashes and eyebrows, malformed low set ears and temporomandibular joint ankylosis. (Morais et al., 2009)

Histological studies of the skin revealed reduced epidermal layers and flattened dermo-epidermal junctions. Connective tissue showed abnormally dense parallel collagen bundles, and the dermis showed a near to complete absence of elastic fibers, resulting in the rigidity of the skin leading to respiratory failure. (Navarro et al., 2004)

As discussed previously, Lamin A isoforms are produced through a series of post-translational modifications. The enzyme involved in both proteolytic cleavage events is the zinc metalloproteinase ZMPSTE24. ZMPSTE24 is the only protease known to be capable of executing the second proteolytic cleavage in prelamin A post-translational modifications, and therefore its inactivation through biallelic inactivation of the gene leads to the phenomenally devastating consequences associated with RD. (Smigiel et al., 2010)

On the basis of common phenotypes with other premature ageing disorders, Navarro et al. screen the LMNA gene, and found one of the nine patients studied contained a mutation commonly found in classical HGPS patients. However the other eight patients were found to have a homozygous or compound heterozygous 1bp insertion in exon 9 (c.1085_1086insT), (Navarro et al., 2004) resulting in the truncation and inactivation of ZMPSTE24 and consequently the complete absence of mature lamin A proteins. (Navarro et al., 2005).

The loss of the emzymatic activity of the prelamin A specific metalloproteinase leads to the accumulation of permanently farnesylated prelamin A which is claimed to be toxic to cells. From

the protein sequence it is predicted that the truncated enzyme lacks the seventh transmembrane domain alongside the protein's cytoplasmic C-terminal catalytic domain.

On a cellular level, similarly to HGPS numerous and major nuclear deformities are observed including blebs or herniations as well as increase nuclear size. (Navarro et al., 2004) Obvious prelamin A staining was observed in RD fibroblast nuclei in nucleoplasmic foci, whereas emerin was normally localized indicating a specific deficit in prelamin A processing. (Moulson et al., 2005) Cellular deformities increase with the number of population doublings in culture as prelamin A accumulates. The large amounts of prelamin A present in RD may explain its increased severity compared to laminopathies with LMNA mutations. (Moulson et al., 2005, Navarro et al, 2005)

Mouse models are increasingly being used to phenocopy such rare and distressing syndromes. However cautions must be taken in that there is an obvious difference between mice and humans and therefore clinical symptoms may be somewhat different and hence conclusions must be made with great prudence.

As discussed previously, Zmpste24-deficient mice phenocopy human HGPS (Pendas et al., 2002) and not restrictive dermopathy and therefore show some different genetic and epigenetic control between mouse and human.

However, a mouse model of RD has been reported. This was created by the targeted disruption of the FATP4 gene, a gene encoding for a fatty acid transport protein. Fatp4 knockout mice mimicked RD by being neonatal fatal with hyperproliferative and hyperkeratosised skin, alongside facial dysmorphia and joint contractures (Herrmann et al., 2005) suggesting this may be a worthwhile model to pursue for further investigations.

1.2.4 Other Laminopathies

Autosomal Dominant Emery Dreifuss Muscular Dystrophy (AD-EMDM)

EDMD is a muscle wasting disorder caused by a mutation in one of two genes. The X-linked variant of EDMD is caused by a mutation in the inner nuclear membrane protein Emerin, whilst the autosomal dominant form is caused by a mutation in LMNA representing the first mutation in a nuclear lamina protein to cause a muscular dystrophy (Broers et al., 2006). It is characterised by a progressive wasting of muscle, early joint contractures and eventual cardiac involvement, frequently dilated cardiomyopathy in its most severe form (Bonne et al., 2000).

The mutations associated with AD-EDMD are both numerous and diverse, which makes an association between genotype and phenotype difficult to comprehend.

Dilated cardiomyopathy with conduction system (DCM-CD)

The vast majority of mutations in LMNA which cause DCM-CD are heterozygous and affect the α-helical rod domains of lamin A/C. These mutations cause progressive symptoms in the conduction system, such as sinus bradycardia, atrioventricular conduction block and atrial arrhythmias. This can often result in sudden deaths from heart failure (Fatkin et al., 1999, Broers et al., 2003).

Limb-girdle muscular dystrophy type B1 (LGMD1B)

LMNA mutations were identified as a cause of this syndrome and have mainly been shown to be missense mutations and are allelic to AD-EDMD. This dystrophy is associated with proximal muscle weakness and wasting often coupled with dilated cardiomyopathy. Alike DCM-CD sudden death is also frequent (Bonne et al., 2000; Broers et al., 2003)

Charcot-Marie-Tooth syndrome.

This LMNA mutation causes a muscle wasting phenotype in the upper and lower limbs with reduced motor and sensory nerve function due to reduced motor nerve velocities (De Sandre-Giovannoli et al., 2002). Mice null of LMNA show signs of a similar phenotype, highlighting the differences between the effects of LMNA mutations on mouse and human.

Familial Partial Lipodystrophy of Dunnigan type (FPLD)

Dunnigan-type familial partial lipodystrophy (FPLD) is a rare monogenic form of insulin resistance characterised by the loss of subcutaneous fat from the extremities, trunk, and gluteal regions beginning at the onset of puberty (Hegele, 2002). The loss of adipose tissue from these areas is accompanied by an accumulation of adipose tissue in the face and neck, axillae and back (Broers et al., 2006).

Patients suffering from FPLD are predisposed to a variety of metabolic disorders such as type II diabetes mellitus and glucose intolerance (Garg et al., 1999; Hegele et al., 2000).

LMNA was confirmed as the causative gene in FPLD (Cao and Hegele, 2000) with the majority of mutations causing FPLD being confined to the R482 amino acid (R482Q, R482W, R482L) affecting amino acid residues at the surface of the LMNA Ig domain (Broers, 2055).

Mandibuloacral dysplasia (MAD)

MAD type A is caused by a homozygous R527H mutation (Novelli et al., 2002), located at the C-terminal globular domain of lamin A, and like RD leads to the accumulation of the prelamin A at the nuclear envelope, along with the presence of intranuclear prelamin A foci and a loss of heterochromatic markers (Filesi et al., 2005). The syndrome is characterised by phenotypes observed in other laminopathies. Patients with MAD frequently have partial lipodystrophy and insulin resistance, which are features seen in FPLD (Novelli et al., 2002). Affected individuals present often with milder phenotypes of HGPS, leading to the theory that HGPS and mandibuloacral dysplasia may represent a single disorder with varying degrees of severity (Plasilova et al., 2004).

Aytypical Werner syndrome

The typical form of Werner syndrome is caused by mutations in RECQL2 encoding RecQ a DNA helicase involved in DNA repair. It results in a premature ageing syndrome and is associated with increased cancer predisposition. Phenotypic symptoms includes amongst others sclerodermal-like skin in extremities, cataract, subcutaneous calcification, premature arteriosclerosis and osteoporosis.

Another form has also been found to be caused by missense mutations in LMNA, especially in the rod domain and C-terminal domain and results in a more severe form of the syndrome.

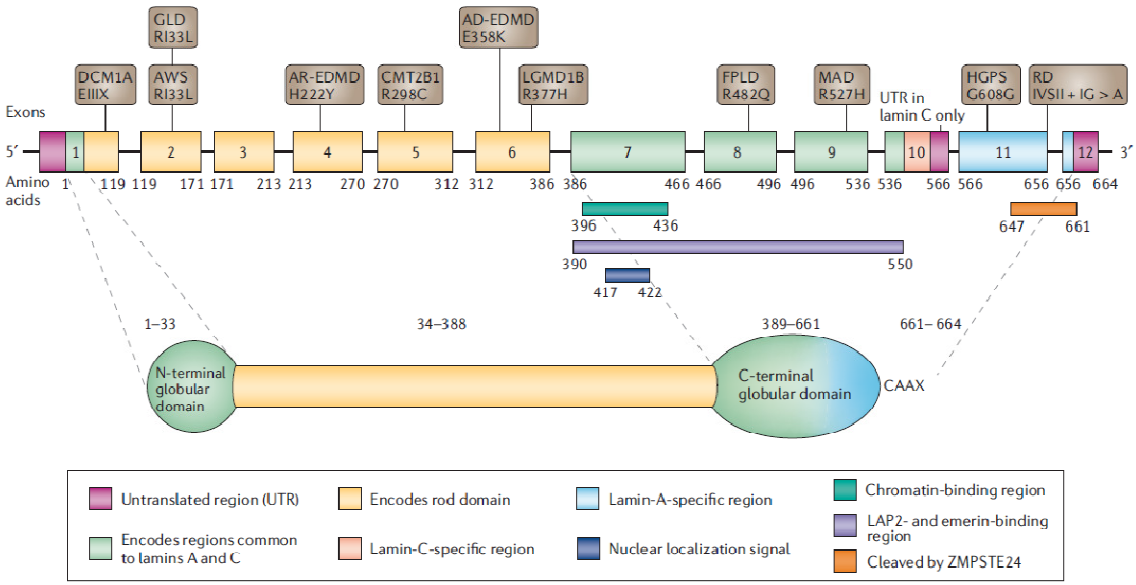


Figure 5: Diagram showing the locations of mutations in *LMNA* and the resulting diseases (Adapted from Capell and Colins, 2006)

1.3 Models of Laminopathies

1.3.1 The Structural Hypothesis.

One of the most difficult and intriguing questions to ask with regard to laminopathies is how a ubiquitously expressed nuclear envelope protein gives rise to a myriad of tissue-restricted diseases.

A number of hypotheses have been proposed which have attempted to causally link the pathophysiology of laminopathies to both the structure and the molecular functions of both lamins and the lamina as a whole.

One of the earliest hypotheses proposed was the structural hypothesis. This proposal was based on the opinion that the most important function of the lamina is to maintain the structural integrity of the cells. The lamina has been shown to act as a tensegrity element (Hutchison and Worman, 2004), protecting the genome from physical strain. Therefore it was proposed that mutations in A-type lamins or emerin would give rise to a weakened nuclear envelope, cellular fragility and ultimately cellular senescence or death.

This model was supported by studies that show that *Lmna* null mouse embryonic fibroblasts show a structurally compromised nuclear envelope. This was shown by measuring the bursting point of a cell under mechanical strain and visualised using a DNA intercalating dye. This showed that the force required to burst Lmna knockout cells was significantly lower than wild type counterparts (Broers et al., 2004).

It was also shown *Lmna*$^{-/-}$ fibroblasts deform isotropically indicating the nucleus is attached to the lamina (Broers et al., 2005). Mutations affecting this connection are manifested by abnormally shaped nuclear envelopes or gaps within the nuclear envelope, which accumulate with age (Goldman et al., 2004). Mutations resulting in cytoskeletal disorganisation may also act to compromise striated muscle function in the Group 1 laminopathies, such as EDMD. Consistent with these findings, *in vitro* ultrastructural investigation of striated muscle from patients with EDMD revealed widespread damage to the nuclear envelope allowing chromatin to leak into the cytoplasm (Beaudouin et al., 2002).

This is further supported by the demonstration that compared to wild type cells, emerin null and lamin null fibroblasts show a reduction in the up-regulation of stress response genes when undergoing physical strain (Lammerding et al., 2004). This helps to explain the biopsies of EDMD patients, in which the continuing strain on the nuclear envelope from muscle contractures leads to a progressive syndrome as more and more cells undergo necrosis.

Whilst I have outlined a number of supporting lines of evidence for the structural hypothesis it is clearly difficult to understand how mechanical weakness of the lamina could cause adipocyte redistribution as seen in FPLD or the premature aging phenotype as observed in HGPS. Therefore further theories have been proposed.

1.3.2 Gene Expression and Cell Proliferation Hypotheses

The structural hypothesis tends to lend itself to the inability to respond to stress to adequately explain muscle wasting diseases seen in laminopathies. However it cannot account for some of the other features of laminopathies such as the selective loss of subcutaneous fat and the redistribution of adipocytes around the face and neck in lipodystrophies.

It has been demonstrated that A-type lamins stabilise differentiated phenotypes by influencing transcription by directly or indirectly binding to transcription factors. These observations led to the gene expression hypothesis, suggesting that mutations in lamins can affect gene expression by several mechanisms.

Global gene expression patterns have been investigated in HGPS and RD cell lines and have consistently shown increases in transcription factors (Gruenbaum et al., 2005), which act as negative regulators of mesodermal tissue proliferation, coherent with the clinical observation that the syndrome affects tissues from of mesenchymal origin (Huang et al., 2008).

For example, changes in lamin-Rb interactions may affect cell cycle progression and cellular differentiation. Rb regulates passage through the G1 phases of the cell cycle. The phosphorylation status of Rb determines whether the cell progresses to S phases, begins a path of terminal differentiation or enters a quiescent state. Hypophosphorylated pRb forms a complex with the E2F family of transcription factors and prevents E2F from activating cell cycle inhibitor genes, resulting in continued cell proliferation. Whereas when pRb is hyperphosphorylated, it is unable to bind E2F. Cell cycle inhibiting genes are therefore induced, resulting in exit from the cell cycle.

In order to function as a cell cycle regulator, Rb must be tethered to the nucleus. This is achieved by a complex of A-type lamins and LAP2α (Markiewicz et al., 2002), thereby assisting in E2F repression. Mutations in A-type lamins lead to the loss of this regulation. As shown in Lmna-/- mice, Rb is undetectable as it is continuously degraded by the proteasome resulting in unrestrained cell cycling and loss of homeostasis in the tissue (Johnson et al., 2004). Laminopathies, however are not caused by lamin A/C nulls, however consistently proliferative and replicative activities are decreased in fibroblasts with LMNA mutations, independent of

apoptosis. Dominant negative lamin A/C mutations also cause LAP2α to redistribute into aggregates (Naetar et al., 2008). Knockdown of LAP2α also results in cell cycle arrest, indicating that lamin A dependent expression and organisation of LAP2α is required to maintain cells in a proliferative state (Markiewicz et al., 2002).

Cyclin dependent kinase inhibitors $p16^{INK4a}$ and $p21^{WAF-1}$, which mediate cell cycle arrest through Rb phosphorylation are found to be overexpressed in fibroblast carrying LMNA mutations. (Caron et al., 2007). This correlates with the observation that early passage fibroblasts from HGPS and RD patients show rapid proliferation, but also demonstrate high levels of apoptosis (Bridger and Kill, 2004).

The fact that a cancerous phenotype is not observed in HGPS is surprising, however the rapid progression through the cell cycle and the resulting accumulation of DNA damage does not activate a cancerous phenotype as the pathway for cell cycle arrest and apoptosis is activated instead. Rb is not only involved in cell proliferation, but also is necessary for differentiation of mesenchymal cells including skeletal muscle and adipocytes, the cells primarily affected in laminopahic diseases. Along with Rb, adiopogenesis is regulated by perioxisome proliferator activator receptor gamma (PPARγ), which is activated by SREBP1 (Kim and Spiegelman, 1996). Recently is has been shown that prelamin A binds preferentially over mature lamin A to SREBP1 (Lloyd et al., 2002)

In fibroblasts for a variety of lamiopathic patients such as those with FPLD, MAD and atypical Werner's syndrome, prelamin A accumulates at the nuclear envelope, sequestering SREBP1, which has been proposed to reduce the pool of activated PPARγ, thereby inhibiting adipogenesis. (Capell et al., 2005) This helps to explain the loss of adipose tissue in many laminopathies, including progeria.

However, once again there are complications with this argument, this theory would suggest that the loss of Rb regulation would hence result in a failure of muscle and adipocyte differentiation at an early stage of development. However, contrary to this, lmna-/- mice are born normally, and only display severe growth retardation and muscle wasting after birth (Sulivan et al., 1999).

Tumour growth factor-β (TGF-β)/Smad pathway are known to regulate mesenchymal tissue homeostasis. A-type lamins have been found to associate with TGF-β induced Smads 2 and 3 and Rb and acts to repress TGF-β dependent gene expression in MEFs (van Berlo et al., 2005). Lmna deficient mice show mesenchymal stem cells with a predisposition for adipocyte differentiation as opposed to osteoblast differentiation. This phenotype is reflected in senile osteoporosis with reduced TGF-β signalling. Additionally, the lamin A binding partner MAN1 interacts with R-

Smads, and when mutated leads to increased bone density as a result of antagonising TGF-β signalling. Therefore deregulated TGF-β signalling may be a consequence of lamin A mutation (Pekovic and Hutchison, 2008)

1.3.3 Stem Cell Hypothesis

As Lmna-/- mice are born normally, it is proposed that A-type lamins are dispensable for prenatal development. However, after birth the mice display severe growth retardation, muscle wasting and generally die as a result of heart failure at around 4 weeks (Sulivan et al., 1999)

This led to the suggestion that lamin A/C may be involved in tissue maintenance, and the preservation of mesenchymal stem cells responsible for the tissue renewal (Pekovic and Hutchison, 2008) This would account for the compromised tissue function in laminopathy diseases due to a failure to maintain cells in a differentiated state and/or loss of regenerative potential (Gotzmann and Foisner, 2006). A-type lamins are therefore suggested to act as intrinsic modulators of aging in adult stem cells and thus contribute to the determination of organismal lifespan (Pekovic and Hutchison, 2008).

It has been proposed that disease causing mutations in A-type lamins could have an important impact on the stem cell compartment. The finding of a reduced regenerative capacity of myoblasts from *lmna-/-* and *emerin-/-* mice show that the satallite stem cells arrest in a primitive state as there is no evidence of upregulation of downstream effectors of muscle differentiation such as MyoD. (Frock et al., 2006). The satellite stem cells still demonstrate the ability to self renew but lack the ability to differentiate, which is mirrored in AD-EDMD as the mutant lamin A prevents differentiation in mouse satellite cells, yet allows amplification (Markiewicz et al., 2005).

This observation may explain the late onset and progressive nature of muscle wasting disorders caused by mutations in lamin A. Only when the muscle cells are injured and need replacing does the disease manifest in symptoms, as the affected stem cells are unable to differentiate into new muscle cells (Gotzmann and Foisner, 2006). Patients suffering from HGPS show conditions manifested in tissues which require regeneration including atherosclerosis, cardiovascular disease, lipodistrophy, alopecia and defects in nails and teeth. Tissues that are exempt from ongoing damage such as the brain are absent from dysfunction in HGPS. As tissue restricted patterns of symptoms are observed in HGPS it is tempting to speculate that HGPS patients suffer from premature stem cell exhaustion (Halashek-Wiener and Brooks-Wilson, 2007), thereby effecting the tissues requiring repair. The absence of a cancerous phenotype can also be

explained, as premature stem cell exhaustion from continued proliferation and increased apoptosis will act to diminish stem cell pools and prevent malignant transformation (Halashek-Wiener and Brooks-Wilson, 2007).

Espada et al. (2008) found that a Zmpste24 null progeroid mouse models shows a perturbation in the number and proliferative capacity of epidermal stem cells in the hair follicle. This effect can be rescued in Zmpste24-/- Lmna +/- mutant mice which do not show evidence of progeroid symptoms, thus indicating a causal relationship between mutations in LMNA and stem cell dysfunction.

Several layers of transcriptional control have been implicated in this alteration of adult stem cell regulation. These include Rb regulation (Pekovic and Hutchison, 2008), β-catenin signalling (Markiewicz et al., 2006) and TGF-β signalling (van Berlo et al., 2005), all of which are associated with adult stem cell proliferation and differentiation. A deregulated Rb pathway that is resultant from lamin A mutation could lead to premature stem cell exhaustion from unrestrained proliferation compounded by the accumulation of DNA damage (Dorner, 2006).

The canonical Wnt/β-catenin pathway is vital in mesenchymal tissue proliferation and differentiation. A study has found an overall decrease in β-catenin levels in lamin A mutant mice as well are a significantly marked reduction in cyclin D1 levels; a direct proliferative target of the Wnt pathway (Espada et al., 2008).

Furthermore it has been demonstrated that the expression of progerin in immortalised human mesenchymal stem cells negatively impacts their differentiation potential by perturbation of the Notch signalling pathway; a vital pathway in stem cell regulation(Scaffidi et al., 2008).

In conclusion, loss of regulation of a number of pathways vital for adult stem cell maintenance and regeneration may be the foundation of the pathophysiology associated with mutations within LMNA.

1.4 Ageing

1.4.1 Cellular Senescence

Most somatic cells cannot divide indefinitely; they permanently stop dividing after a finite number of cell divisions and enter a state known as cellular or replicative senescence. This lifespan of replication was first described by Hayflick, and is often termed the 'Hayflick limit' (Hayflick and Moorhead, 1961). Tumour cells and certain stem cells are exceptions to the Haylick

limit and can continue to divide and evade senescence. Cellular senescence acts as a barrier to prevent cancerous phenotypes, averting the accumulation of mutations and therefore plays an important role in tumour suppression (Ohtani, Mann and Hara, 2009).

Recent evidence suggests that replicative senescence occurs *in vivo* also and therefore also plays an important role in ageing (Campisi, 2005). A number of proposals have been put forward to explain the mechanisms leading to senescent arrest, which most likely are both contributing factors. Data has shown that replicative senescence is induced by critically short telomeres, which act therefore as a mitotic clock, counting the number of cell divisions as they progressively shorten with each division (d'Adda di Fagagna et al., 2003) This theory gained support from evidence showing the addition of hTERT (the active subunit of telomerase) can immortalise human somatic cells (Bodnar et al., 1998).

However, telomere independent cellular senescence also occurs *in vivo.* Stress induced premature senescence (SIPS) occurs in response to various stresses including oxidative stress and non-telomeric DNA damage (Toussaint et al., 2000; Gorbunova et al., 2002).

It is also important to point out that telomere dependent replicative senescence is also influenced by cellular stress, in that telomere attrition is affected by the level of oxidative stress in the cell (von Zglinicki et al., 1995).

Cellular senescence is characterised by an impaired redox state, irreparable DNA damage and a build up ox oxidatively damaged proteins (von Zglinicki et a., 2005), and phenotypic markers such as expression of senescence associated β-galactosidase (Dimri et al.,1995), aberrant cellular morphology and expression of senescence associated genes are practically identicial (von Zglinicki et al., 2005) between replicative senescence and SIPS. This indicates that both types of cellular senescence (telomere dependent and telomere independent) may be a common mechanism in a stress response pathway contributing to both tumour suppression and ageing.

1.4.2 DNA Damage and DNA Repair

The genome of cells is under a constant barrage from both environmental agents such as UV, ionizing radiation and genotoxic chemicals and from within the cell. Reactive oxygen species formed as a by-product of metabolising cells causes a wide variety of damage to the genome (Houtgraaf, Versmissen and Giessen, 2006). In order to survive this damage, cells must be able to repair the damage, or apoptose to ensure it is not passed onto progeny.

In order to achieve this genomic integrity cells have evolved the DNA damage checkpoint and repair programme. This acts to arrest the cell cycle in response to DNA damage, to provide the cell with time to carry out repair. DNA bases are highly vulnerable to damage, whereby lesions can be converted into mutations if the repair pathway fails. These modifications are then permanent and passed onto daughter cells (Hoeijmakers, 2009). A variety of lesions can occur in DNA, including single and double stranded breaks, and therefore a variety of repair pathways have evolved, as summarised in figure 6 (Houtgraaf, Versmissen and Giessen, 2006).

In order to repair the damaged DNA, it must firstly be recognised. However the proteins that initially sense the anomalous DNA are largely unknown. Candidate sensors include Rad1, Rad9 and Hus1, it is suggested that form a ring structure (9-1-1 structure) that is able to encircle the damaged DNA, and form a scaffold for the downstream processes. This structure is proposed to be loaded onto the lesion by a protein complex (Rad17-RFC_{2-5}) consisting of four RFC subunits and Rad17. From mutations in RFC, it is thought that this complex acts to maintain the replication checkpoint during S phases (Noskov, Araki and Sugino, 1998).

From the initial sensor of the damage, this signal must then be transduced, a process which is much more fully understood. Two functionally related proteins are key in this part of this early response pathway (Elledge, 1996), Ataxia telangiectasa (AT) mutated (ATM) and ATM and Rad3 related (ATR). ATM and ATR are protein kinases belonging to the phosphatidylinositol 3-kinase like (PIKK) family of serine/threonine protein kinases.

ATM plays a key role in response to ionizing radiation, and mediates phosphorylation of p53, Mdm2, BRAC1, Chk2 and Nbs (Zhou and Elledge, 2000). ATR is more vital in response to UV and chemical modifications of DNA. ATM and ATR act to phosphorylate proteins known as mediators in the DNA damage response pathway. These mediators are mostly cell cycle specific and help as transducers. These include p53 binding protein (53bp1), topoisomerase binding protein (TopBP1) and mediator of DNA damage (MDC1).

Effector proteins Chk1 and Chk2 act to phosphorylate target proteins that ultimately result in cell cycle arrest. Chk1 and Chk2 phosphorylate and inactivate cell cycle proteins Cd25A, Cdc25B and Cdc25C, preventing cell cycle progression (Houtgraaf et al., 2006), however alterations in cell cycle arrest depend upon the type of DNA damage.

DNA repair pathways consist of an intricate network of repair systems, each designed specifically to target a different subset of lesion. Four important mechanism include base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR) and DSB repair.

1. *Base excision repair*

DNA base damage is primarily the result of reactive oxygen species or ionising radiation. The damaged base is removed by a DNA glycosylase and APE1 endonuclease. The resulting DNA either side of the site is shortened by poly(ADP-ribose) polymerase. The single-stranded gap is filled by DNA polymerase-β and ligased by the Ligase3/polymerase-β complex (Houtgraaf, Versmissen and Giessen, 2006)

2. *Nucleotide excision repair*

The NER pathway is utilised to repair large damages caused by UV radiation, genotoxic chemicals or reactive oxygen species. The DNA double helix is unwound by Transcription Factor IIH which is constituted of the helicases XPB and XPD. A third xeroderma pigmentosum protein XPA signals the presence of DNA damage. XPG acts to remove the damaged strand 3' of the lesion whilst the XPF/ERCC1 complex cleaves the strand 5' of the lesion creating a single stranded gap. This is subsequently filled by DNA polymerase/ε (Houtgraaf, Versmissen and Giessen, 2006)

3. *Mismatch repair*

Mismatch repair is utilised when replication errors occur resulting in incorrectly matched bases. Msh2-6 proteins recognise and bind to the erroneous DNA. Proteins Mlh1 and Pms2 are recruited to the site allowing the cleavage of the mismatched bases, which are consequently replaced by DNA polymerase-α (Houtgraaf, Versmissen and Giessen, 2006)

4. *DSB repair*

Caused by ionising radiation, reactive oxygen species and genotoxic drugs, double strand breaks in DNA can potentially be very damaging. As both strands of DNA are damaged, chromosomal fragmentation, deletions and transclocations can easily occur. One of the observable first responses is extensive phosphorylation of H2AX by ATM and other kinases. This activates one of two repair pathways. Firstly homologous recombination, which requires homology between the sister chromatid. Rad51, a member of the Rad52 epistasis group, searches for the intact gene on the sister chromatid, this is then copied into the damaged site. Other proteins involved in this repair pathway include Rad52, Rad54, Rad50, Mre11, Nbs1, Brac1 and Brac2, although their precise roles are unclear. The second pathway is non-homologous end joining (NHEJ) which does not necessitate homology. The broken DNA is simply joined together by the DNA ligaseIV/Xrcc4

complex . Once again other proteins are involved (Ku70, DNA-PK_{CS}, and the Rad50/Mre11/NBS1 complex) yet their exact roles are yet to be elucidated (Houtgraaf, Versmissen and Giessen, 2006)(Figure 6).

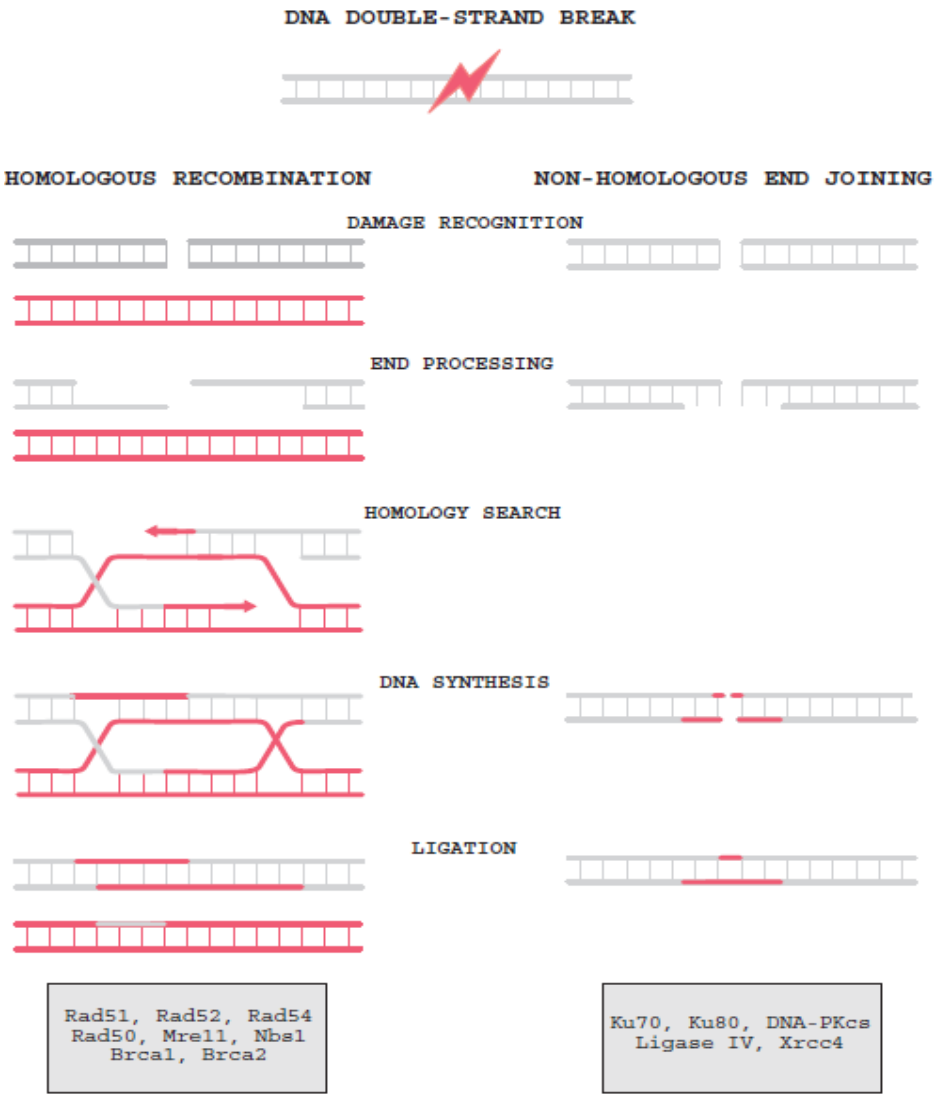


Figure 6: Mechanism of DSB repair by homologous recombination or non-homologous end joining. (Taken from Houtgraaf, Versmissen and Giessen, 2006

1.4.3 Reactive Oxygen Species

Reactive oxygen species (ROS) is a collective term used to describe oxygen derived free radicals including the superoxide anion (O_2•−), hydroxyl (HO•), peroxyl (RO_2•) and alkoxyl (RO•) radicals, as well as nonradical species including hydrogen peroxide (H_2O_2) (Halliwell and Cross, 1994). These species are inevitably formed during the metabolism of oxygen, especially in the reduction of oxygen by the electron transfer chain (ETC) system in the mitochondria, mainly at complex I and complex II. Mitochrondia is considered the major source of intracellular ROS and it is predicted that 1-2% of mitochondrial O_2 consumed goes on to form ROS (Circu and Aw, 2010).

To date, around ten sources of oxygen radicals within the mitochondrial alone have been identified, including α-ketoglutarate dehydrogenase, a Krebs cycle enzyme (Starkov et al., 2004), $p66^{Shc}$ (Migliaccio, Giorgio abd Pellicci, 2006) and monoamine, an outer membrane enzyme (Andreyev et al., 2005). Other organelles also contribute to levels of ROS, including peroxisomes and the endoplasmic reticulum, as well as external sources such as UV radiation, toxic chemicals and drugs.

The formation of superoxide anion radical creates a cascade leading to the formation of other ROS. Superoxide anion is converted to hydrogen peroxide and oxygen by the enzyme manganese superoxide dismutase, and transformed furthermore to the highly reactive hydroxyl ion via Fenton reactions (Andreyev et al., 2005). The hydroxyl radical is able to react instantaneously with any biological molecule (RH) from which it can abstract a hydrogen atom. The resulting free radical (R•) is more stable and hence longer-lived than the hydroxyl radical.

ROS, and in particular the hydroxyl radical are able to interact with all biological macromolecules (lipids, proteins and nucleic acids) and inflict damage. There is now growing support for the free radical theory of ageing, by which changes in biological function over time are caused at least in part by the accumulation of cellular oxidative damage (Harman, 1972). During ageing there is a progressive increase in the percentage of oxidatively damaged proteins, lipids and nucleic acids (Stadtman, 2001).

ROS modify both the structure and function of proteins. Metal-catalyzed protein oxidation results in addition of carbonyl groups or cross-linking or fragmentation of proteins. Lipid (peroxidation) aldehydes can react with sulfhydryl (cysteine) or basic amino acids (histidine, lysine). Similarly, modification of individual nucleotide bases, single-strand breaks and cross-linking are the typical effects of reactive oxygen species on nucleic acids.

The most common protein modification by ROS is the addition of carbonyl groups on arginine, lysine, threonine and proline residues. ROS is also able to modify cysteine residues as well as methionines to create methione suphoxide and its sulphone (Hipkiss, 2006). Oxidatively damage proteins are generally less active, less thermostable and expose hydrophobic residues at their surface (Friguet, 2006). It can also lead to proteins that are preferentially degraded by the proeatsome, or other forms that are resistant to proteolysis, or forms that inhibit the action of proteasomes to degrade other incorrect protein conformations (Stadtman, 2001).

In regard to lipids, on oxidative attack they are peroxidised. The high concentration of allylic hydrogens in the unsaturated fatty acids of phospholipid membranes makes them susceptible to oxidation by ROS (Bokov et al., 2004).

Finally DNA is susceptibe to damage by ROS, especially the highly damaging hydroxyl radical. The main products of oxidative DNA base damage are thymine glycol (Wang, Kreutzer and Essigmann, 1998) and 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) (De Bont and van Larebeke, 2004). Thymine gycol has a low mutagenicity, while 8-oxodG has the ability, albeit with low frequency to cause G>T transversions upon replication (Alexeyev, 2009).

1.4.4 Antioxidant Enzymes

Cells are under a constant barrage from ROS, contributing to damage in the cell, however they are equipped with a vast array of defences to prevent the accumulation of damage to macromolecules. When all these defences fail, the apoptotic pathway is induced to remove the damages cell (Hiner et al., 2002)

The cell acts to buffer the effects of ROS by actively reducing intracellular ROS levels, one of the key defences against ROS toxicity is a series of antioxidant enzymes, with the rate of ROS generation and the levels of antioxidant defences determine the overall level of oxidative stress in the cell. This will be referred to as ROS buffering capability throughout.

The main antioxidant enzymes are superoxide dismutase (SOD), of which there are two forms; the copper and zinc containing SOD (CuZnSOD) and manganese-containing SOD (MnSOD), catalase (CAT) and glutathione peroxidise (GPX).

SODs work to reduce the ROS level by catalysing the breakdown on the superoxide anion into oxygen and hydrogen peroxide, rendering the molecules safe (Zelko I, Mariani T, Folz R, 2002). The copper and zinc containing forms of the enzymes are found throughout the cytosol, whereas the manganese SOD is present in the mitochondria, and appears to be the most biologically

important of the class, as mice lacking the mitochrondrial form die soon after birth. (Nozik-Grayck, Suliman, Piantadosi, 2005).

Catalases catalyse the conversion of hydrogen peroxide to water and oxygen by using either an iron or manganese cofactor (Chelikani Fita, Loewen, 2004). The cofactor is oxidised by a hydrogen peroxide molecule, and then transfers the oxygen to a second molecule of hydrogen peroxide, restoring the cofactor to its original state (Hiner et al., 2002). Although it has important functions in hydrogen peroxide elimination, patients with acatalasemia, a disease characterised by a deficiency of catalase, show few phenotypic symptoms (Mueller, Riedel, Stremmel, 1997) but can be manifested by recurrent infection or ulceration of the gums and mouth.

Finally, the antioxidant enzyme glutathione peroxidase acts to catalyse the breakdown of hydrogen peroxide and organic hydroperoxides, by oxidation of its selenium cofactors. There are at least four different glutathione peroxidase isozymes present in vertebrates (Brigelius-Flohé, 1999). Glutathione is key for maintaining reducing conditions within the cell. Glutathione peroxidase 1 is highly abundant and is the most efficient of the glutathione peroxidases in scavenging hydrogen peroxide, while glutathione peroxidase 4 is effective in the breakdown of lipid hydroperoxides. Interestingly, glutathione peroxidase 1 is not essential, as mice with a deficiency for this enzyme have normal average lifespans, (Ho et al., 1997) but yet are hypersensitive to oxidative stress (de Haan, 1998). It is well established that cellular glutathione levels fall as a function of age (Hipkiss, 2006).

1.4.5 Oxidative Stress Theory of Ageing

A theory that has gained a large amount of interest over the years is the Oxidative Stress Theory of Ageing. In 1972, Harman proposed a Free Radical theory of ageing, by which free radicals generated via Haber-Weiss chemistry as a product of normal reactions would damage biological structures, including DNA, causing mutations. According to his theory, the deterioration in biological function over time is due to the progressive accumulation of free-radical induced cellular damage (Harman, 1972). Since then, it has been shown that ROS, some of which are not free radicals also play a role in oxidative damage, including hydrogen peroxide, thus generating the revised Oxidative Stress Theory of Ageing (Beckman and Ames, 1998).

Much evidence over time has supported the Oxidative Stress Theory of Ageing, critically at the molecular level, the accumulation of altered proteins, both within cells and extracellularly has been shown to increase with age (Rosenberger, 1991). Most cells have a wide range of mechanism designed to both prevent the transcription of altered proteins and to recognise and

dispose altered proteins, hence preventing their accumulation (Hipkiss, 2006). Therefore it is thought that ageing is associated with either an increase in generation of altered macromolecules, a decline in their removal, or both.

The Oxidative Stress Theory lies on three basic assumptions which will be discussed in turn.

1. *Levels of oxidatively damaged biomolecules increase with age.*

It has been shown that protein oxidation increases with age. The most widely studied measure of protein oxidation is the additional of a carbonyl group, which can render proteins inactive (Levine, 1983). There is an increase in protein carbonyls in cultured fibroblasts from donor's from individuals aged 60 plus (Oliver et al., 1987). This is further supported by the finding that global protein carbonyl levels in human tissues including the human brain increase exponentially with age (Moskovitz et al., 2002). Oxidative damage to specific proteins has also been demonstrated using antibodies designed to detect oxidized residues, all of which have been shown to accumulate oxidative damage as a function of age. These include glucode-6-phosphate dehydrogenase (Agarwal and Sohal, 1993), adenine nucleotide translocase (Yan and Sohal, 1993) and mitochondrial aconitase (Yan et al., 1997).

Interestingly, the accumulation of protein carbonyl groups is not a linear function of age, there is a marked tendency to increase in the last third of life in many organisms, and can affect one in every three proteins (Hipkiss, 2006).

Lipid peroxidation has also been shown to increase with age in rats (Roberts and Reckelhoff, 2001), as does DNA oxidation, as measured by levels of oxo8dG in several tissues (Hamilton et al., 2001).

It is thought that mitochondrial dysfunction leads to increased levels of ROS. Lowered ATP production by mitochondria can cause a compensatory increase in glycolytic activity, this in turn will raise levels of methylglyoxal. These rasied levels could result in reduced glutathione peroxidise activity, increasing the potential for ROS damage (Hipkiss, 2006).

2. *Manipulations that increase lifespan with reduce oxidative damage to biomolecules.*

Calorie restricted rodents have been shown to have an increased lifespan (Merry, 2002). Calorie restriction in rats has supported the Oxidative Stress Theory of Ageing, as it has been shown to

reduce levels of lipid oxidation (Rao et al., 1990), carbonyl content of proteins (Lass et al., 1998), and oxidative damage of DNA (Hamilton et al., 2001).

Calorie restriction seems to reduce the amount of ROS being produced by the mitochondria (Gredilla et al., 2001), accounting for the reduction in oxidative damage. This could be further attenuated by the finding that calorie restriction may improve the repair and turnover of altered biomolecules, including DNA. This is shown by the increased repair of UV induced DNA damage in calorie restricted rodents (Guo et al., 1998).

Genetic manipulations to model species are also consistent with the oxidative stress theory of ageing. Mutations that increase the lifespan of C.elegans (*age-1* and *daf-2* amongst others) have all shown to increase the resistance of worms to several induced stresses, including heat shock, UV and most importantly ROS (Johnson et al., 2002), this permits the assumption that the increased lifespan on these mutant worms is due to increased stress resistance, including oxidative stress (Bokov et al., 2004).

Mice with a targeted $p66^{shc}$ knockout show a significantly increased lifespan. It is thought that $p66^{shc}$ is a downstream target of p53, and is critical for the transduction of stress-activated signals in the apoptotic response. One of the standout phenotypes of this mutant is a vastly greater resistance to oxidative stress compared to wildtype littermates. They show lower basal ROS levels and reduced oxidatively damaged DNA (Trinei et al., 2002).

Therefore the data from these species are with Harman's mechanistic concept of ageing. However there is some evidence contrary to this. Naked mole rats are the longest lived rodents, and show very little age-related deterioration of biological function. In comparison to age-matched mice, they showed greatly increased levels of lipid damage, DNA damage and protein carbonyl content, thus suggesting that a continuing robust defence against oxidative stress is not critical for a long lifespan in the naked mole rat (Andziak et al., 2006). However it must be considered that this species may be 'an exception to the rule', and whilst oxidative stress many not play a major role in determining the lifespan of the naked mole rat; it may be critical in determining longevity in others.

3. *Manipulations that alter the ROS balance will influence lifespan*

Establishing a causal relationship between oxidative stress and longevity, instead of a correlative relationship, rests upon the ability of experimental manipulation of ROS to impact longevity.

However the evidence here is less definite, thus causing uncertainty over the Oxidative Stress Theory of Ageing.

By using pharmacological manipulations, some researchers have shown that increased levels of synthetic antioxidants EUK-8 and EUK-134 to alter the flux of ROS, extended the mean and median lifespan of C.elegans (Melov et al., 2000). Whereas subsequent studies have been unable to replicate these findings, with some even suggesting that increased EUK-8 levels reduced lifespan and fertility (Keaney and Gems, 2003). Genetic manipulations have also been investigated with mixed results. Overexpression of the antioxidants Cu/ZnSOD or catalase in *Drosophila* was first shown to increase maximum lifespan by 14-34% (Orr and Sohal, 1994), but was compromised by the method used to cause overexpression, which was shown to increase lifespan independently (Kaiser et al., 1996). Subsequently, using a larger number of flies to mitigate methodology issues, no increase in lifespan was observed (Orr et al., 2003). Improved protocols using yeast inducible systems have shown that overexpression of Cu/ZnSOD alone resulted in a increase in maximum lifespan of upto 48%, whilst overexpression of catalase has no beneficial effect on lifespan (Sun and Tower, 1999), yet no investigations were made concerning the effect on oxidative damage.

In mice, reduced expression of MnSOD (*Sod2*$^{+/-}$*)* are more susceptible to oxidative stress and display higher levels of damaged DNA than control littermates, yet did not have a reduced lifespan (Van Remmen et al., 2003).

While there is data to support the first two predictions, the evidence is inconsistent when considering the third assumption. Little has been done to ascertain a causal relationship between increased/decreased antioxidant levels and accumulated macromolecular damage. Until these experiments have been tested, doubt still reigns over the oxidative stress theory of ageing.

1.4.6 Oxidative Stress and Premature Ageing Laminopathies

The concept that oxidative stress might contribute to the pathogenesis of laminopathies has been quickly gaining support. As discussed accumulation of ROS-mediated oxidative damage is a key characteristic of ageing, whereby a disturbance in the pro-oxidant/antioxidant balance results in macromolecular level impairments including DNA strand damage. When defences fail, cellular senescence is initiated and is characterised by an impaired redox state, irreparable DNA damage and a build up of oxidized proteins and contributes to organismal ageing.

Evidence has shown that HGPS fibroblasts show a 1.6 fold increase in the basal levels of ROS, compared to age matched controls, this is also correlated with an increase in the levels of protein carbonyl groups, showing a 1.7-4 times increase to age and passage matched control fibroblasts. The carbonyl content rose throughout passage numbers, indicating the damaged proteins could not be repaired (Viteri, Chung and Stadtman, 2010).

Mitochondria are considered to be the main source of ROS, defective mitochondria produce ROS is excessive quantities. Mitchondrial DNA encoded subunit II of the cytochrome oxidase complex IV (COX2) is significantly decreased in HGPS fibroblasts (Caron et al., 2007). ATP can also be used as a measure of functionality of mitochondria, of note, ATP levels of progeria fibroblasts were reduced by approximately 50% of age matched controls. This may be a sign of mitochondrial dysfunction, leading to increase ROS, and may also be insufficient energy for the proteasomes to degrade oxidatively damaged proteins (Viteri, Chung and Stadtman, 2009). Mitochondrial dysfunction producing excess ROS can lead to a vicious cycle, whereby ROS might act to reinforce the mitochondrial defect, hence producing greater quantities of ROS available to cause damage within the cell (Caron et al., 2007). As progeria is characterised by a defective DNA repair response, they are not able to repair the damage induced by excessive ROS.
Proteasomal activity showed a 40% decrease in patient fibroblasts (Viteri, Chung and Stadtman, 2009), most likely due to the accumulation of oxidised proteins, this is likely to impair processes including cell-cycle progression, apoptosis and DNA damage repair (Alexeyev. 2009).
It appears that the permanent farnesylation of prelamin A can account for excessive ROS production and the increase in senescent fibroblasts, as HGPS cells treated with a FTI showed a consistent reduction in ROS overproduction. This suggests that the toxicity of progerin causes increased oxidative stress within HGPS cells (Caron et al., 2007).

With regard to antioxidant levels, it was demonstrated that progeria cells have decreased levels of the mitochondrial enzyme MnSOD compared to age-matched controls, along with an impaired upregulation of antioxidant enzymes in response to chronic stress, implying a deficiency in stress signalling pathways (Yan et al., 1999). Patient fibroblasts also show reduced levels of catalase and most critically glutathione peroxidise (30% of normal protein levels) (Yan et al., 1999).
However, a more recent study found contradictory results. Analysis of purified mitochondria (as opposed to whole cell lysate) showed an increase in mRNA and protein levels for MnSOD,

dependent upon passage number (Viteria, Chung and Stadtman, 2009). The divergence of these results requires continuing research.

It has been shown that mature lamin A can act as a redox sensor. It contains three conserved cysteine residues in the C-terminal tail, which are able to form reversible disulphide bridges in response to oxidative stress, and mediate the induced stress response. The disuphilde bridges activate cell cycle arrest, allowing time for DNA damage repair and other checkpoints to prevent inheritable mutations, however with increased replicative age, the cysteine residues become progressively less accessible for disulphide bond formation (Pekovic et al., unpublished data). The presence of a cysteine-deficient lamin A leads to nuclear disorganisation and premature senescence. Therefore mutations in lamin A may lead to the impairment of its ability to act as a 'free radical sink', and also prevent the redox sensitive activation of the stress response pathway including NFκB (Pekovic et al., unpublished data). It has been proposed therefore, that the conserved cysteine residues in the lamin A tail may be a governing factor of cellular ageing, and loss of function through irreversible oxidation or mutations results in nuclear deformations, increased ROS accumulation and premature cellular senescence (Pekovic et al., unpublished data).

1.5 Aims of the Thesis

Fibroblasts from HGPS and RD patients accumulate DNA double strand breaks (DSBs). Initially it was thought that the presence of progerin (HGPS) or farnesylated pre-lamin A (RD) in some way impairs the rate of double strand break repair. However other studies have shown that treatment with FTIs does not decrease the load of DSBs. It has also been shown that progerin fibroblasts sustain raised levels of intracellular ROS. Since ROS can act as an endogenous mutagen and is considered to be involved in the accumulation of unrepairable DSB in aged cells, we therefore speculated that these high levels of ROS may be responsible for the accumulation of DSB within fibroblasts from premature ageing syndromes.

The overall aim of this thesis therefore is to contribute to the understanding of the mechanisms and dynamics involved in DNA damage by oxidative stress in cultured fibroblasts with premature ageing syndromes.

To meet this overall aim, my specific aims within the thesis are to:

a) Examine the differences between levels of intracellular ROS from cell lines from young, old, HGPS and RD donors, as well as examining the dynamics of ROS recovery after treatment with varying degrees of oxidative stress.

b) Determine the variation in the proliferation indices of the cell lines by a Ki67 index after treatment with oxidative stress and the ROS scavenger N-acteyl cysteine.

c) Discover if the previously shown accumulation of abnormally shaped nuclei in HGPS and RD fibroblasts is correlated with increased DNA damage after oxidative stress.

d) Gain a deeper understanding of both the DNA damage and repair dynamics of the cell lines by measuring and analysing double strand break foci after DNA damage induction by both oxidative and non-oxidative stress. This will be achieved via the application and analysis of a statistical model.

e) Assess whether treatment with N-acetyl cysteine may be beneficial in combinatorial therapy to HGPS patients

Chapter 2: Materials and Methods

2.1 Cell Culture

Human dermal fibroblasts were cultured in Dulbecco's Modified Eagles Medium DMEM (Sigma) supplemented with 10% heat inactivated foetal bovine serum (FBS) (Invitrogen) and penicillin/streptomycin. Cells were maintained in 12.5cm^2, 25cm^2 or 75cm^2 flasks depending on the cell type and confluence, and kept in a 37°C, 5% CO_2 incubator. Cells were passaged at approximately 70% confluence. Old media was aspirated off before adding versene (2ml for a 25cm^2 flask, 3ml for a 75cm^2 flask). The versene was removed, and a combination of versene and trypsin added (same quantities as versene previously). The flask was then placed in the incubator for 3-4 minutes. Fresh media was then added (same quantity as versene/trypsin solution) to stop the action of trypsin. The cell containing solution was then placed in a universal tube, and placed in the centrifuge for 5 minutes, at 4°C at 1000RPM. The supernatant was carefully removed and the cell pellet resuspended in fresh media in the correct dilutions and returned to the incubator. Fresh media was replaced every 3-4 days.

When freezing cells, the same procedure was followed. When the supernatant was removed after centrifugation, the pellet was resuspended in fresh media, 0.9ml per cryovial, and 0.1ml of DMSO added per cryocial and placed into the -80°C freezer and subsequently transferred to -150°C freezer.

Cell Line	*Description*	*Passage number at time of culture*	*Split Ratio*	*Place of purchase*
GMO3348	Healthy human dermal fibroblast from young male (8 years old)	P10	1:3	Human Genetic Cell Repository
GMO9558	Healthy human dermal fibroblast from young male (7 years old)	P7	1:3	Human Genetic Cell Repository
AG13129	Healthy human dermal fibroblast from elderly male (89 years)	P9	1:3	Human Genetic Cell Repository
AG11695	Healthy human dermal fibroblast from elderly male (82 years)	P6	1:3	Human Genetic Cell Repository
RD30178	Human dermal fibroblast from patient with restrictive dermopathy. Homozygous Zmpste24 null.	P13	1:2	Corielle Cell Repository
AGO6917	Human dermal fibroblast from male patient with HGPS (3 years). Classical C>T mutation.	P13	1:2	Corielle Cell Repository
AG11513	Human dermal fibroblast from female patient with HGPS (8 years). Classical C>T mutation.	P9	1:2	Corielle Cell Repository
R453W	Fibroblast with a R453W mutation resulting in AD-EDMD.	P10	1:2	Corielle Cell Repository

2.2 Immunofluorescence

Dermal fibroblasts were seeded on glass coverslips at a density of 50,000 cells per well of a 6 well plate (Sigma) and allowed to reach 70% confluence. Cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. The coverslips were then washed three times in fresh 1x PBS for five minutes each. Cells were then permeabilised with 0.5% Triton X-100 (Sigma) in PBS for 15mins at 4°C, washing 3 times with PBS repeated again.

1% NCS in PBS was added to block unspecific binding of the antibodies for a minimum of 45mins at room temperature. Coverslips were then incubated with primary antibodies in NCS for 1 hour at room temperature in the dark in moist staining chambers. After the hour, cells were dipped five times sequentially in 5 beakers of fresh PBS and transferred to secondary antibodies for 1hr at room temperature in the dark in staining chambers. After incubation with secondary antibodies, cells were once again dipped five times sequentially in four beakers of PBS and lastly five times in deionised water. Coverslips were then mounted onto glass slides in 30% glycerol containing Mowiol (Sigma), 2mg/ml 4,6-diamindino-2-phenylindole (DAPI) and 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO; Sigma). Images of stained cells were taken using a Bio-Rad

Radiance 2000 confocal laser scanner attached to a Zeiss Axioskop. Images were viewed and saved using AxioVision, before importing to ImageJ and Adobe Photoshop 7.0.

Primary Antibodies used;

Anti-phospho-Histone H2AX (Millipore) – Mouse monoclonal antibody against Histone H2A.X phosphorylated at Ser139.
Anti-Lamin A (Sigma) - Rabbit monoclonal antibody Anti-Lamin A (C-terminal)
Ki67(Abcam) - Rabbit polyclonal antibody. Synthetic peptide conjugated to KLH derived from within residues 1200 - 1300 of Human Ki67.
XPA (FL-273) (Santa-Cruz) - Rabbit polyclonal antibody against amino acids 1-273 in human XPA
Rad 51 (Santa-Cruz) - Rabbit polyclonal antibody against amino acids 1-92 in human Rad51
53BP1 (Abcam) - Rabbit polyclonal antibody against exons 11-12 in human 53BP1

Secondary Antibodies used:
Donkey anti-mouse IGg conjugated to either tetramethylrhodamine B isothicyanate (TRITC) or fluorescein isothiocyanate (FITC).
Donkey anti-rabbit IGg conjugated to either TRITC or FITC at a working ratio of 1:50 NCS/PBS (both Stratech Scientific Ltd).
Secondary antibodies were tested to ensure unspecific binding. All results showed no unspecific binding.

2.3 Fluorescence Activated Cell Sorting Analysis

Fibroblasts (GMO3348, AG13129, RD30178 and AG11513) were seeded in a six well plate, at a density of 50,000 cells per well. The cells were grown in DMEM supplemented growth media and allowed to reach approximately 70-80% confluence. Once at confluence, old media was removed and replaced with 1ml of phenol free, low glucose DMEM.
Dichlorfluorescein-diacetate (DCF) (Cell Signalling) dye used to measure intracellular ROS levels was diluted in 86µl DMSO to allowing permeation of membranes. 5µl of this solution was added per 1ml of phenol free DMEM and cells incubated for 1 hour in the dark at 37°C, 5% CO_2. Cells then received one of the six following treatments.

Untreated Controls –No DCF dye added, 5µl DMSO added instead to ensure DMSO had no effect on ROS levels.

Basal- DCF dye added to measure basal ROS levels

Low Stress – 20 mins before the end of incubation, 150µM H_2O_2 was added.

High Stress – 20 mins before the end of incubation, 350µM H_2O_2 was added.

NAC- Before incubation, 0.0816g of NAC was dissolved in 1ml sterile deionised H_2O. NAC treated cells had 20µl of NAC solution added per 1ml phenol free DMEM at the same time as DCF-dye.

NAC recovery – Same as described for NAC treated cells above, but 20 minutes before incubation, 150 or 350µM H_2O_2 was added.

After the full hour incubation, media containing H_2O_2 was removed, cells were washed twice with 1x PBS, carefully scraped and suspended in 500µl PBS in a FACS vile. All results were based on triplicate results over two separate assays. Controls were used to determine autofluorescence and deducted from all experimental results. Cells are sorted based on DA-DCF fluorescence using FACScaliber using CellQuestPro software and results analysed in FlowJo.

Alternative protocols in which treatments are added either added concurrently or prior to DA-DCF dye (Eruslanov and Kusmartsev, 2010) could have been used and may have provided more consistent results.

2.4 Cell Treatments with Hydrogen peroxide, Etoposide and NAC

Fibroblasts were cultured on glass coverslips in 6 well plates (4 coverslips per well), and allowed to reach 80% confluence in supplemented growth media. To induce DNA damage two procedures were used;

1. Etoposide Treatment.

Growth media was removed from cells, and 1ml phenol free, low glucose DMEM media was added to each well. Untreated controls did not receive any further treatment. To treated cells, 4µl of 20µM etoposide (Sigma) was added, and cells were incubated in the dark for 20mins at 37°C, 5% CO_2. After incubation, the media and etoposide were removed and replaced with supplemented 10% growth media and immunofluorescence procedures followed.

2. Hydrogen Peroxide Treatment.

Growth media was removed from cells, and 1ml phenol free, low glucose DMEM media was added to each well. Untreated controls did not receive any further treatment. Hydrogen peroxide comes in standard 8.8M stock solution and was diluted as follows;

11.37µl 8.8M H_2O_2 in 988.63µl sterile dH_2O → 100mM

40µl of 100mM H_2O_2 in 960µl sterile dH_2O → 4mM

For low stress treatment, 37.5µl of the 4mM solution was added per 1ml growth media (37.5µl).

For high stress treatment, 87.5µl of the 4mM solution was added per 1ml growth media (87.5µl).

Cells were incubated in the hydrogen peroxide in the dark for 20mins at 37°C, 5% CO_2. After incubation, the media and etoposide were removed and replaced with supplemented 10% growth media and immunofluorescence procedures followed.

3. NAC pre-treatment

0.0816g of NAC dissolved in 1ml sterile dH_2O to give 10mM solution. 20µl was added per 1ml growth media and incubated for 1hr at 37°C, 5% CO_2.

2.5 Statistical Methods

All comparison treatments were against paired controls unless otherwise stated. A paired, two tailed student's T-test was used throughout to quantify statistical significance at a 5% probability level.

Chapter 3: Reactive Oxygen Species Levels and DNA Damage in Progeroid Syndromes

3.1 Introduction

In order to determine the effect of oxidative stress on premature ageing syndromes it is vital to first determine the levels of ROS within the cells under different levels of stress. Preliminary assays were used to investigate reactive oxygen species levels in cells and their recovery after oxidative assault. This would subsequently allow further investigations into the role of oxidative stress and reactive oxygen species in some of the characteristic phenotypes observed in syndromes caused by *LMNA* mutations. The phenotypes investigated included nuclear envelope structural abnormalities, altered proliferative status, increased cellular senescence and DNA damage repair defects.

From these results it should be possible to determine the contribution of oxidative damage to the phenotypes observed, and therefore indicate whether or not a known antioxidant such as NAC would be appropriate as a form of therapeutic treatment for HGPS.

3.2 Optimisation of FACS assay

Preliminary investigations were performed in order to optimise subsequent assays. Fibroblasts from a healthy young subject (GMO3348) were used to determine the optimal incubation time required for both the DA-DCF ROS reporter dye and hydrogen peroxide.

Fibroblasts were grown to confluence in 6 well plates and were treated with 5µM of DA-DCF solution and incubated for 20, 30, 40 or 60 minutes in the dark, this was repeated in triplicate wells to gain accuracy. The DA-DCF dye reports the level of intracellular ROS at a given time. From figure 7A it can be seen that incubation for 20 and 30 minutes produced a range of peaks over the triplicate wells showing inconsistent results over 3 magnitudes. The results show a double peak over the short incubation times suggesting a population of cells with higher ROS levels. Over 40 minutes and 60 minutes, triplicate results showed greater consistency with each other, however those incubated for 40 minutes produced a wider peak, indicating a wider range of ROS levels within the cells. 60 minutes incubation time however provided both consistency across triplicate results and the narrowest peak of all the incubation times, indicating reduced

variability and therefore was chosen to be the appropriate length of incubation for DA-DCF dye. The peak that was present in the shorter incubation times is not present at 40 and 60 minutes incubation. It is thought that this peak may represent an excess of DCF-DA dye in the media and not intracellular ROS.

Only GMO3348 fibroblasts were used to optimise this assay and optimal protocols were then applied to additional cell lines.

Incubation time with hydrogen peroxide was then investigated to determine the effect of hydrogen peroxide on intracellular ROS (Figure 7B). Once again dermal fibroblasts from a young healthy subject were used and results applied to additional cell lines. Fibroblasts were incubated with DA-DCF ROS reporter dye for 60 minutes, and with 150μM hydrogen peroxide for either 20, 40 or 60 minutes in the dark. Triplicate results were taken and combined in Figure 7B. Intracellular ROS declines with incubation time, suggesting that the rise in ROS occurs within the first 20 minutes of H_2O_2 incubation, with minimal cellular buffering to reduce ROS levels.

3.3 Laminopathic Fibroblasts show reduced resistance to induced oxidative stress compared to healthy controls.

Next, I determined the levels of intracellular ROS across young, old, RD and HGPS fibroblasts in order to establish the levels of oxidative stress within ageing and laminopathic cells. It was hypothesised that healthy fibroblasts from older individuals may show higher basal levels of ROS than fibroblasts from younger individuals, as the machinery in the cell used to reduce the pool of ROS may degenerate over time (Agarwal et al., 1993). It was also thought that laminopathic cell lines from HGPS and RD patients might have higher basal levels of ROS. As lamins A/C and Zmpste24 mutations tested resulted in premature ageing syndromes, it was predicted that the levels of ROS would more likely reflect those observed from individuals of old age, instead of fibroblasts from children nearer to their own chronological age. If these hypotheses were true, it would help to support the Oxidative Stress Theory of Ageing.

The four cell lines were treated with DA-DCF ROS reporting dye and then either left untreated, treated with high levels of oxidative stress (350μM H_2O_2), low levels of oxidative stress (150μM H_2O_2 for 20 minutes, or pre-treated with the antioxidant NAC for an hour, and then treated with high or low stress for 20 minutes.

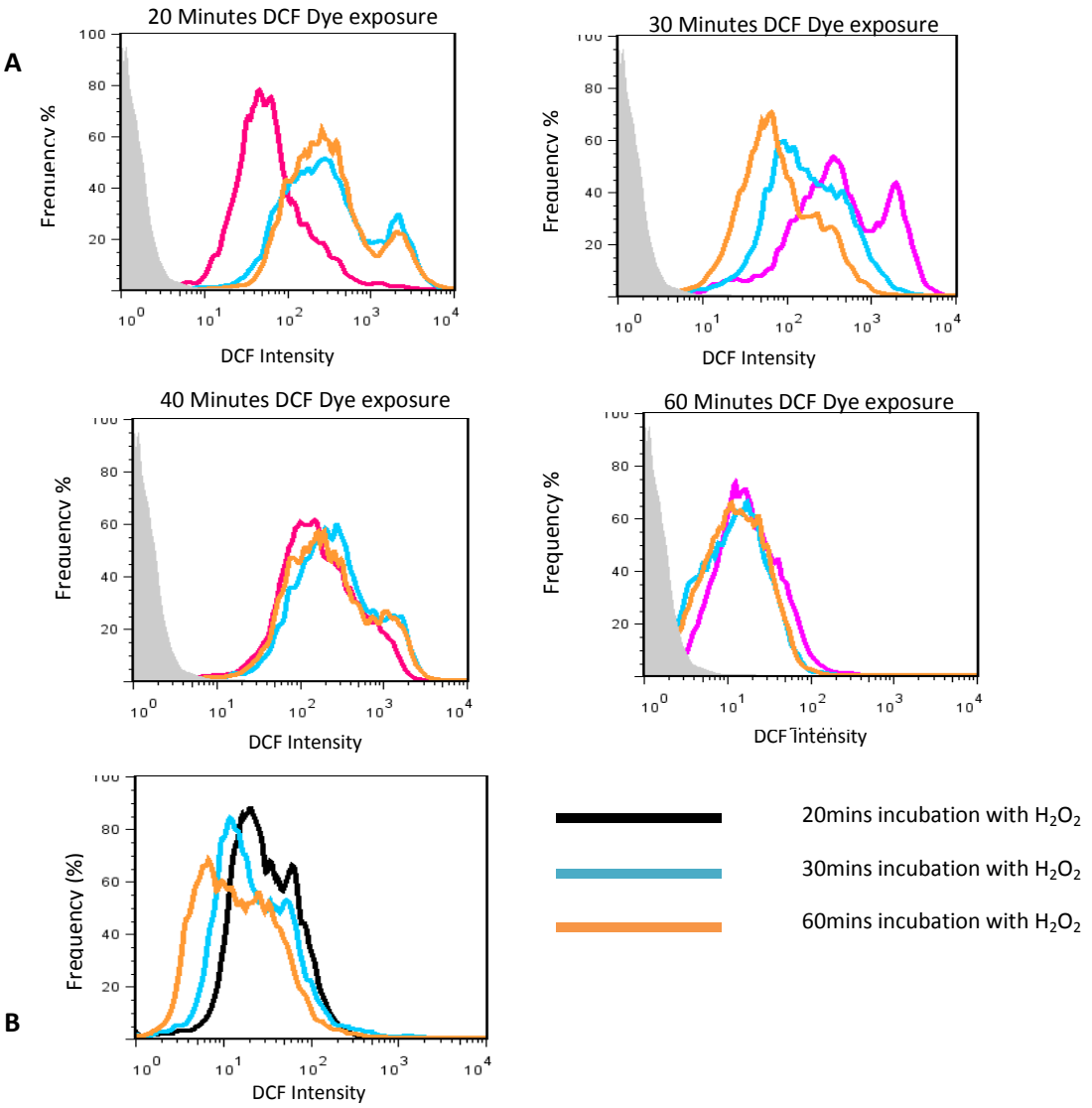


Figure 7: A Optimisation of incubation time of ROS reporting DA-DCF dye. Grey areas represent autofluorescence. 60 minutes incubation with DA-DCF dye gave rise to the most consistent results over triplicate wells to measure ROS levels as shown by a shift to the right indicating higher ROS levels.

B: Optimisation of incubation time with 150µM H_2O_2. 20 minutes with 150µM H_2O_2 raise ROS levels to the highest levels over the tested time points, and therefore used throughout.

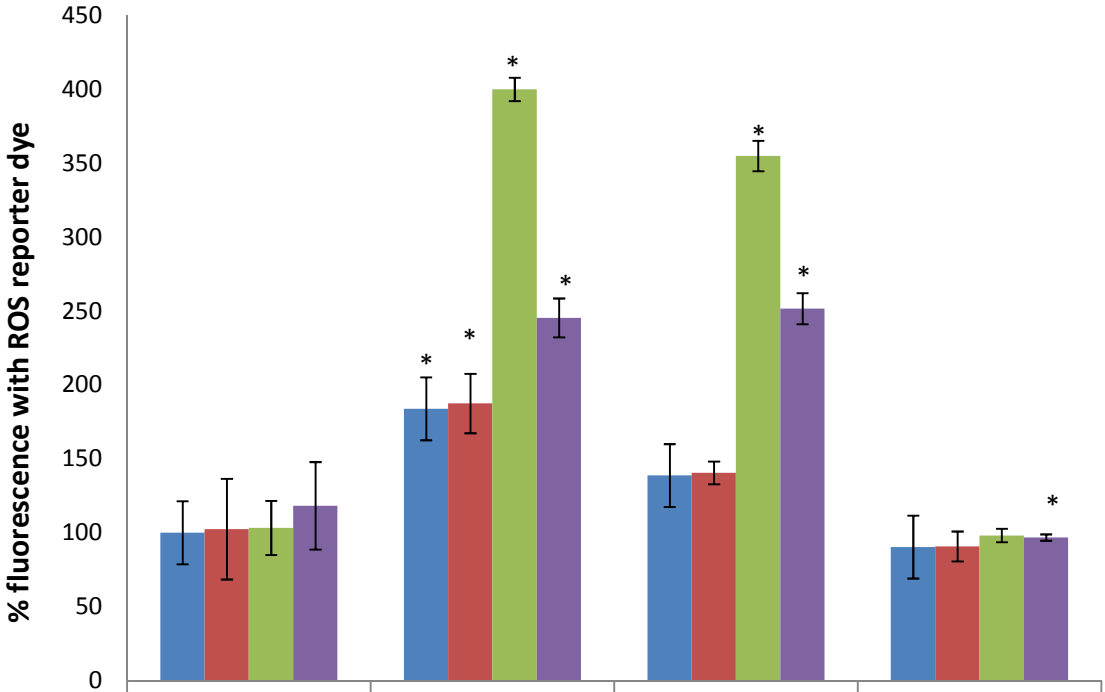
The results obtained showed that fibroblasts from young (GMO3348) and old (AG13129) donors demonstrated very little difference in either basal levels of ROS, or levels of ROS after either high (350µM hydrogen peroxide) or low (150µM hydrogen peroxide) ROS stimulation (Figure 8). Fibroblasts from a HGPS patient show slightly higher levels of basal ROS levels (18.3% increase), yet in response to induced oxidative stress, show dramatically higher levels compared to healthy control fibroblasts. There was a statistical significant 33.5% increase when compared to GMO3348 fibroblasts in response to high oxidative stress and an 81.3% increase in response to

low stress. Fibroblasts from a patient with restrictive dermopathy also showed interesting results. Whilst there was only a modest 3% increase in basal ROS levels compared to GMO3348, there were dramatic increases when oxidative stress was induced with hydrogen peroxide (117.5% increase with high stress and 155.8% increase upon low stress both statistically significant). These results show that the fibroblasts from both RD and HGPS patients, despite showing similar basal levels of ROS compared to control fibroblasts were unable to cope with even modest amounts of oxidative stress, as induced with hydrogen peroxide (Figure 8).

As a control condition, N-acetylcysteine (NAC), a well tolerated antioxidant *in vivo* and *in vitro* was used as it reacts with oxidant species including the hydroxyl radical, hydrogen peroxide and hypocholorous acid. It is able to act as an oxidative sink as it is preferentially oxidised, thereby protecting biological macromolecules (Aruoma et al., 1989). NAC treatment in all cell lines revealed that it acted as an effective antioxidant and was able to reduce ROS levels intracellulary to below basal levels (9.68% in GMO3348, 11.44% in AG13129, 18.18% in HGPS fibroblasts and 4.93% in RD fibroblasts) after mild oxidative stress.

The level of recovery after oxidative stress was also measured to determine if the fibroblasts have the ability to reduce the levels of ROS. Fibroblasts were treated with hydrogen peroxide and NAC as described previously, reseeded and then allowed to recover for 24 hours before levels of intracellular ROS were measured using DA-DCF reporter dye.

From Figure 9 it can be seen that fibroblast from an old donor AG13129 show comparable basal ROS levels to young dermal fibroblasts, and also consistently show lower ROS levels after hydrogen peroxide treatment, and are also show an ability to recover to near basal levels (5%) after reseeding and recovery after hydrogen peroxide treatment. The results indicate that although HGPS and RD fibroblasts show increased sensitivity to both severe (both RD and HGPS show a >50 fold increase) or mild (RD a 43 fold change and HGPS a 35 fold change to basal levels) oxidative stress, they still displayed an ability to recover following ROS stimulation to 13.6% and 45.2% of basal levels for RD and HGPS respectively. (Figure 9).



	Basal	High Stress	Low Stress	NAC + Low Stress
Young	100	183.87	138.7097	90.32258
Old	102.4758	187.41	140.451	90.7485
RD	103.2258	400	354.8387	98.14
HGPS	118.2774	245.416	251.6129	96.77419

Figure 8: Graph showing the percentage of DA-DCF (ROS reporter dye) fluorescence in response to differing treatments. GMO3348 (fibroblasts from young healthy donor) basal levels were normalised to 100%, and other results extrapolated from this. Autofluorescence measurements were deducted from all results. Error bars represent standard deviation from the mean from triplicate results. Statistical significance from the particular cell line's basal condition is denoted by * as measured by a Student's paired, two tailed T-test at the 5% significance rate from the average of triplicate results.

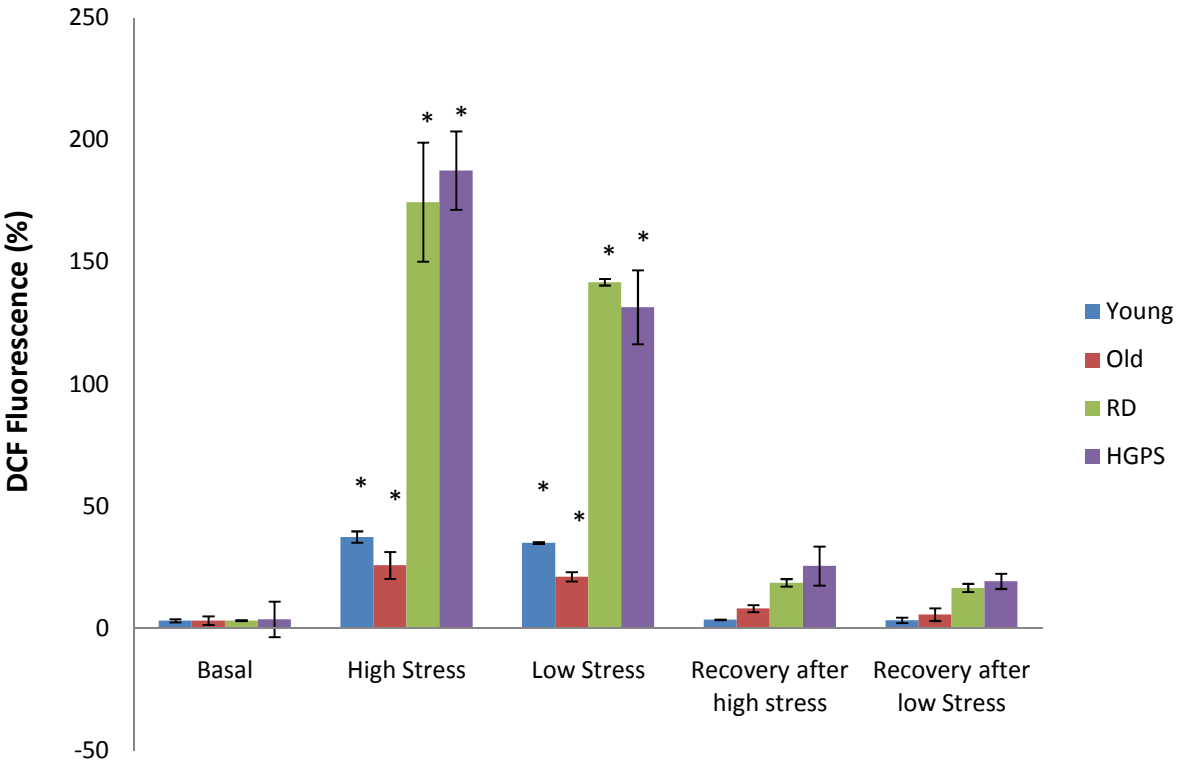


Figure 9: Graph showing levels of ROS within four different cell lines GMO3348 (p10), AG13129 (p9), R30178 (p11) and HGPS AG11513 (p11). Cells were treated with a ROS reporting dye (DA-DCF) to measure ROS levels. Controls were not treated with DCF dye to determine autofluorescence which was deducted from all results. Fibroblasts were subjected to either high (350µM H_2O_2) or low (150µM H_2O_2) oxidative stress for 30mins, measured and then allowed recovery for 24 hours. Errors bars represent standard deviation from the mean, from triplicate results. Statistical significance from the particular cell line's basal condition is denoted by * as measured by a Student's paired, two tailed T-test at the 5% significance rate from the triplicate results. All cell lines show statistically significant rises in ROS levels after both high and low stress, interestingly none show any significant difference from the basal condition after recovery.

3.4 Fibroblasts from HGPS and RD patients show increased nuclear abnormalities, including nuclear blebbing.

Fibroblasts from HGPS (AGO6917) and RD (RD30178) and control fibroblasts (GMO9558) were stained with anti-lamin antibody in order to investigate the structure of the nucleus. Nuclei were judged to be normal if the nuclear envelope was of ellipsoid shape or with intra-nuclear staining. Cells were deemed abnormal if they showed any of the following: Intra-nuclear aggregates of lamin-A/pre-lamin A or progerin, blebbing or invaginations of the nuclear envelope, micronuclei (small lamin A positive structures next to the 'parent' nuclei) or trans-nuclear structures (shown by tubular lamin A positive staining inside the nucleus). (Figure 10)

Levels of nuclear abnormalities in both GM and AG fibroblasts remained low over all treatments. However, AG fibroblasts harbour a higher number of nuclear abnormalities compared to GM fibroblasts, suggesting that an increase in nuclear abnormalities may be associated with normal physiological ageing.

Basal levels of nuclear abnormalities were dramatically raised in both laminopathic patients. HGPS fibroblasts showed a > 900% increase compared to control fibroblasts, and RD nuclei show a 675% increase in nuclei aberrations. This confirms that expression of either pre-lamin A (in the case for RD) or progerin (in HGPS) causes structural defects in the nuclear envelope. It was interesting to note the differences between the nuclear abnormalities in HGPS and RD. HGPS fibroblasts consistently displayed a higher proportion of nuclear defects than fibroblast from RD patients. This may suggest that accumulation of progerin HGPS cells causes greater nuclear envelope defects than the accumulation of pre-lamin A.

As ROS has been shown to be increased in laminopathic cell lines (Figure 8 and 9), it was assumed that increased ROS sensitivity may correlate with an increase in nuclear structure abnormalities. However upon treatment with low stress (150μM H_2O_2) for 30 mins, and 24 hours recovery, neither HGPS, RD or control cells showed significant increases in nuclear defects. This was also the case after high stress was induced (350μM H_2O_2), no significant increase in structural defects was observed in any of the cell lines.

Pre-treatment with NAC in the absence of H_2O_2 failed to reduce the proportion of nuclear abnormalities, suggesting that changes in ROS levels do not have any impact on the abnormal nuclear morphology.

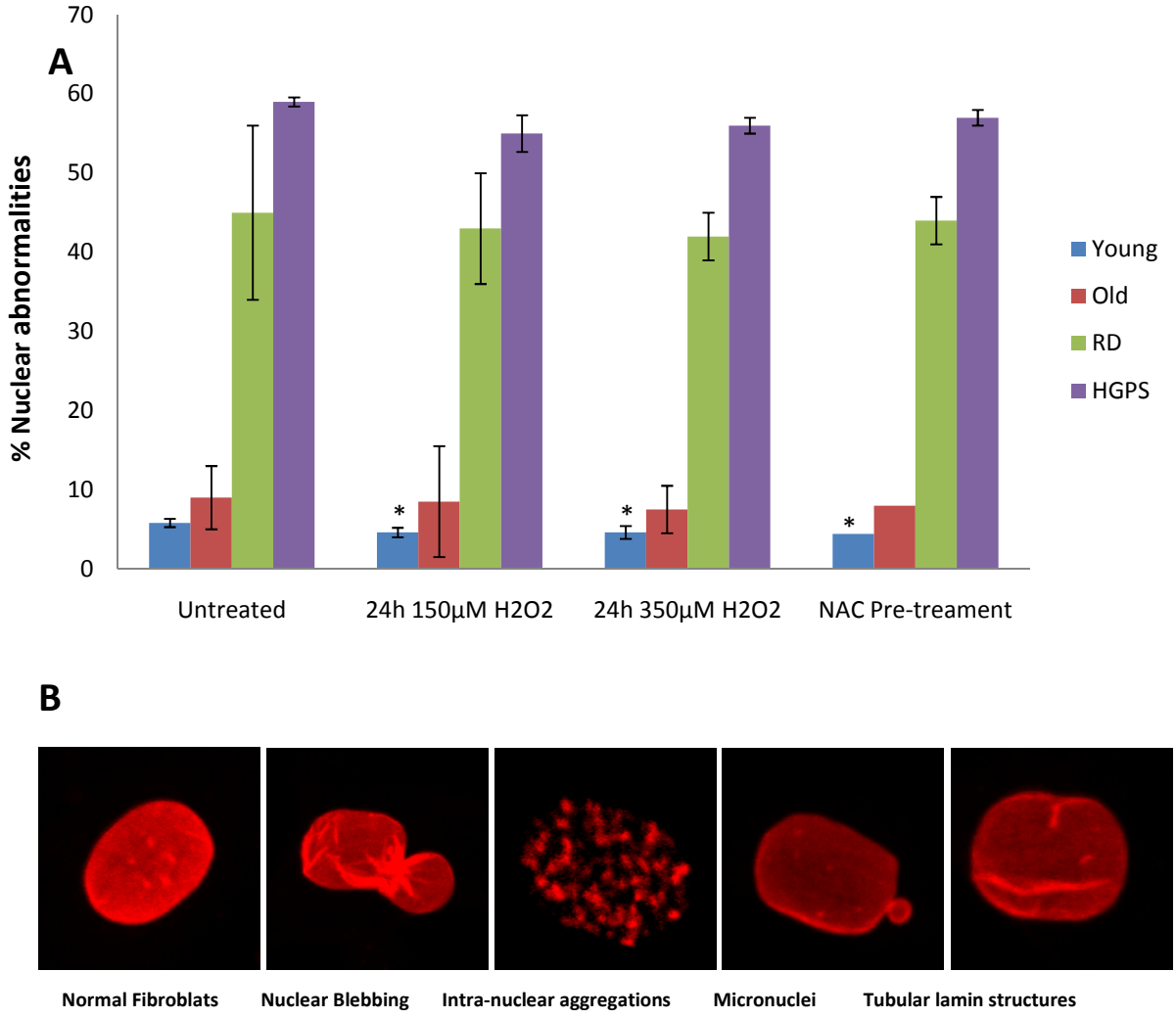


Figure 10A: Analysis of nuclear abnormalities found in fibrblobasts harbouring mutations associated with premature ageing. All cells were between passage 10-12. Cells were classified as abnormal based on thier nuclear envelope structure. Fibroblasts from young individuals (GMO3348) show low levels of abnormalities in all condidtions (average 5% of nuclei containing abnormalities). Error bars represent the standard deviation in a smaple size of 200, with triplicate results. Statistical significance from the particular cell line's untreated condition is denoted by * as measured by a Student's paired, two tailed T-test at the 5% significance rate.

Figure 10B: Micrographs of types of nuclear abnormalities found in premature ageing syndromes. Nuclei are stained with anti-lamin A antibody which detects mature lamin A, pre-lamin A and progerin. Nuclei are categorised as abnormal if any one or more of these defects are present.

3.5 Classical HGPS (G608G) shows a higher proportion of dysmorphic nuclei than a lamin A mutation causing Autosomal Dominant Emery Driefuss Muscular Dystrophy (R453W).

In order to evaluate whether increased nuclear abnormalities is associated with premature ageing or any lamin A mutation I compared the levels of dysmorphic nuclei from premature ageing fibroblasts (AG11513) with fibroblasts harbouing a R453W mutations resulting in AD-EDMD (R453W). Fibroblasts were either subjected to either 150μM H_2O_2 or left untreated. They were subsequently reseeded for 24 hours and stained for lamin A and DAPI. Dysmorphic nuclei were classified as described previously. The first thing to note, was that lower passage HGPS fibroblasts show lower percentages of nuclear abnormalities than those of a higher passage, as seen previously. This implied that the progressive accumulation of progerin within cells, leads to escalating proportions of nuclear abnormalities.

Second it was noted that the proportion of nuclear abnormalities was much lower in fibroblasts carrying the R453W lamin A mutation when compared to those with a classical HGPS mutation, yet was higher than in control GMO3348 fibroblasts. Presumably the presence of mutant progerin in HGPS cells leads to dysmorphic nuclei, whereas in AD-EDMD the aberrant lamin A association with emerin leads to slight nuclear fragility. However this again does not change in relation to treatment with oxidative stress (13.2% and 12.2% in untreated and treated respectively.)

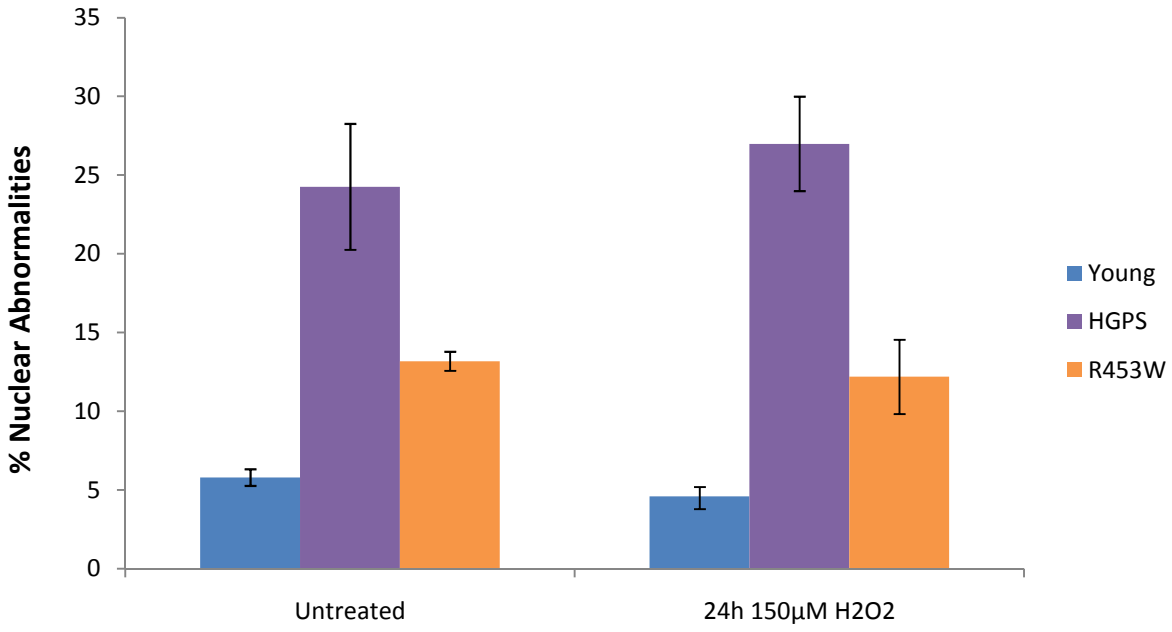


Figure 11: Percentages of nuclear abnormailities found in fibroblasts from AD-EDMD (R453W), HGPS and control (GMO3348) patients. Passage numbers are matched between p9-p14. Cells were counted after either no treatment or after 30mins treated with 150µM H_2O_2 and reseeded and allowed to recover after 24 hours. None of the results gained were statistically significant between any cell line's untreated condition to the oxidatively stressed condition as measured by a Student's paired, two tailed T-test at the 5% significance rate. Error bars represent standard deviation from the mean, sample size 200 with triplicate results.

3.6 The proliferation associated marker anti-Ki-67 is reduced in fibroblasts harbouring lamin A mutations.

Anti-Ki-67 is a monoclonal antibody that reacts with nuclear antigens and is widely used as a proliferation marker. Fibroblasts from four cell lines were either left untreated, treated for half an hour with 150μM hydrogen peroxide, or treated for half an hour with 150μM H_2O_2 and then reseeded for 24 hours before staining. All cells were between passage 9-13.

Fibroblasts from healthy dermal fibroblasts GMO3348 showed 40% actively proliferating cells in culture, which decreases to approximately 33% after treatment with mild oxidative stress. This indicates that the cell cycle arrested in G0, presumably to allow time for induced damage to be repaired. 24hours recovery after reseeding, the proportion of healthy dermal fibroblasts active in the cell cycle returned to pre-treatment levels.

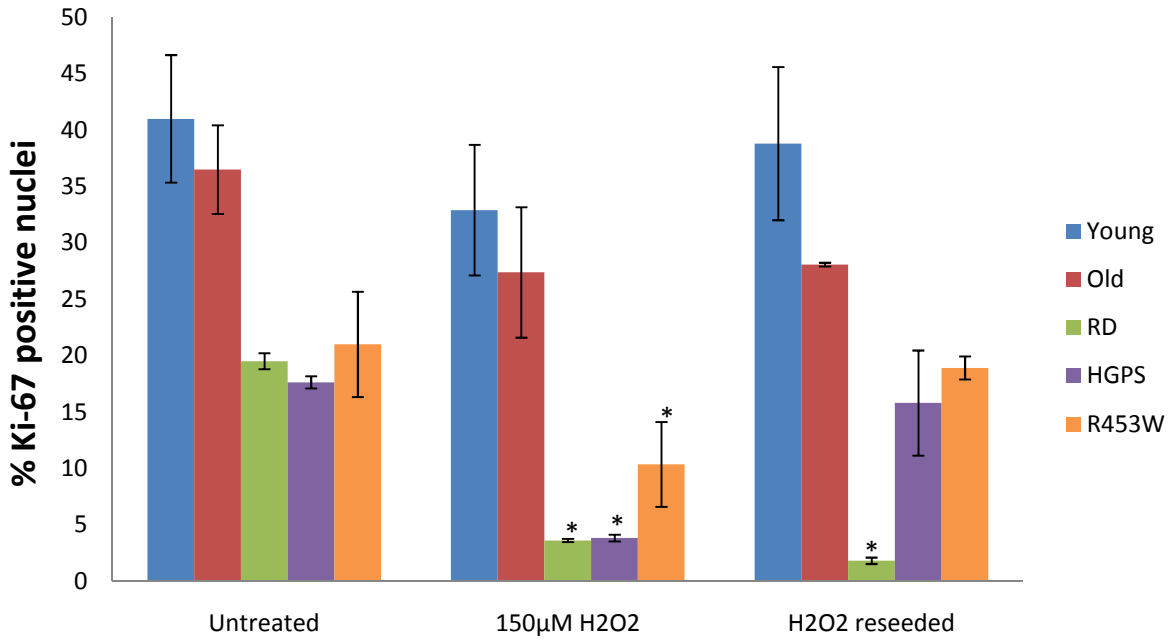
AG13129 fibroblasts from an elderly donor showed reduced proliferative capacity compared to those from a young donor, and showed a similar decline of active proliferation after mild oxidative stress (27% Ki-67 positive). Yet, compared to GMO3348, recovery 24 hours after reseeding was not as complete as Ki67 levels stayed 8% less than untreated controls.

In regard to fibroblasts with lamin A mutations the proportion of actively proliferating cells was dramatically lower even before oxidative assault. Upon mild oxidative stress only a little over 10% of R453W cells were proliferating, however after 24hr recovery and reseeding proliferation levels returned largely to pre-treatment levels (18.9%).

The most dramatic changes in proliferation status however occur in fibroblasts from patients with premature ageing syndromes. Both RD and HGPS fibroblasts displayed low levels of proliferation under normal cell culture conditions (19% and 18% Ki67 positive respectively), over 20% lower than controls. However the most striking result was upon mild oxidative stress where proliferation levels for RD and HGPS dropped to 3.7% and 4.0% respectively. This indicates that upon even mild levels of oxidative stress many cells arrest the cell cycle in an attempt to repair the oxidative damage. This could imply that RD and HGPS fibroblasts are more susceptible to mild oxidative stress than control cells, or that these cells enter cell cycle arrest at lower levels of damage. 24 hours after reseeding and recovery, the proliferation index in both cells strains increased to 11.8% for RD and 18.0% for HGPS. Fibroblasts from HGPS return to within 2% of their pre-treatment levels, whereas RD fibroblasts only return to within 7.2%. This could imply that HGPS fibroblasts are more likely to repair DNA damages induced by mild oxidative stress, and re-enter the cell cycle, whereas fibroblasts from patients suffering from RD show a reduced

likelihood of transient cell cycle arrest, whereby they regain their proliferating status, and maybe more likely to enter a state of cellular senescence.

A



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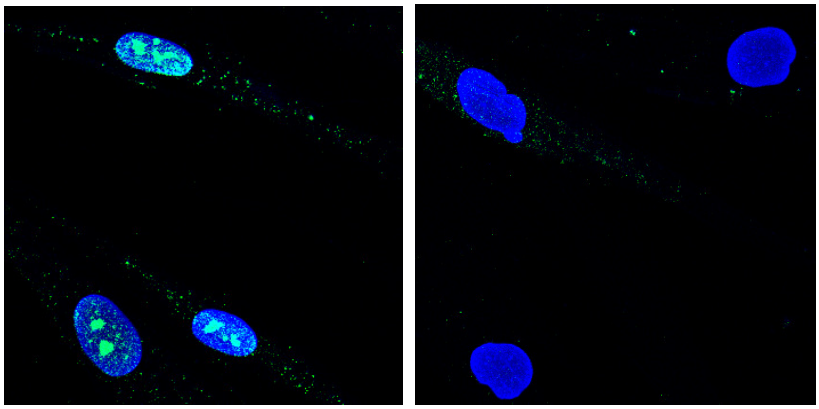

A **B**

Figure 12A: Immunofluorescence results of proliferation antigen Ki-67 in two lamin A mutated cell lines, a RD cell line and control. 200 cells of each treatment and cell line were counted under a randomly selected microscopic view. Cells were either untreated, treated with 150µM H_2O_2 for 20 mins, or treated with 150µM H_2O_2, reseeded and allowed to recover for 24 hours. Fibroblasts were classed as Ki-67 positive if medium-strong staining was shown. Weak Ki-67 staining was classed as Ki-67 negative. Errors bars calculated as standrad deviation from the mean. Statistical

significance from the particular cell line's untreated condition is denoted by * as measured by a Student's paired, two tailed T-test at the 5% significance rate. Mutant fibroblasts (HGPS, RD and R453W) show a significant difference in Ki67 staining after oxidative treatment, whereas after reseeding and recovery, only RD fibroblasts show a statistical difference in Ki67 staining from the untreated control.

Figure 12B: Micrograph showing positive (A) and negative (B) staining for Ki-67 antibody. Cells were counterstained with DAPI. Positive controls showed no association between fibroblasts and secondary antibodies (not shown.)

Chapter 4: Development of a mathmatical model to analyse DNA damage repair defect in premature ageing syndromes.

4.1 Introduction

DNA damage repair is known to be impaired in RD and HGPS fibroblasts. The aim of these investigations was to determine the contribution of defects in ROS buffering capability and DNA damage reapir to the phenotypes observed. This was tested by treating fibroblasts from four individuals with different DNA damaging agents. Firstly etoposide was used to directly induce DSBs in the DNA. Etoposide, an anti-cancer drug acts that forms a ternary complex with DNA and inhibits the enzyme topisomerase II, thereby preventing ligation of DNA strands. The other damage inducing agent used was two differing concentrations of hydrogen peroxide (150µM (mild) and 350µM (severe)). H_2O_2 acts to raise the intracellular levels of ROS, which in turn acts to cause lesions in DNA.

All treatments were performed either in the presence or absence of the antioxidant NAC. After treatment with the damage inducing agent samples were taken at times 0, 1, 2, 4, 7,and 24 hours. The sample fibroblasts were fixed and stained with anti γ-H2AX, counterstained with DAPI, imaged. γ-H2AX foci were counted in 200 cells per samplein triplicate slides Over three replicate experiments and divided into groups of 0, 1-2, 3-5, 6-10, 11-15 or 16+ foci per cell.

The six treatments (Etoposide + NAC, Etoposide – NAC, 150µM H_2O_2 + NAC, 150µM H_2O_2 – NAC, 350µM H_2O_2 + NAC and 350µM H_2O_2 – NAC) allow examination of the contribution of DNA damage repair defects and deficient ROS buffering capability to the phenotype observed in RD and HGPS. If cells show a defect in DNA repair, they will not show a reduction in γ-H2AX foci after 24 hours with any treatment, however if the primary defect is a defect in ROS buffering capability, the cells should be able to repair etoposide induced DNA damage but show difficulty in repairing damage induced by ROS (H_2O_2).

For the data obtained, please see appendix A

4.2 Cells from premature ageing syndromes are characterised by an inability to reapir induced DNA damage.

Previously studies have shown a defect in DNA damage repair in both HGPS and RD fibroblasts, and have suggested a failure to recruit the correct repair factors to the site of induced damage (Liu et al., 2005).

In order to investigate this defect preliminary, fibroblasts from the four cell lines (GMO3348, AG13129, AGO6917 (HGPS) and RD30178) were subjected to either low (150µM $H_2O_{2)}$) or high (350µM H_2O_2) oxidative stress for 20 minutes to induce DNA double strand breaks. Fibroblasts were then removed at varying intervals during the recovery (untreated controls, 30 mins, 1 hour, 2 hour, 4 hours, 7 hours and 24 hours) and the amount of DNA damage was analysed using anti-γH2AX which associates with the induced double strand breaks in DNA and counterstained with DAPI. Controls for secondary antibody association were performed and no unspecific secondary antibody staining was found (not shown).

From the figure below it can be seen that none of the cell lines had high levels of basal DNA DSBs in the untreated condition, as there are few γH2AX foci. It can also be observed that hydrogen peroxide is a potent inducer of DNA DSBs as shown by the high level of anti-γH2AX staining half an hour after the treatment had been applied. The level of staining is fairly even across all four cell lines, suggesting that HGPS and RD fibroblasts are no more susceptible to hydrogen peroxide induced damage than healthy dermal fibroblasts.

However what can be observed is that after 24 hours recovery fibroblasts from young and old healthy donors showed significant reductions in anti-γH2AX staining, indicating that they are able to repair the induced DNA damage. Conversely fibroblasts from laminopathic patients show persistent γ-H2AX foci after 24 hours recovery, showing that some of the DNA damage induced has not been repaired (Appendix B)

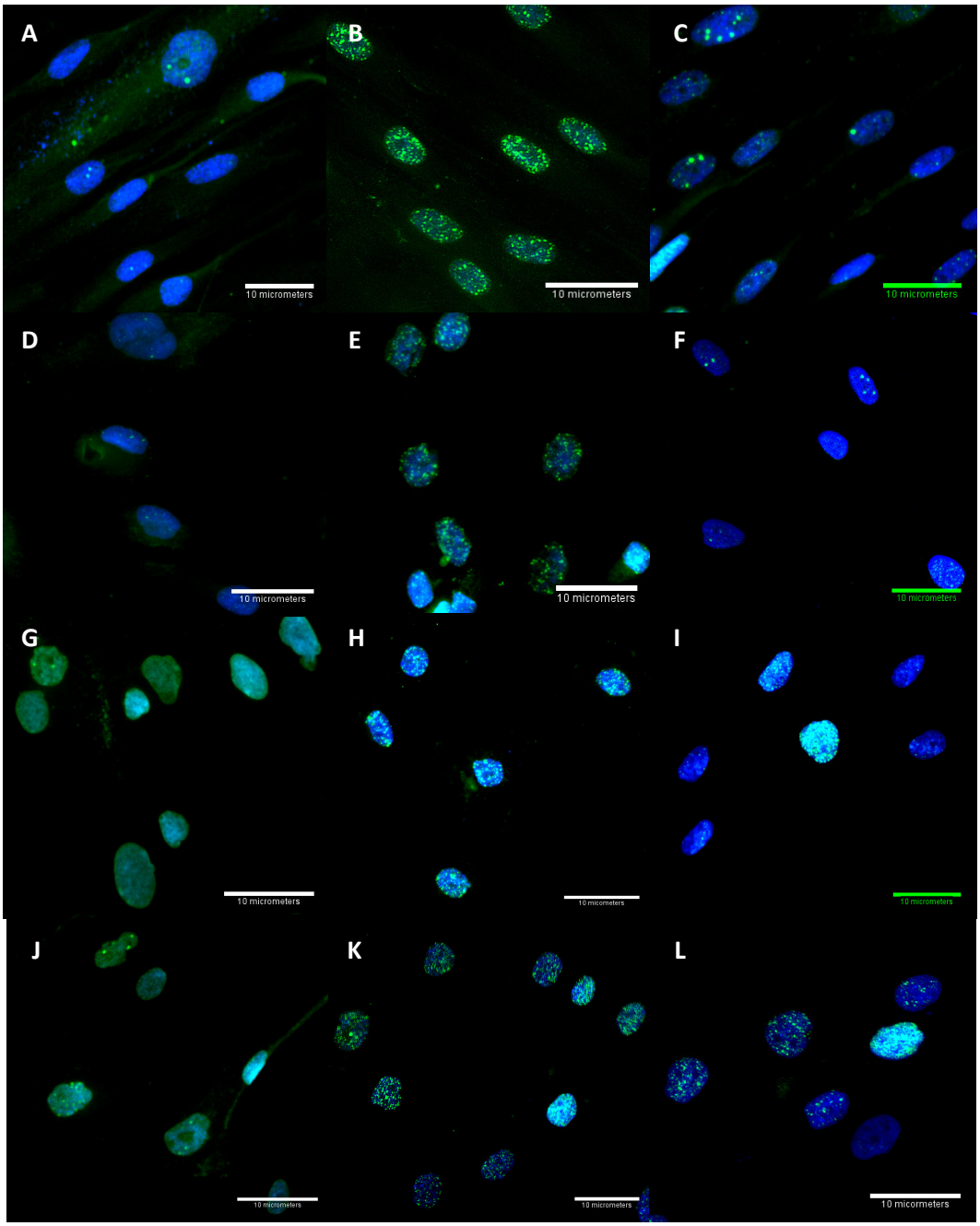

Figure 13: Micrographs showing immunofluorescence results of fibroblasts stained by anti-γ-H2AX and counterstained with DAPI. A-C shows fibroblasts from a young donor (GMO3348) from untreated (A), recovered 30 minutes after oxidative stress (B) and recovered 24 hours after oxidative stress (C). D-F show the same treatment from fibroblasts from an old individual (AG13129). G-I show the same for RD fibroblasts and J-L, HGPS fibroblasts. γ-H2AX staining (green) shows sites of DNA double strand breaks.

4.3 Development of a mathematical model to investigate DNA repair defects and ROS buffering defects in premature ageing syndromes.

Due to the difficulty in precisely counting the number of foci per cell, the previous groupings were used as described on page 70. This created difficulty in analysing the results statistically, therefore a mathematical model was developed and used to further understand the data obtained.

The model was based on the multinomial distribution, a generalisation of the binomial distribution. It is a categorical distribution where each trial results in exactly one of some fixed finite number of possible outcomes.

The model is based on the following;

- let *n* be the the number of γ-H2AX foci per cell
- breakages occur at rate *m*
- repair occurs at rate *r*
- let time be *t*
- let *n(t)* be the number of γ-H2AX foci per cell at time *t*

Therefore;

$$\frac{dn}{dt} = m - rn$$

This means that the rate at which *n* is changing with *t,* is dependent upon the total rate of 'breakages' minus the total rate of repair *r.*

This leads to the following equation,(for proof see appendix.)

$$n(t) = n_{\infty} + (n_0 - n_{\infty})e^{-rt}$$

It is assumed that there is a basal level of DNA damage in a cell before any treatment. This is n_b. After the DNA damage inducing treatment (etoposide or hydrogen peroxide) there is new total number of γ-H2AX foci within the cell at time 0, this is refered to as n_0. Over 24 hours, the cell is

assumed to repair the damage in the cell to an eventual steady state of DNA damage, this is refered to as $n_{\infty,}$ which is equal to the rate of damage divided by the rate of repair.

Thus, the model assumes a negative exponetial distribution, as shown below.

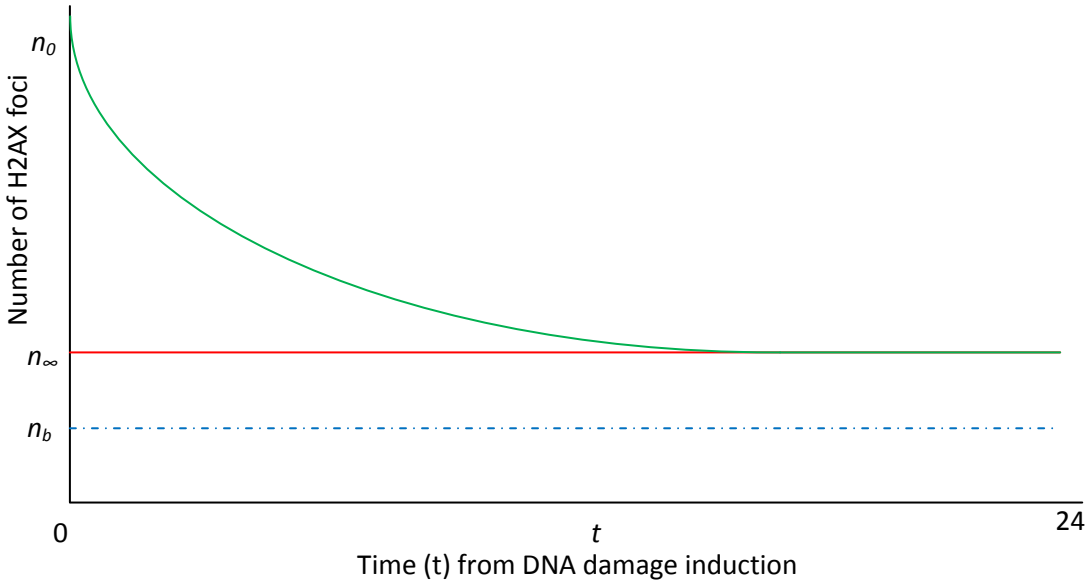

Figure 14: Graphical representation of the assumed mode of action of DNA damage induction and repair over time. The blue dashed line represents the basal level of DNA damage in a given cell. At time 0 (the time after the 30mins with DNA damage inducing agent) the level of DNA damage is n_0. Over time the level of DNA damage will reduce and level off to an asymptote at n_∞.

4.4 Choice of the best model to fit the data by Maximum Likelihood estimation modelling.

The model used maximum likelihood modelling, which is a statistical method used for fitting a statistical model to data, and providing estimates for the model's parameters. Maximum likelihood picks the values of the model's parameters that make the data more likely than any other values. (Richards, Whittingham, and Stephens, 2011) This is achieved using Excel Solver add in.

To correctly account for variation among cells caused by unmeasured sources, for each cell line and treatment, we assumed that the number of DSB observed among cells varied about the modelled mean according to a negative-binomial distribution. The model parameters (m, the number of repairable foci induced; n, the number of non-repairable foci induced; and r, the rate at which repairable foci were repaired), were estimated using maximum likelihood and uncertainty in these parameters was estimated using the profile likelihood method (Richards et

al., unpublished data). Model selection incorporating Akaike's Information Criterion (AIC) was then used (AIC Burnham and Anderson, 2002) to detect evidence that the NAC treatment affected each of the three model parameters; namely, the number of non-repairable and repairable foci induced, and the rate at which repairable foci were repaired.

The following table shows the models trialed to produce best estimates of the model's parameters.

Model	***Log Likelihood***	***No.of parameters (K)***	***AIC***	***Δ***	***Accept/ Reject***
Full Model- Every variable has an effect on the outcome	-12756.97	21	25555.64	1.14	Reject
The type of tretment (Etoposide, 150μM H_2O_2 or 350μM H_2O_2) has an effect, NAC has no effect	-12997.28	11	26016.56	462.06	Reject
The type of treatment has no effect. Only NAC effects outcome	-13325.05	9	26668.11	1113.61	Reject
Treatment effect, NAC effects rates of reapir only	-12877.87	14	25783.74	229.24	Reject
Treatment effects outcome, NAC only effects rates of repair and n0	-12800.60	17	25635.20	80.70	Reject
Treatment effects outcome, NAC effects rates of repair, n0 and ninf	**-12757.25**	**20**	**25554.50**	**0**	**Accept**
Treatment effecst outcome, NAC effects rates of reapir, n0 and nb	-12800.32	18	25636.65	82.15	Reject
Treatment effects outcome, NAC effects rates of repair and ninf	-12780.39	17	25594.78	40.28	Reject

Table 15: Table showing the log likelihood of different models. Excel solver is used to maximise log likelihood by changing varying parameters as described by the model. AIC is a measure of *the best model* and is required to be as low as possible. It is calculated using the formula **AIC= -2 x log likelihood + 2 x K**. Delta (Δ) is calculated by the subtraction of the AIC of the best model from the AIC. Any Δ 6 points away from the best model is automatically rejected. Any within 6 points of the best AIC is considered, if it is a more complicated model (higher number of parameters) it must have a lower AIC. The best model for DNA damage data collected suggests that the type of DNA inducing agent affects the outcome, as does NAC for the rates of repair, the number of DSBs initially after treatment (n_0) and the asymptote steady state damage level of damage (n_∞), but there is no evidence to show NAC affects the basal level of damage (n_b).

4.5 The statistical model predicts the number of γ-H2AX foci in a cell at a given time for the four cell lines investigated.

From the statistical model developed it is possible to estimate the expected number of γ-H2AX foci in any one of the four cell lines (GM, AG, RD or HGPS) at any given time upto 24 hours for any one of the six treatments (Figure 16). From these plots it is then possible to analyse the data and the effect of the treatment type and the cell type on the repair of induced DNA damage.

With regard to GM (fibroblasts from a young donor) it can be seen that the plots generally follow the negative exponential. All the treatments induce between 12.1 and 20.5 γ-H2AX foci per cell. Both etoposide treatments (+/- NAC) and both 350μM treatments (+/-NAC) induce a similar number of DNA DSBs. Interestingly, treatment with 150μM H_2O_2 seems to induce both the lowest (12.1 γ-H2AX foci in the +NAC treatment) and the highest (20.5 γ-H2AX foci in the NAC – treatment) amount of damage. Yet, over the 24 hours, the GM fibroblasts seem able to cope with DNA damage assault and repair the damage to a low steady state, which appears around 7 hours. The steady state asymptote ($n\infty$) estimated by the model is similar for all treatments (between 1.3 and 2.4 foci per cell) (Figure 16).

The situation is similar with regard to fibroblasts from an elderly donor (AG). The amount of damage induced seems to be fairly independent of the method used for induction of damage. Only treatment with etoposide (-NAC) induces more γ-H2AX foci than any other treatment (24.7 foci per cell at t=0). Interestingly there is minimal difference in the amount of damage induced by 150μM H_2O_2 and 350μM H_2O_2. Therefore it should be questioned whether or not 150μM is a low enough dose of H_2O_2 to induce mild oxidative stress. However, alike GM, AG fibroblasts are able to repair DNA damage to a steady state asymptote with a fairly low level of DNA lesions by 24 hours (1.6-3.8 foci per cell).

Conversely, with reference to the graphs in figure 17 it is obvious that a clear difference exists between healthy fibroblasts and those with a premature ageing syndrome. Both RD and HGPS show differential amounts of induced DNA damage in response to different treatments, the highest being Etoposide – NAC producing 27.1 and 21.4 foci per cell in RD and HGPS fibroblasts respectively. The addition of a NAC pre-treatment has more of an effect on these laminopathic fibroblasts than those from healthy individuals. It reduces the amount of induced DNA damage by 10.3% and 6.2% on average for RD and HGPS fibroblasts respectively.

However over the 24 hours recovery time, the amount of repair achieved is much more differentiated over the treatment types when compared to the healthy fibroblasts. When treated

with 350µM H_2O_2 the level of repair is much reduced, with the asymptote reaching only 13.9 and 14.2 foci per cell for RD and HGPS respectively. Both RD and HGPS cells appear more able to reduce the number of foci induced by etoposide treatment than foci induced by hydrogen peroxide. This would suggest that these cells suffer less from a DNA damage defect, but more from an inability to cope with oxidative stress.

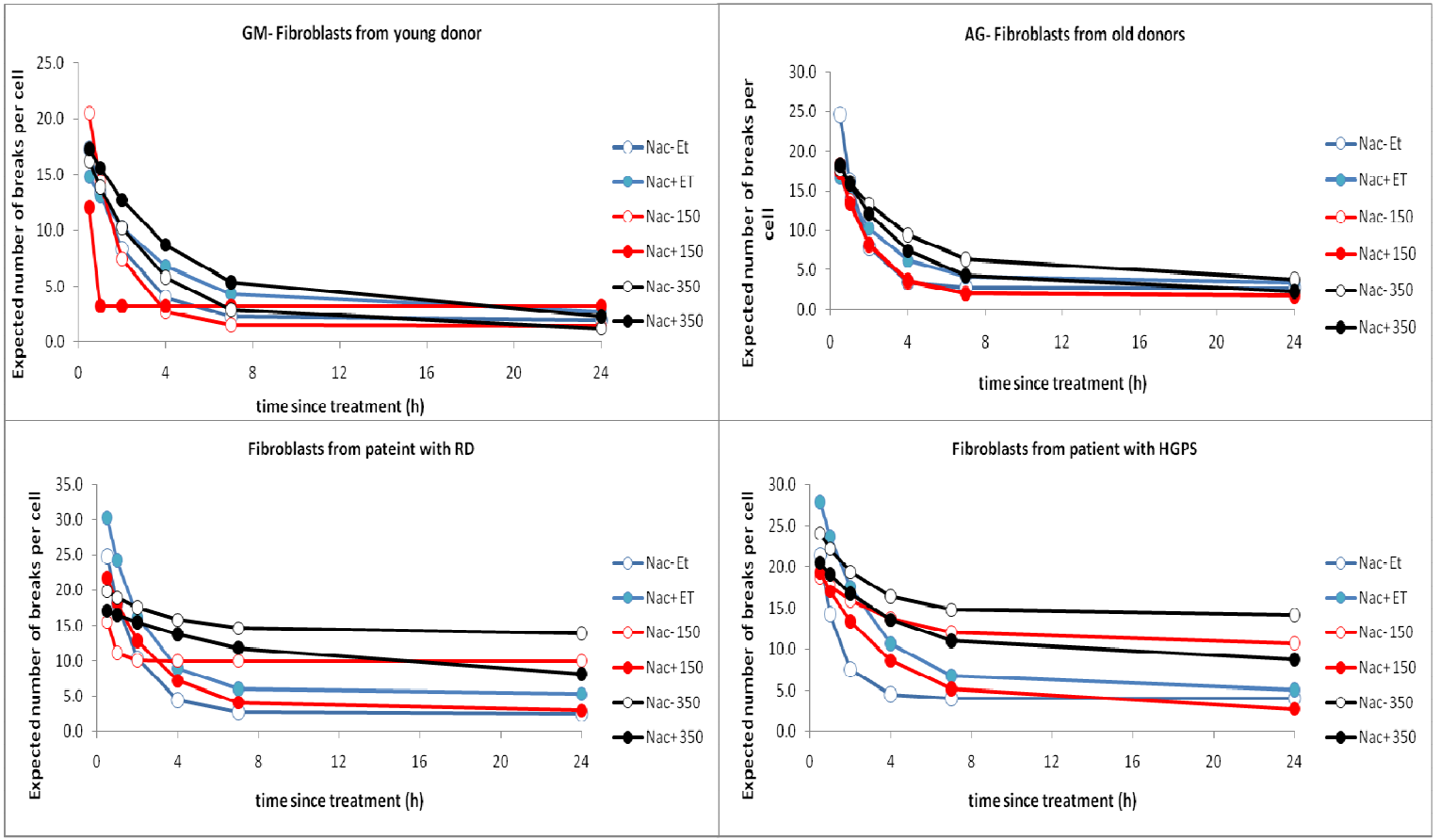

Figure 16: Graphs showing the average number of DSBs as measured by γ-H2AX foci (and statistically modelled) over time and six different treatments (as shown in the legend). Top left and top right are from fibroblasts from a young donor (GM) and an old donor (AG) respectively. Bottom left and bottom right show RD and HGPS fibroblasts respectively. Note premature ageing syndromes show some DNA repair, yet when damaged using hydrogen peroxide(150µM and 350µM show a reduction in the amount of repair over 24 hours (higher n∞).

4.6 Fibroblasts from patients with premature ageing syndromes show increased levels of irreparable DNA damage when induced with hydrogen peroxide.

The amount of irreparable damage was calculated by deducting the asymptotic level estimated by the statistical model ($n\infty$) by the basal level of DNA damage in the untreated controls (n_b). This leaves the number of DNA foci per cell that are not repaired by the cell's machinery.

When treated with etoposide both in the presence and absence of the antioxidant NAC, the vast majority of induced DNA DSBs are repaired over all cell lines (Figure 17). Consistently HGPS fibroblasts show a higher number of irreparable foci, approximately 2.5 more foci than control cells in both conditions. Healthy fibroblasts from older individuals are also modelled to have higher levels of irreparable DNA damage compared to those from a child, suggesting that as ageing occurs, DNA damage repair machinery becomes less effective. Interestingly, pre-treatment with the antioxidant NAC seems to increase the number of irreparable foci when treated with etoposide as modelled by the AIC. It would be interesting to research whether or not NAC interferes with the mechanism of etoposide, making it more potent. It appears to have the greatest impact on RD fibroblasts which show 3.18 irreparable foci per cell in the presence of NAC, but only 0.51 in its absence.

When fibroblasts are treated with the ROS inducing H_2O_2, dramatic differences between the cell lines become apparent. When treated with mild oxidative stress (150μM) the presence or absence of NAC is critical. In its presence, fibroblasts from all cell lines are able to repair the vast majority of induced damage, returning close to basal levels (GM: 1.6 foci/cell, AG: 0.47foci/cell, RD: 0.81 foci/cell, HGPS: 1.47 foci/cell). However in the absence of NAC, RD and HGPS fibroblasts have a drastic increase in the number of foci that are deemed irreparable, reaching 8.0 and 9.4 foci per cell for RD and HGPS respectively as estimated by the statistical model. Therefore, it can be assumed that the presence of NAC under mild oxidative stress is effective in reducing the proportion of irreparable DNA damage.

When treated with severe oxidative stress (350μM) the presence or absence of NAC is equally significant. From the model, control GM fibroblasts show an inverse NAC effect, with a higher number of irreparable foci in the NAC + condition than the NAC – condition, however the difference between the two is marginal suggesting no effect of NAC. Fibroblasts from the AG cell line show a small NAC effect presenting 1.1 and 2.7 un-reparable foci/cell in the NAC+ and NAC- conditions respectively.

The model suggests that both the premature ageing cell lines have higher levels of irreparable

DNA damage both in the presence and absence of NAC under high oxidative stress. From the best estimates of the model RD shows 5.5 and 11.9 unrepaired foci/cell in the NAC+ and NAC- conditions respectively. HGPS fibroblasts present 7.5 and 12.9 unrepaired foci/cell. This suggests that NAC is effective in reducing the proportion of irreparable DNA lesions when induced with oxidative stress.

The demonstration that the premature ageing fibroblasts are able to repair damage induced by the drug etoposide, yet show defects in repairable damage induced by oxidative stress suggest these cells less suffer from a defect in DNA damage repair, but more from a deficient response to the presence of ROS.

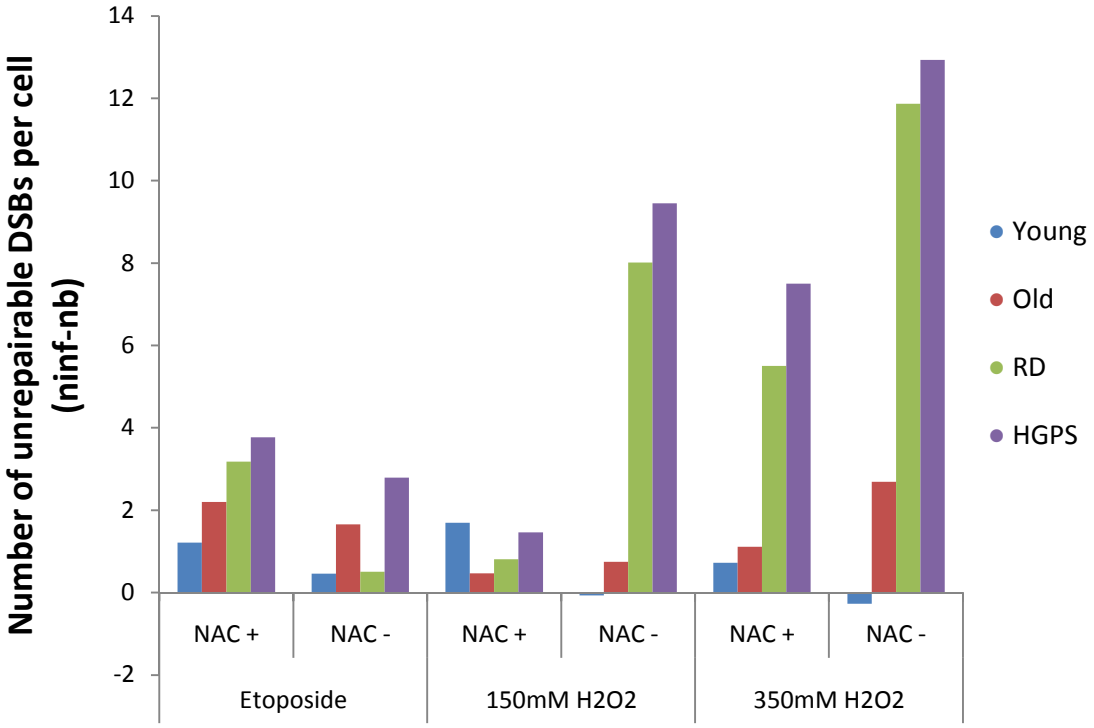

Figure 17: Graph showing the amount of irreparable DSBs as measured by presence of γH2AX foci and modelled using the AIC model. From the model's best estimates of n_b (the number of DSBs per cell before any treatment) and n_∞ (the final steady state level of DSB damage), the difference between the two figures reflects the number of DSBs that the cell is unable to repair.

4.7 Fibroblasts from patients with RD and HGPS show reduced rates of DNA damage repair when treated with oxidative stress.

From the model it was therefore possible to estimate normalised repair rates. The best estimates of the repair rates did not take into account the level of induced damage and the final level of damage as estimated by n_∞. The figures therefore were calculated by dividing the best estimates of rates of repair (*r)* by n_∞ to give a standardised rate of repair, negating the differences in the steady state level of damage after recovery.

From these estimates it can be observed that over the etoposide treatments, the rates of repair are fairly constant over the four cell lines. Interestingly rates of repair over the etoposide NAC-treatment seem to be faster than those pre-treated with NAC. This is further evidenced from the higher proportion of irreparable DNA damage, suggesting that NAC may act to prevent efficient repair of DNA damage induced by etoposide.

After treatment with 150μM H_2O_2 differences between the four cell lines can clearly be observed. GM fibroblasts show the highest rate of repair, with AG 26% slower than control fibroblasts in the NAC+ treatment, thus suggesting that with age, fibroblasts are less able to cope with oxidative stress, and either ROS buffering capability or DNA repair machinery are less effective. RD and HGPS show a 70% and 78% decreased in repair rates compared to control fibroblasts. Similar results are found in the absence of NAC with regard to RD, however the absence of NAC appears to have an impact on the repair rates of HGPS fibroblasts. The presence of NAC increases repair rates substantially for HGPS, yet no effect is observed with regard to RD.

After severe oxidative stress (350μM H_2O_2), rates of repair over all four cell lines are slower than those after mild oxidative stress, thus showing that rates of repair of DNA damage are not independent of the type of damage inflicted. Control fibroblasts show a 43% reduction in the NAC+ treatment and a 77% reduction in the NAC – treatment. However when combined with the proportion of irreparable DNA damage, these results show that although the time taken to reach $n\infty$ may be longer after severe oxidative stress, the level of DNA damage after recovery is similar. AG fibroblasts show a similar reduction in rates of repair after a higher level of oxidative stress. Unlike previously, the presence or absence of NAC appears to have a greater effect on repair rates, doubling GM and AG repair rates when in the presence of NAC. This may imply that NAC is more effective at buffering ROS at higher levels, and may have a minimum level of ROS at which it is an effective antioxidant.

Both premature ageing fibroblasts show greatly reduced repair rates when treated with severe

oxidative stress, thereby resulting in high intracellular ROS levels. Both show incredibly low repair rates, mainly due to the high proportion of irreparable DNA strand breaks as shown previously under this given treatment. The effect of NAC is also minimal at this level, hardly influencing repair rates at all.

The small effect of NAC over the two H_2O_2 concentrations seems to suggest that there is a narrow range of ROS which is effective in aiding DNA damage repair.

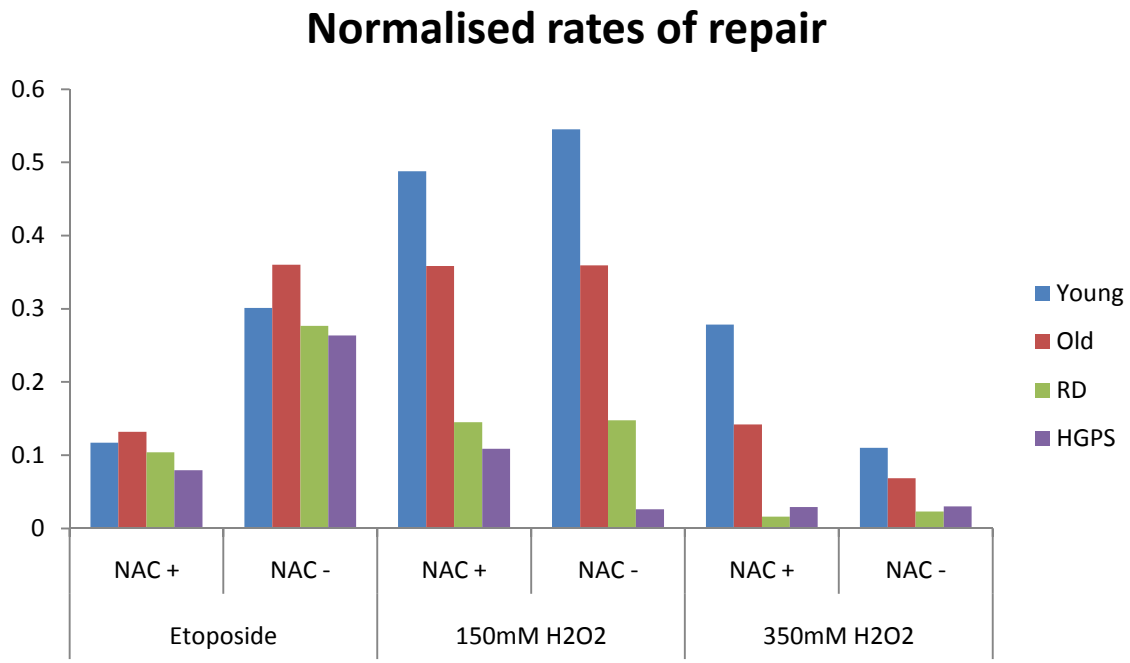

Figure 18: Showing normalised rates of repair of DSB as measured by γ-H2AX foci at time intervals over four cell lines and six treatments. Normalised rates of repair are calculated using the model's best estimates of rates of repair divided by the model's best estimates of n∞..

Chapter 5: Discussion and Conclusions

5.1 Fibroblasts from RD and HGPS patients show defects in both DNA damage repair and ROS buffering ability.

Studies have identified the accumulation of double strand breaks (DSBs) is characteristic in both HGPS and RD cells, and is thought to contribute to genomic instability, cellular senescence and therefore contributing to the premature ageing phenotype (Liu et al., 2005). Here in support of previous data (Liu, 2005) HGPS and RD fibroblasts show increased DNA damage as indicated by the increased presence and more importantly persistence of γ-H2AX foci compared to GM control fibroblasts.

Here, it has been shown from both the raw data and the mathematical model that fibroblasts from patients suffering either HGPS or RD show defects in DNA damage repair and ROS buffering ability (Figures 17 and 18). This supports a wide body of research suggesting a similar phenomenon. (Lui et al., 2006; Caron et al., 2008; Contantinescu et al., 2010) However, what is novel is the approach to further understand the contribution of each of these defects to the phenotypes observed. Previously it has been assumed that the defective DNA repair response in these diseases is the primary cause of the accumulation of DNA damage, here we have shown that the defect is primarily an ineffective means of dealing with increased ROS intracellularly. Results show a close to normal DNA repair response when damage was directly induced with the anti-cancer drug etoposide, thereby suggesting that the machinery used to repair DNA DSBs is relatively intact. This contradicts research from other groups who have suggested that the DNA repair response is impaired in HGPS and RD fibroblasts due to the mis-localisation of the NER protein XPA, thereby preventing correct localisation of Rad50 and Rad51 (Lui, 2008).

From my results, I propose that the accumulation of DNA damage likely to be due to a major defect in the ability to effectively deal with intracellular reactive oxygen species. This is supported by my results showing a failure to repair DNA damage induced by the oxidising agent hydrogen peroxide (Figure 17). The fibroblasts from RD and HGPS are both unable to cope with even mild levels of oxidative stress, supported by evidence showing both vastly increased levels of irreparable DNA damage, and reduced repair rates after treatment with 150μM hydrogen peroxide. This reflects an either overproduction of ROS or an inability to process or effectively remove induced ROS within the cell, or both. Evidence from the DCF ROS reporter dye suggests it

is more an ineffectual ROS buffering ability, rather than ROS overproduction. Furthermore, this defective response is reduced when the fibroblasts are pre-treated with the antioxidant NAC, thus suggesting that the reduction in the levels of intracellular ROS results in a more effective DNA repair response, helping the prove that the high levels of ROS are the primary cause of the DNA damage.

However, depletion of XPA by siRNA knockdown is able to partially restore the recruitment of Rad50, Rad51 and Ku70 to γ-H2AX chromatin containing DNA DSBs (Liu et al, 2008). This supports the claim that the binding of XPA to DSBs in HGPS and RD cells causes disruption to the recruitment of factors normally involved in their repair, contributing to genome instability. However, what is difficult to understand is how rates of repair in progeroid cell lines are not vastly different than control cell lines when the damage is induced by etoposide. Recently, it has come to light that XPA does not localise to camptothecin (CPT)- induced DSBs, suggesting that XPA-localised DSBs may be functionally different (Constantinescu et al., unpublished data). It is therefore feasible to suggest that etoposide induced DSBs do not result in XPA mislocalisation, and although repair is significantly slower, this may be due to other compounding factors.

Iit is very tempting to speculate that the presence of progerin or prelamin A results in an inability to effective deal with ROS, resulting in the production of DNA DSBs. These DSBs are not effectively removed due to XPA mislocalisation and a failure to localise correct DNA repair proteins, causing repair defects leading to genomic instability and premature senescence.

Results from my experiments suggesting that the presence of prelamin A or progerin negatively affects a ROS buffering capability support the hypothesis that mature lamin A is able to act as a redox sensor. The conserved cysteine residues in the carbonyl tail are able to form reversible disulphide bridges in response to oxidising conditions to mediate the stress response. Mutations in lamin A may therefore result in a impairment in its ability to act as a 'free radical sink' and prevent the appropriate redox sensitive NFκβ stress response pathway (Pekovic et al., unpublished data). Therefore mutated lamin A would lead to an increase in intracellular ROS levels, and therefore increased levels of DNA damage as visualised by γ-H2AX foci, as supported by the collected data.

It has been identified that progeria patients have shown signs of both mitochondrial and proteasomal dysfunction, both of which would result in increased levels of ROS intracellularly (Caron et al., 2007; Viteri, Chung and Stadtman, 2010).. As well at causing DNA damage within

the cell, ROS also act to damage other macromolecules including proteins involved in the DNA damage repair response pathway, and it would be interesting to measure the carbonyl content of these proteins in progeria fibroblasts. It has been proposed that the presence of progerin or prelamin A in the case of RD results in increased ROS production and a decrease in proteasomal activity, both of which act to antagonise each other. This results in increased oxidative damage to macromolecules, critically including DNA, causing cellular dysfunction and senescence. (Viteri, Chung and Stadtman, 2010)

The data collected supports the proposition that the presence of mature lamin A may act as one of several governing factors of cellular ageing, and therefore loss of function through mutation results in increased ROS accumulation and premature cellular senescence, contributing to the observed progeria phenotype.

5.2 RD and HGPS fibroblasts more readily enter a state of cellular senescence when exposed to oxidising conditions.

Here it is shown that upon induction of DNA damage by the oxidising agent hydrogen peroxide, fibroblasts from premature ageing syndromes show a high proportion of irreparable DNA. This represents persistent γ-H2AX foci within the cell, estimated by the statistical model.

However, when the cell lines were treated with the DNA damage inducing agent etoposide both in the presence and absence of the antioxidant NAC, the vast majority of induced DNA DSBs are repaired over all cell lines. This seems to suggest that the amount of repair is dependent upon the treatment used to induce the damage.

Intriguingly the addition of NAC to the etoposide treatment consistently creates a higher number of DNA damage foci that are unable to be repaired, over all four cell lines, including healthy ones. NAC is a well studied antioxidant, and is well tolerated; however the data shown suggests that its presence makes etoposide damage less available for repair. Factors required for DNA damage repair may require protein modification for their mode of action, and pre-treatment with NAC may act to prevent their modification.

It may be proposed that the mutated cell lines are more affected by the hydrogen peroxide and etoposide treatment, however the treatment effect size is fairly consistent over all four cell lines, showing equal amounts of induced damage.

One point to consider is that the action of etoposide also results in single-strand breaks (SSBs), as well as DSBs. In addition, recent evidence indicates that topoisomerase II-linked DSBs remain

undetected unless topoisomerase II is removed to produce free DSBs (Muslimović et al.,). The action of hydrogen peroxide is somewhat different. Treatment of cells with oxidative stress by H_2O_2 produced DNA lesions including oxidised bases, and both SSB and DSBs via Fenton Chemistry. The prevailing idea is that free iron within the lysosomes may act as a key player in oxidative dependent cell damage.

What is of note however, is that the proportion of SSBs to DSBs is different depending on the type of damage induction. Only DSBs within the cell induced with either etoposide or hydrogen peroxide are visualised by the appearance of γH2AX foci, a robust DSB marker. However, SSBs and oxidised bases are not recognised as damage within the cell. Therefore if a cell contained high number of SSBs but no or few DSBs it will appear to be healthy with no or few γ-H2AX foci.

By examining the numbers of senescent fibroblasts this issue can be examined.

The six times increase in senescent fibroblasts from a HGPS patient compared to control fibroblasts under basal conditions show that over a fifth of HGPS fibroblasts in culture were already senescent. When DSBs were induced therefore, around a fifth of these cells were completely unable to repair any DNA damage as they were in a senescent state. However, due to the increase in senescence after oxidising treatment to a quarter of fibroblasts, it can be assumed that HGPS fibroblasts are more likely to enter a state of senescence than control fibroblasts after treatment with hydrogen peroxide.

Stress induced premature senescence (SIPS) occurs in response to various stresses such as oxidative stress and non-telomeric DNA damage (Pekovic et al., unpublished data). Under cellular stress, the data supports previous knowledge that healthy cells undergo transient growth arrest in order to repair DNA damage and regeneration of oxidised macromolecules. This is supported by the data showing a reduction in Ki67 positive staining in GM healthy fibroblasts after hydrogen peroxide treatment, followed by a return close to original levels after reseeding and recovery.

However, those cells harbouring lamin A mutations show a preference to enter SIPS rather than transient growth arrest. This is thought to arise as lamin A can no longer act as a redox buffer through disulphide bridge formation between cysteine residues. Therefore cells enter a state of senescence characterised by irreparable DNA, an impaired redox balance and a build up of oxidatively modified proteins (von Zglinicki et al., 2005). Data from Ki67 assays shows that upon hydrogen peroxide DNA damage induction, cells with lamin A mutations and Zmpste24 mutations show a marked reduction in the number of actively proliferating cells, showing some recovery after recovery, yet a large proportion do not regain active proliferative status as

detected by positive Ki67 staining, thus indicating an inability to effectively manage transient growth arrest. This supports the finding by Liu et al. (2006) that determined that caffeine inhibition of both DNA damage checkpoint proteins ATM and ATR in HGPS cells results in a significant restoration of replicative activity. Thus, the decreased cellular proliferation observed in aged progeroid cells is a response to the accumulated DSBs mediated by ATM and ATR checkpoint pathways. Therefore the DNA damage to these cells is deemed a critical threat to cellular function, and a senescent state is imposed.

It would be interesting to investigate the proportion of apoptotic progeroid fibroblasts after oxidative stress as this was not investigated. This would provide an insight into the proportion of cells entering senescence and those apoptosing. As both RD and HGPS fibroblasts ain culture are associated with a high level of proliferation followed by a high level of apoptosis, it would be of note to see if oxidative stress increased the number of apoptotic cells, or just those entering SIPs.

A type lamins have been linked to signalling pathways involved in the cellular response to stress including MAPK, p53/21 and NF-κβ (Pekovic and Hutchison, 2008). NF-κβ signalling is involved in multiple cell processes including apoptosis, immunity and stress response. Following cytokine stimulation or imposed mechanical strain, lamin A/C null mouse fibroblasts show an impaired transduction pathway involving NFκβ after nuclear entry (Lammerding et al., 2004, 2005). This may act to contribute to fibroblasts with lamin A/C mutations defective response to stress, especially as NF-κβ shows an upregulation in response to oxidative stress (Schreck et al., 1992). Recently it has been proposed that the NEMO (NF-κβ essential modifier) shuttle protein involved in signalling between DNA damage to NF-κβ activation may be persistently activated in progeroid syndromes due to increased levels of DNA damage. This is proposed to lead to increased stress on the NF-κβ pathway contributing to the premature ageing phenotype by activation of an auto-immune response (Salminen et al., 2008)

The data here tends to support the oxidative stress theory of ageing, showing that an imbalance in ROS intracellularly causes an increase in generation of altered macromolecules or a decline in their removal resulting in the decline of biological function.

5.3 Aberrant nuclear morphology associated with premature ageing syndromes is an independent phenotype of an impaired ROS buffering capability.

It has previously been assumed that the characteristic aberrant nuclear morphology associated with progeroid syndromes was causative of the premature ageing phenotype. Mouse and cell

models of progeria have been used and it was found that this abnormal nuclear shape could be dramatically improved by FTIs in a dose-dependent manner (Capell et al., 2005) and therefore have been trailed in patients as a prospective treatment.

However doubts of their effectiveness began with Liu et al. (2006) demonstrated that several functional defects associated with HGPS are not improved upon FTI treatment. Critically, large numbers of DNA DSBs were shown to accumulate in progeria fibroblasts even though DNA damage checkpoint proteins ATM and ATR were consistently activated both in the presence and absence of FTIs thereby questioning their suitability. Although the FTIs improved nuclear shape, DNA damage repair remained unchanged causing widespread genomic instability, thus leading to the proposition that these manifestations of the syndrome are independent from one another (Liu et al., 2006).

Data from these assays throughout this thesis supports the claims made by Liu et al. (2006). Here it is being claimed that ROS act to cause persistent DNA damage within progeroid fibroblasts, however nuclear morphology remains unchanged by oxidative conditions. Nuclear morphology was not affected by the addition of hydrogen peroxide treatment in either healthy or mutant fibroblasts. Therefore it can be suggested that whilst ROS contributes in a significant proportion to total DNA damage levels in progeria and RD fibroblasts, aberrant nuclear shape is a manifestation of the presence of a permanently farnesylated progerin or pre-lamin A.

Basal levels of nuclear abnormalities were drastically raised in both laminopathic patients confirming that expression of either prelamin A or progerin cause structural defects in the nuclear envelope. It is interesting to note that fibroblasts from HGPS patients consistently show a higher proportion of nuclear defects than fibroblast from RD patients, suggesting that accumulation of progerin within HGPS cells results in a greater number of nuclear envelope defects than the accumulation of pre-lamin A associated with RD.

An additional cell line (R453W) harbouring a mutation resulting in AD-EDMD was used in the following assay. Unfortunately this cell line only became available at a later date and provided a control to demonstrate nuclear abnormalities in another lamin A mutant cell line. R453W showed increased nuclearr abnormality levels compared to controls, but much lower levels than in fibroblasts harbouring premature ageing mutations. It is thought that the increased levels of nuclear abnormalites found in R453W are a result of an impaired ability of the mutant lamin A protein to bind to emerin, thereby leading to increased nuclear fragility. In progeria, however the presence of permanently farnesyalted progerin at the nuclear rim results in dramatic changes to nuclear shape. The hydrophobic farnesyl residue on both progerin and permanetly farnesylated

pre-lamin A cause the protein to have a greater affinity for the INM than mature lamin-A, hence mislocalising progerin/pre-lamin A away from nucleoplasmic foci. This results in deformation of the membrane. However it can be thought that this mislocalisation may also contribute to genomic instability. Lamin A containing nucleoplasmic foci are known to contain PCNA and polymerase δ used for the initiation of replication in S-phase (Musich and Zou, 2009).

The trial of FTIs on HGPS patients was cut short on the finding that some of the laminoptahy conditions persisted, alongside the alternative prenylation by geranylgeranyl. Hypercholesterolemia drugs statins and amino-bisphosphonates are being used to block cholesterol biosynthetic pathway, preventing the addition of prenyl groups. It will be of interest to determine whether the statin/amino-bisphosphonate combination will be more effective in reducing aberrant nuclear morphology and genome instability phenotypes.

However, if ROS and impaired redox buffering abilities are involved in contributing to the phenotype, an addition of an antioxidant to the drug combination may be beneficial.

5.4 HGPS as a model for normal ageing.

Much research has concentrated on HGPS as recent findings have linked normal ageing to the premature ageing syndrome. Cells from older healthy individuals exhibit the same activation of the cryptic splice site and produces low levels of progerin. Results here show that the AG cell line taken from a healthy 89 year old show increased persistence of γ-H2AX foci and also an decrease in DSB repair rates as estimated by the mathematical model.

The modest increase in the number of γ-H2AX foci in fibroblasts from older donors compared to younger donors is consistent with previous research showing that levels of γ-H2AX increases with an individual's age in tissue samples and with passage number of primary cell explants (Scaffidi and Misteli, 2006).

Like HGPS, DNA damage accumulation with normal ageing is not associated with a genetic defect in DNA repair, unlike for example Werner's syndrome. It can be proposed that age associated genome instability is likely to be caused by the product of the sporadically active cryptic splice site (Musich and Zou, 2009). It is tempting to speculate that as individuals age and accumulate more progerin through the use of the cryptic splice site the proportion of lamin A to progerin reduces. Therefore lamin A is less available to act as a free-radical sink hence increasing oxidative damage to proteins and DNA contributing to normal ageing and a decline in biological function. Due to the common expression of progerin in both HGPS and normal ageing, it will be of great

interest for future research to determine whether or not XPA mislocalisation is also observed in normal ageing.

5.5 A potential role for the antioxidant NAC in the treatment of HGPS.

NAC is a widely used powerful antioxidant and is well tolerated and is commonly used as a dietary supplement. It is a derivative of cysteine and is used to synthesise the antioxidant glutathione which actively scavenges ROS. From the results it is feasible to suggest that an effective treatment would be to reduce the amount of ROS in HGPS cells. Unfortunately, no treatment would ever be available for RD as it is neonatal fatal and only genetic counselling could be offered. However, with regard to HGPS, the addition of an antioxidant to treatment programmes may have a beneficial effect on the easing of symptoms.

The mathematical model uses AIC as a measure of 'the best fit' of the data, with log likelihood determining which variables give rise to significant differences in the data. The best model of DNA damage data collected shows that there is evidence from the data to show that NAC pre-treatment affects rates of repair *(r),* the number of DSBs induced by the treatment *(n_0)* and the asymptotic steady state level of damage after repair *(n_∞).* However there is no evidence to suggest that NAC affects the basal level of DNA damage *(n_b).* This seems to suggest that NAC pre-treatment for an hour is unable to reduce the number of DSBs already present in the cell before any induction, but is effective in reducing the damage inflicted by oxidative stress, and improves repair of these induced DSBs.

From the results here, NAC has shown to be effective in reducing the proportion of irreparable DNA damage in HGPS fibroblasts, effectively reducing levels by 48%. It has also been shown to increase repair rates, and actively reducing ROS levels as detected by flow cytometry. This NAC pre-treatment therefore shows beneficial properties in treating certain phenotypes associated with progeria.

However, NAC has no effect on aberrant nuclear morphology associated with progeria patients. It is assumed that nuclear envelope abnormalities and defects in DNA repair pathways are independent and therefore NAC would need to be used in conjunction with statins and aminobisphosphonates to ensure both defects are treated.

Interestingly, it has been suggested that the nuclear structural abnormalities found in HGPS may play a causative role in the DSB repair defect. It has been suggested that fibroblasts with abnormal nuclear morphology are more likely to show defective DSB repair response in both

wild-type and progeria cells than those with a normal nuclear morphology (Constantinescu et al., unpublished data). Contrary to this however, multiple researchers have found that although FTIs are effective in improving nuclear shape, they are not effective in reducing the number of γ-H2AX (Liu et al., 2006).

Further research will need to establish whether or not there is a connection between abnormal nuclear morphology and a defective DNA repair response and effective treatment can then be designed. Nonetheless, from the results here, it can be suggested that NAC or another form of well tolerated effective ROS scavenging may be beneficial to the DNA DSBs repair deficit associated with HGPS and may help to prevent premature senescence and thereby improve the premature ageing phenotype.

5.6 Limitations of the mathematical model and future research proposals.

The mathematical model is based on the negative multinomial using maximum likelihood modelling to estimate the models parameters to ensure the model accurately reflects the data collected and precise calculations can then be made.

However, the model is based on the data collected from GM (young, healthy fibroblasts) and then applied to the other cell lines. The model assumes that the steady state asymptotic level of DSBs (n_∞) after a period of recovery is not dependent on the amount of damage induced (n_0) thus providing a 1st order differential.

With regard to fibroblasts from progeroid patients (HGPS and RD) the steady state level of DSBs appear to be dependent on the amount of damage induced, thus creating a 2nd order differential equation. Yet in order to be consistent, the decision was made to create the model for the healthy fibroblasts and then apply it to the other cell lines, and examine how far from the model, the estimates deviated, thus providing a constant 'yard stick' to which other cell lines could be measured. This however, produces a certain amount of uncertainty surrounding the model with regard to the best estimates. This is caused mainly from the difficulty in counting γ-H2AX foci. It is not possible currently to isolate a single cell and measure γ-H2AX foci at different time points, therefore cells from the same sample are removed and the varying time points and the foci are counted.

Another difficulty arose from the groupings assigned (0 foci, 1-2, 3-5, 6-10, 11-15, 16+ foci). This was required as often the precise number of foci was difficult to assess from the pictures taken, as often two foci would be joined together, or fibroblasts were stained totally with the antibody

and precise numbers were not possible. One possible solution to this issue would have been to use live cell microscopy to actively track γ-H2AX foci in a single cell after etoposide or hydrogen peroxide treatment (Nelson, Buhmann and von Zglinicki, 2009), using Z stacked images. This option was not available for this project, however would have provided more consistent data and would not require the use of a statistical model to interpret the data.

However, despite these difficulties, the model developed took these inconsistencies into account by ascertaining the probabilities of seeing 3 foci, 4 foci or 5 foci in a cell for the 3-5 grouping and manipulating the data into the model to provide estimates of the number of foci in a given cell, at a given time, with a given treatment.

Overall, the model accurately represents that data obtained and provides a viable method for statistical analysis.

Research into premature ageing laminopathies is a vastly expanding body of research, especially since the diseases are so rare, and the genetic mutation was only identified in 2003. Therefore new developments are constantly being formed and investigated.

Recently an association between the classical mitochondrial heat shock protein Hsp70 and the lamin A associated protein LAP2α has been found (Syners and Schofer, 2008), and is not mediated by Rb. Hsp70 functions generally as an ATP-dependent import motor and assists in the correct folding of newly synthesised polypeptides (Doulias et al., 2007). Under stressful conditions in the cell, Hsp70 may associate with damaged proteins to help reconstitute correct protein structures.

In 2007 it was found that knockdown of Hsp70 in *C.elegans* promotes a progeria like phenotype characterised by lower motility, defects in oogenesis and a shorter life-span (Kimura et al., 2007). Furthermore Hsp70 has been found to play a role in the mechanism of hydrogen peroxide induced DNA damage. Cells with depleted Hsp70 were shown to be more sensitive to oxidative stress, whilst cells that overexpressed Hsp70 were shown to be more resistant to hydrogen peroxide induced oxidative stress (Doulias et al., 2007).

From Hsp70's association with LAP2α, it would be of interest to note whether or not Hsp70 expression levels were lower in progeria cells compared to controls, or whether or not they are mislocalised, perhaps due do an aberrant association with LAP2α. It is not difficult to imagine that due to the mutation within lamin A, associations with LAP2α may be impaired, which could theoretically impair further associations with proteins such as Hsp70.

It will be of future importance to also gain a deeper understanding of the effectiveness of FTIs on

the treatment of HGPS, with results to be published soon. Importantly HGPS pathology appears to be an intensification of the normal biological ageing process, especially with regard to DNA and protein damage accumulation, and therefore as well as the criticalness of researching potential treatment for HGPS patients, future research will elucidate the processes contributing to natural ageing.

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Appendix

Appendix A

Proof of Binomial equation

Let n(t) be the number of γ-H2AX foci in a cell at time t.

DSBs occur at rate m

Repair of DSBs occurs at rate r, hence;

$$\frac{dn}{dt} = m\text{-}rn$$

Assume that at time t=0, there are n_0 γ-H2AX foci, hence

$$\int \frac{dn}{m - rn} = \int dt$$

Rearrange;

$$\ln(m - r) = -rt + c$$

$$m - rn = ce^{-rt}$$

$$n = \frac{m}{r} - ce^{-rt}$$

Solve for c at n_0 at t=0

$$c = \frac{m}{r} - n_0$$

Therefore;

$$n(t) = \frac{m}{r} + \ (n_0 - \frac{m}{r})e^{-rt}$$

Substitute,

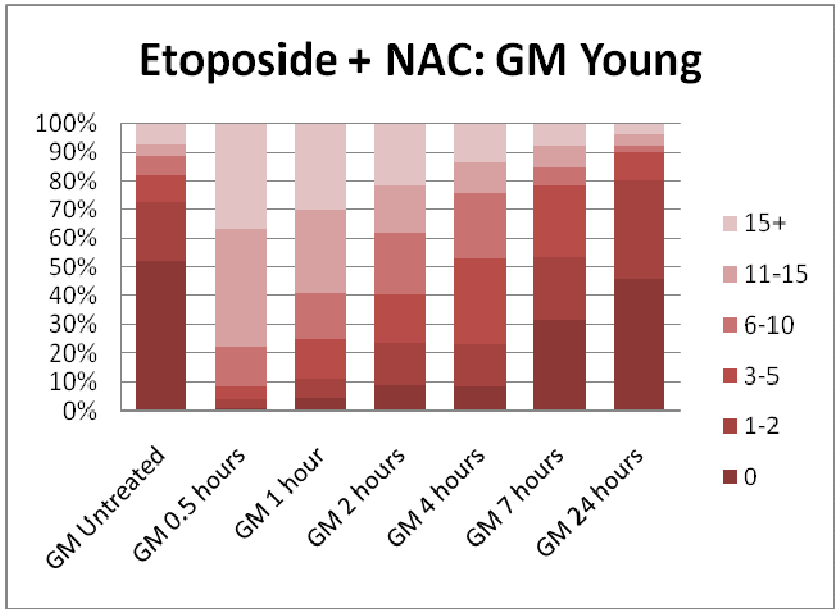
$$\frac{m}{r} = n_\infty$$

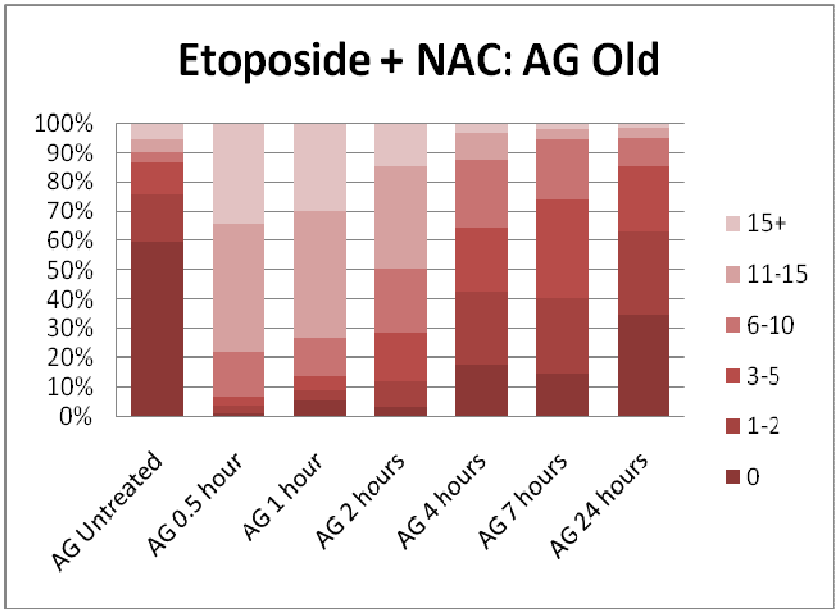
Hence giving;

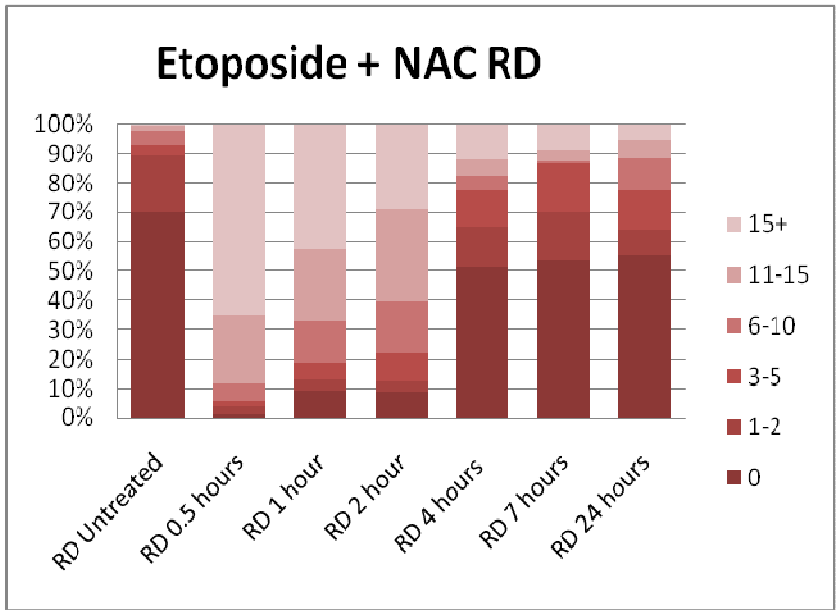
$$\boldsymbol{n(t) = \ n_\infty + (n_0 - n_\infty)e^{-rt}}$$

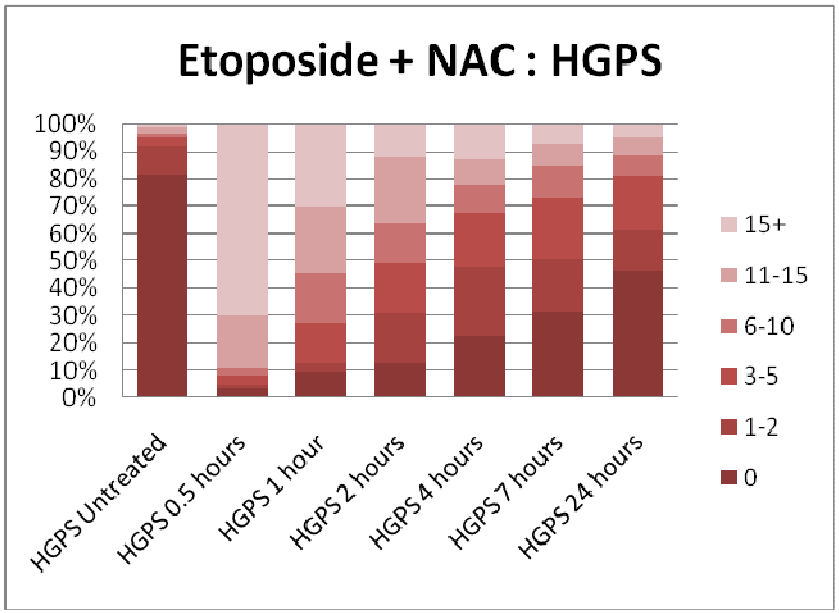
Appendix B

Please see following pages.

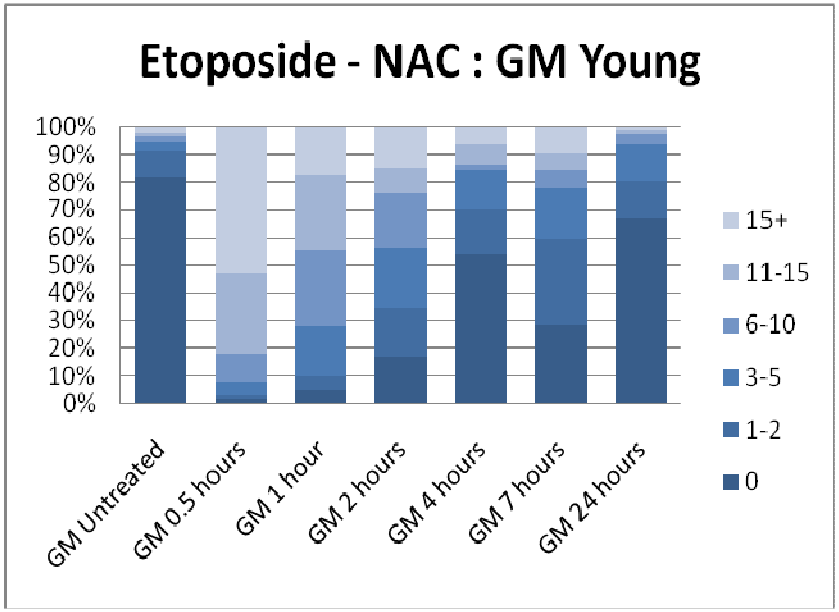


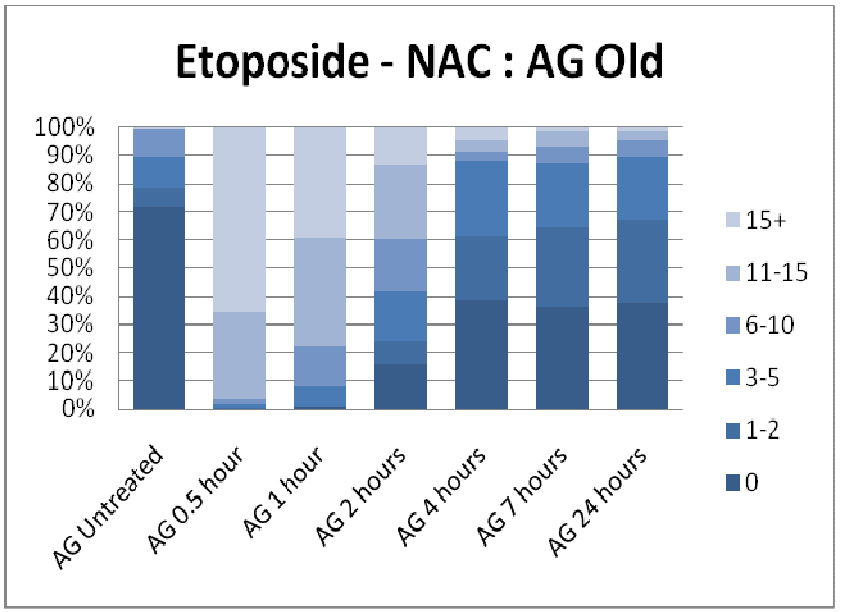


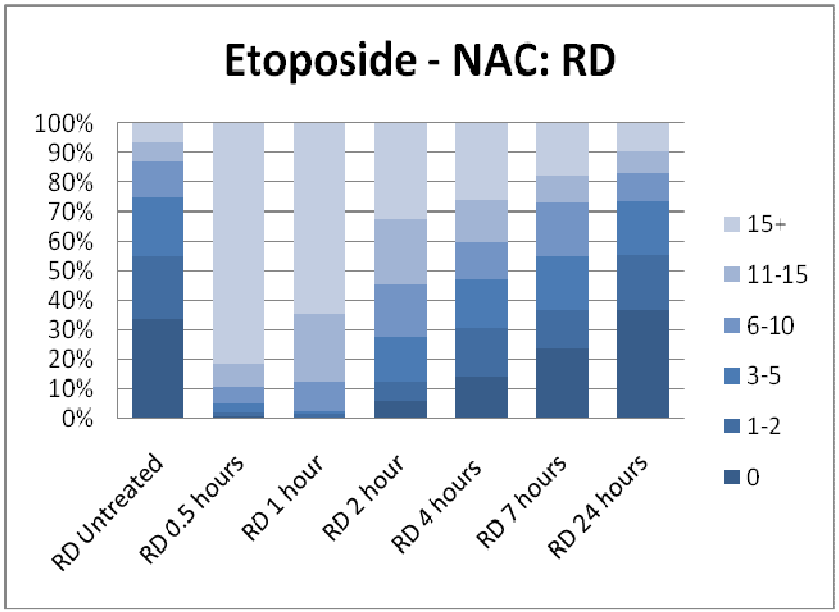


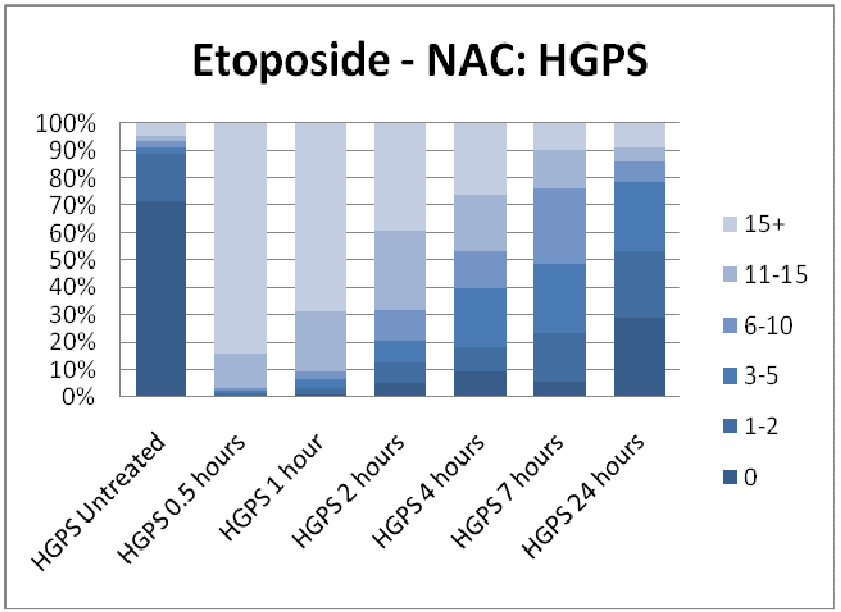


Graphs showing the number of fibroblasts with 0, 1-2, 3-5, 6-10, 11-15 or 15+ γ-H2AX foci after NAC pre-treatment and etoposide damage induction in GM, AG, RD and HGPS cell lines over the given time points.

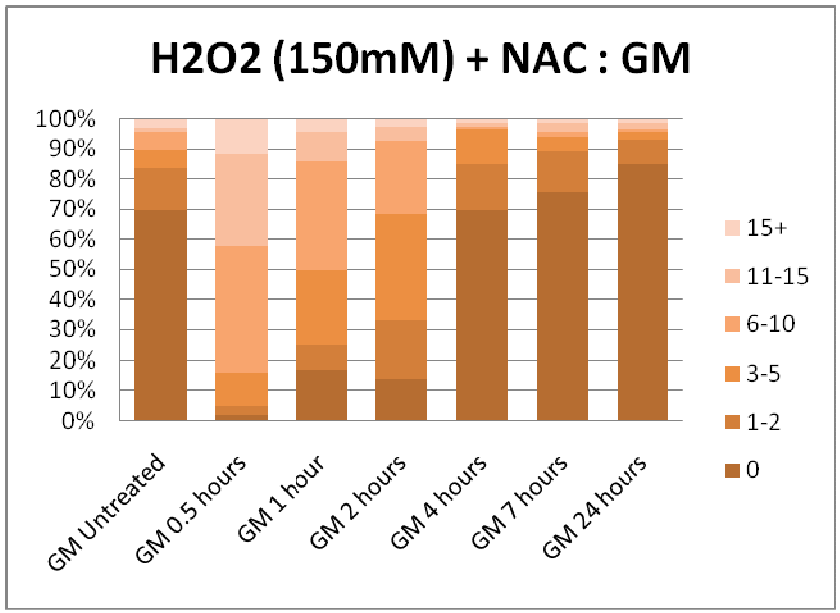


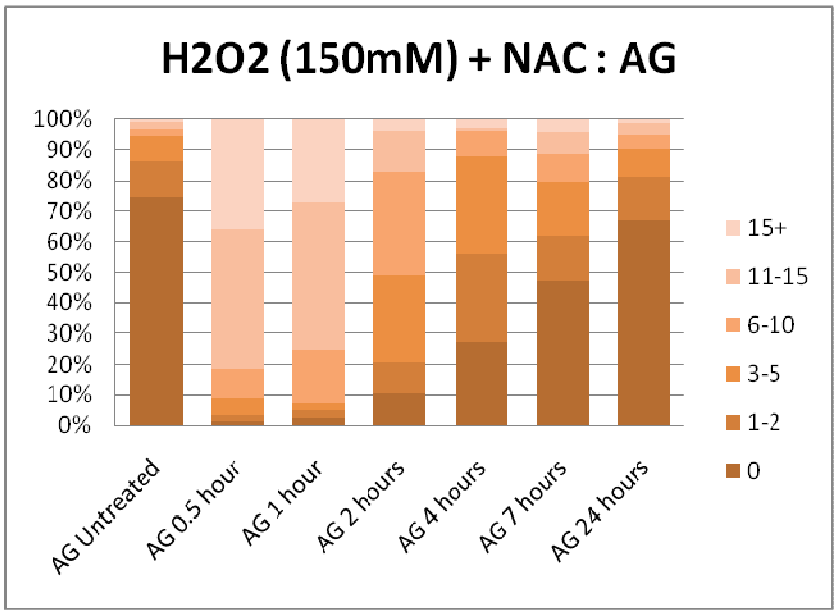


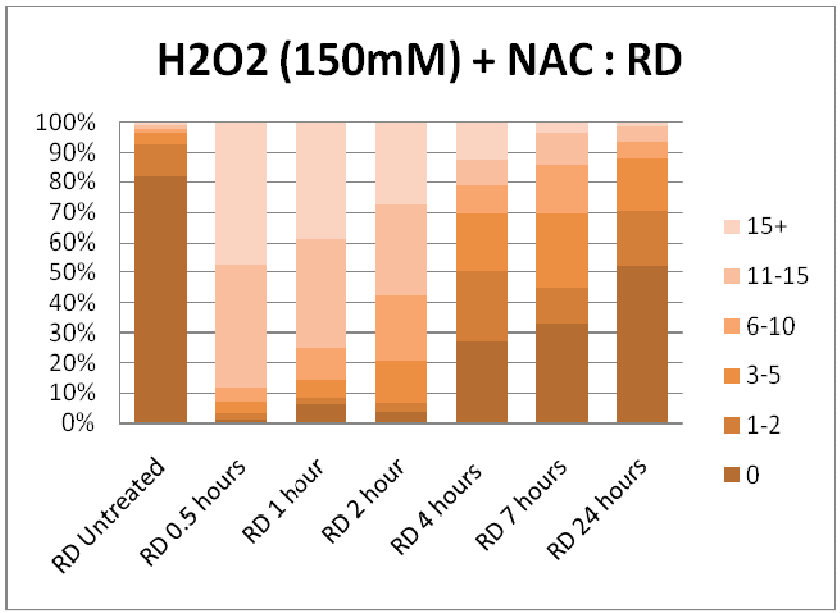


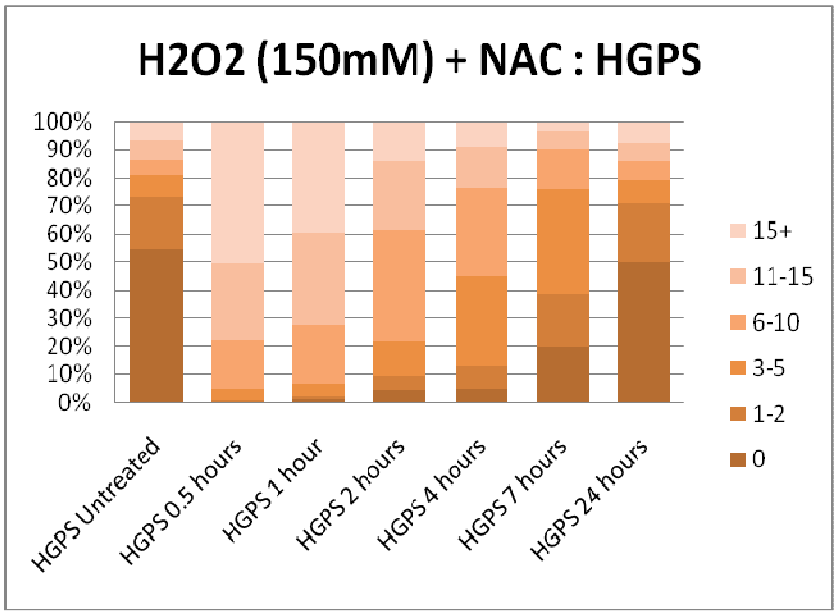


Graphs showing the number of fibroblasts with 0, 1-2, 3-5, 6-10, 11-15 or 15+ γ-H2AX foci after etoposide damage induction in the absence of NAC in GM, AG, RD and HGPS cell lines over the given time points.

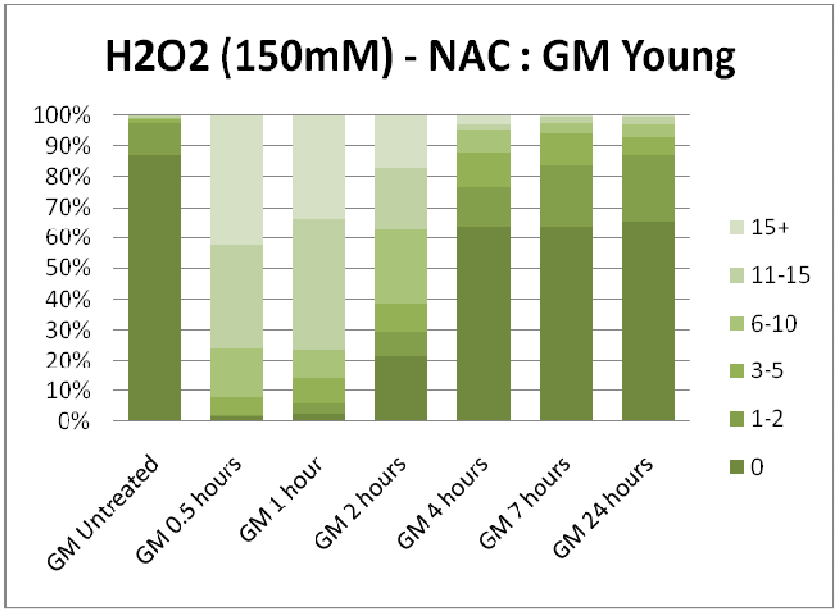


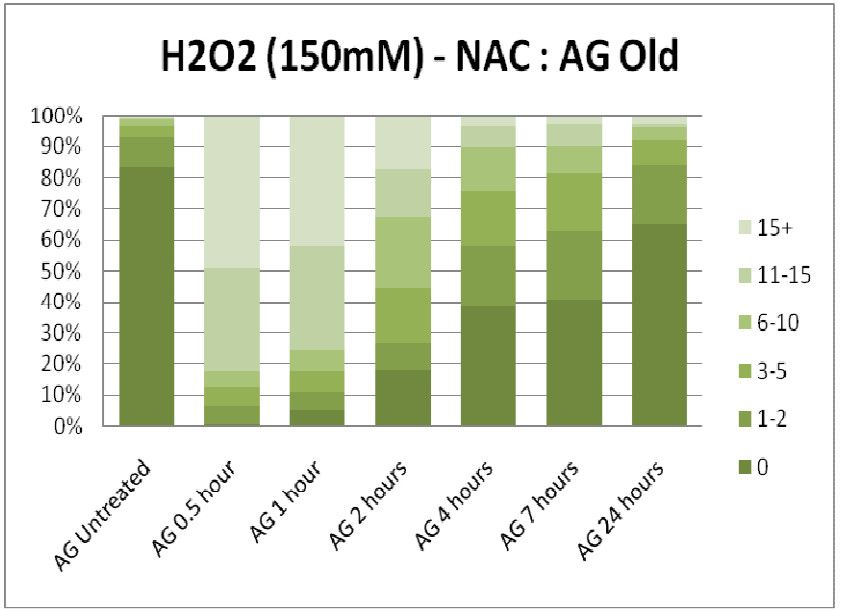


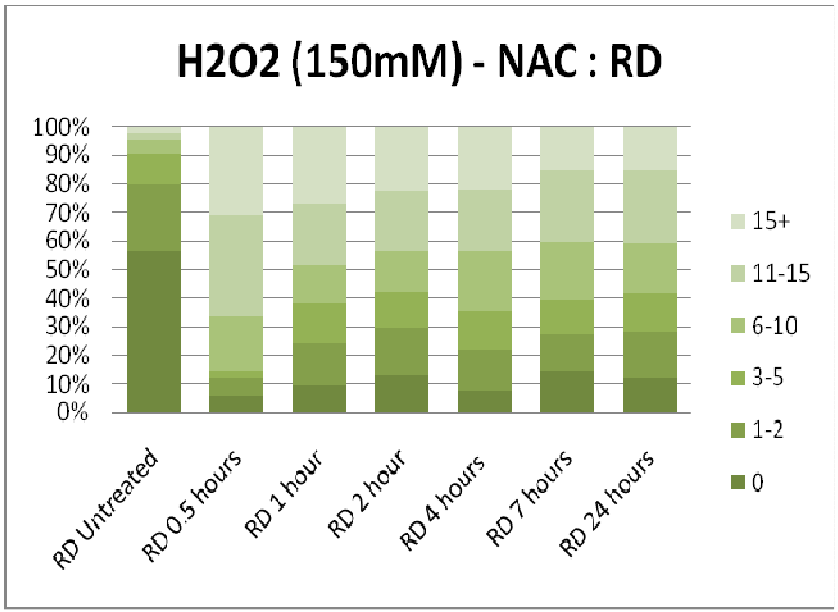


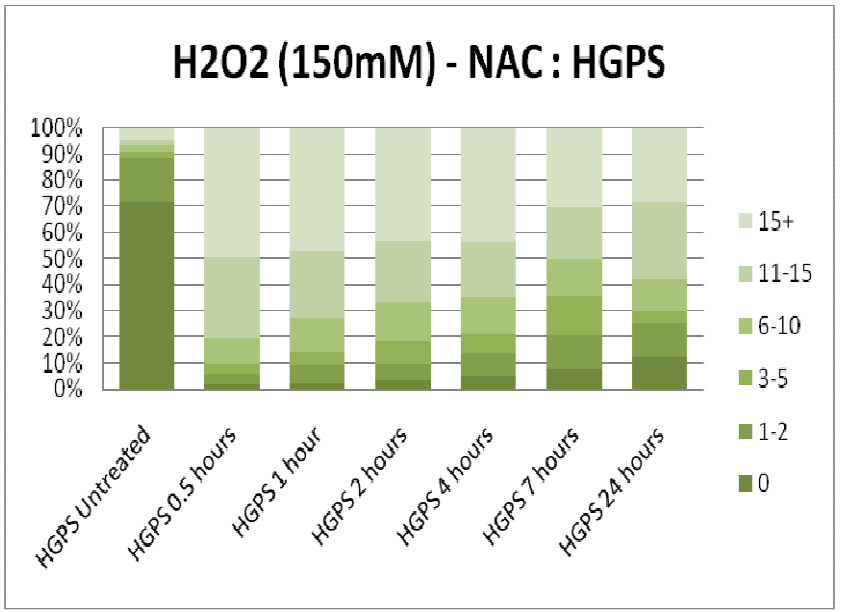


Graphs showing the number of fibroblasts with 0, 1-2, 3-5, 6-10, 11-15 or 15+ γ-H2AX foci after NAC pre-treatment and 150µM H_2O_2 damage induction in GM, AG, RD and HGPS cell lines over the given time points.

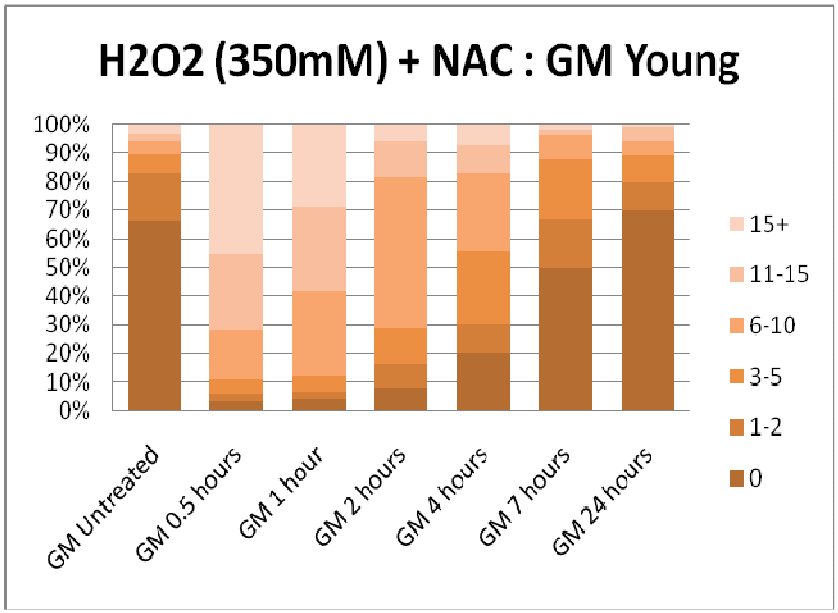


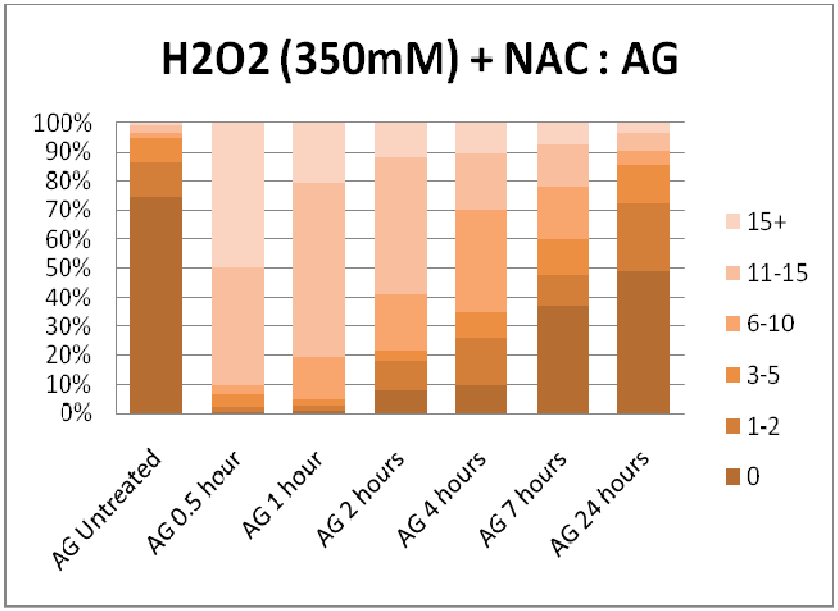


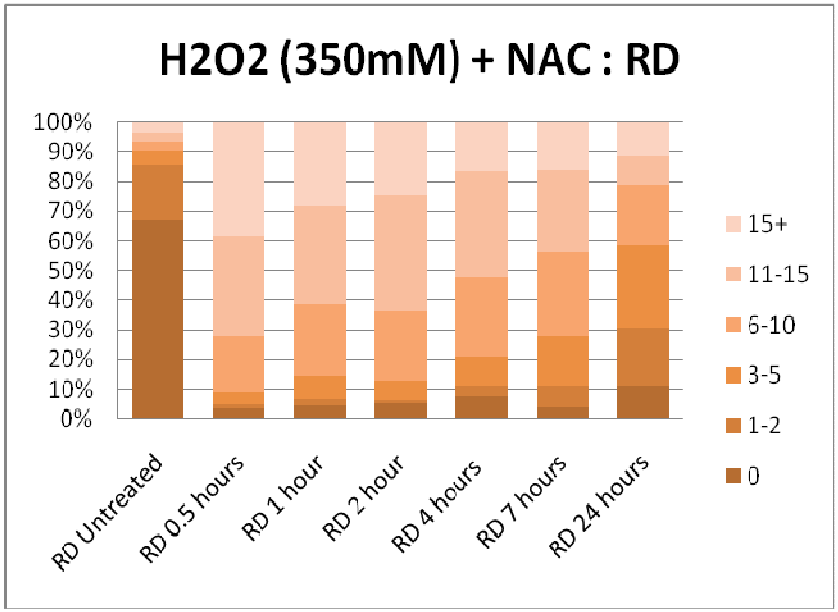


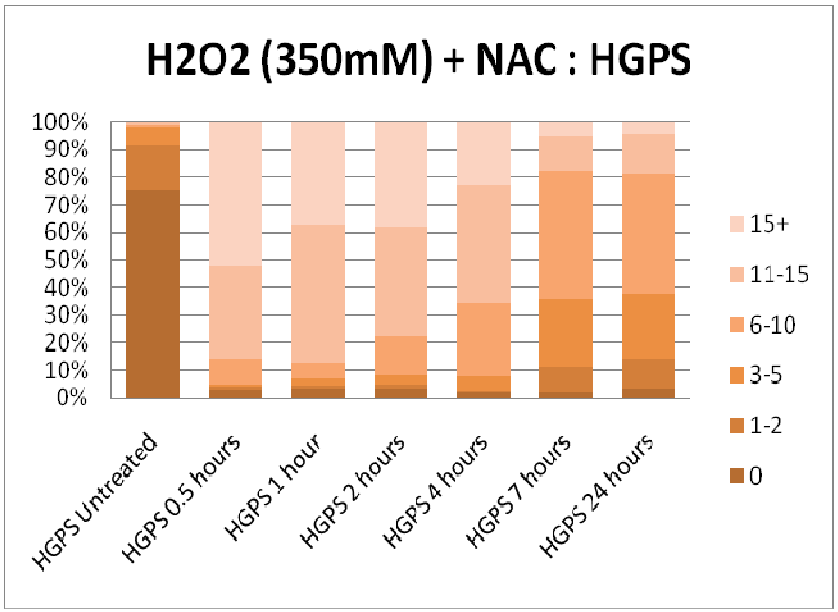


Graphs showing the number of fibroblasts with 0, 1-2, 3-5, 6-10, 11-15 or 15+ γ-H2AX foci after 150µM H_2O_2 induction in the absence of NAC in GM, AG, RD and HGPS cell lines over the given time points.









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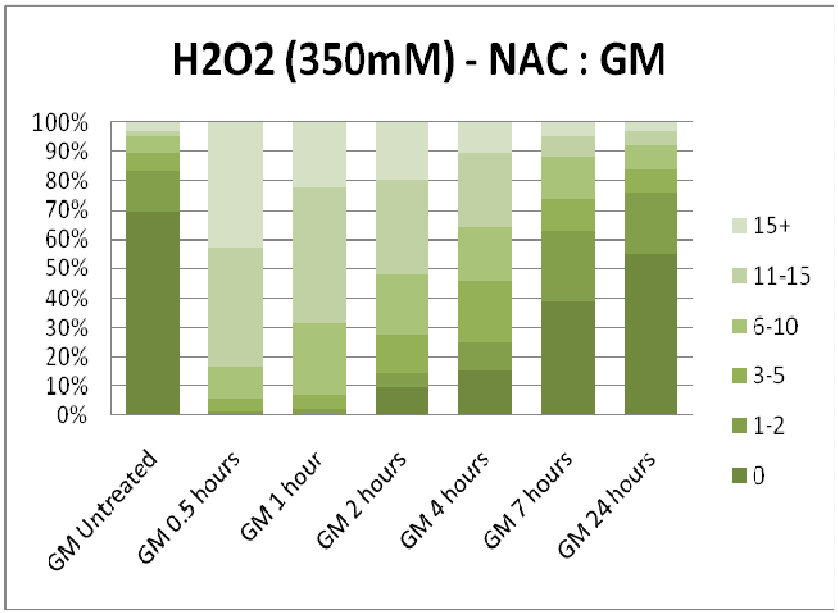

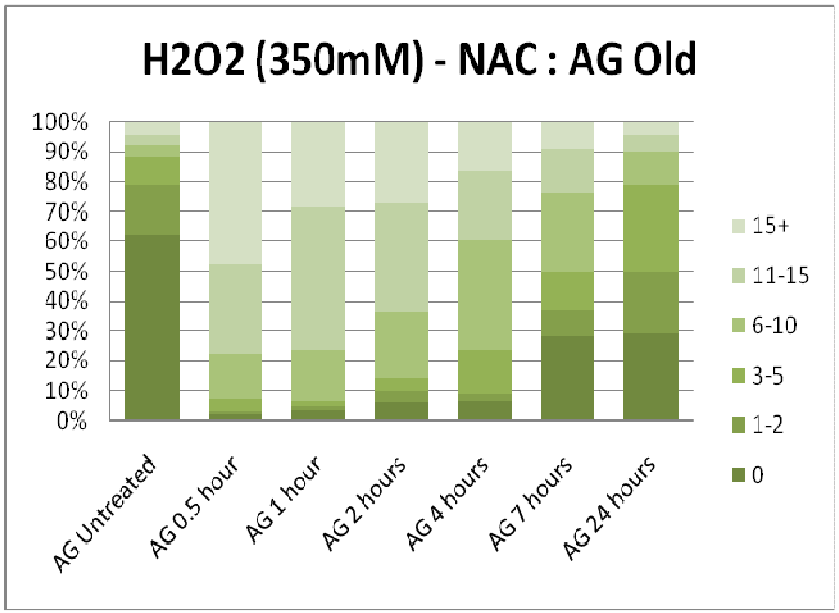

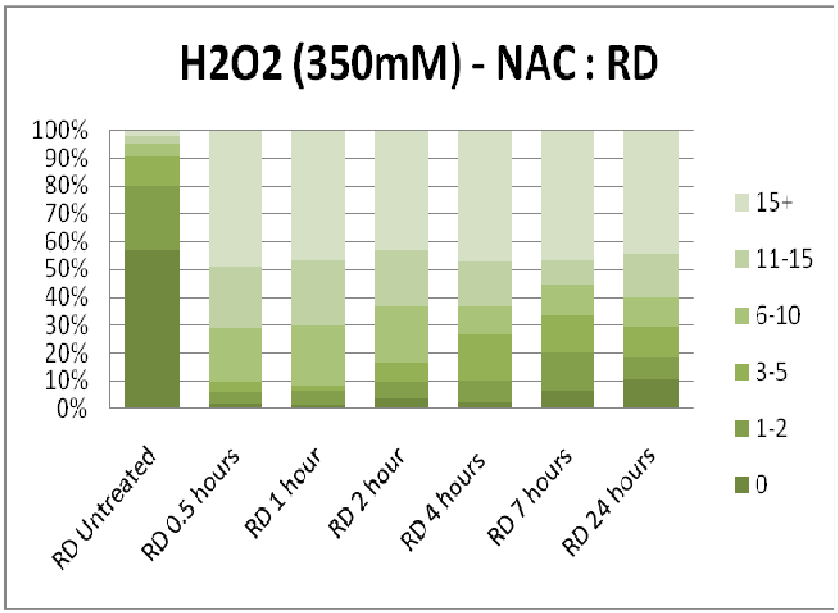

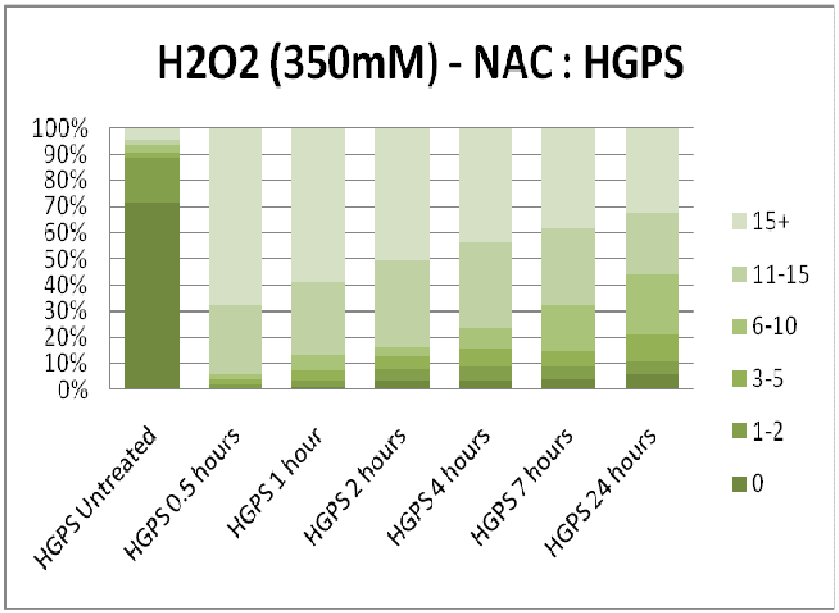

Graphs showing the number of fibroblasts with 0, 1-2, 3-5, 6-10, 11-15 or 15+ γ-H2AX foci after 350μM H_2O_2 induction in the absence of NAC in GM, AG, RD and HGPS cell lines over the given time points.