Role of Oxidative Stress Responses in Clonal Variations in Dental Pulp Stem Cell Ageing and Regenerative Potential

Thesis presented for the degree of
Doctor of Philosophy

Nadia Yousef Alaidaroos, BDS, MSc

Regenerative Biology Group
Oral and Biomedical Sciences
School of Dentistry
Cardiff University
UK

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Dedication

To Yakoub; my beloved brother,
Up there in heaven, I hope you are proud of me…
I miss you every day…

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Thesis Summary

Human dental pulp stem cells (hDPSCs) are increasingly being recognised as a viable cell source in regenerative medicine. However, significant heterogeneity in their *ex vivo* expansion capabilities are well-established, which influences their multipotent differentiation and therapeutic potential. This is partly attributed to contrasting telomere lengths between individual DPSC sub-populations influencing their relative susceptibilities to replicative (telomere-dependent) senescence, which impairs cellular regenerative properties. This has significant implication for regenerative medicine, in terms of obtaining sufficient cell numbers for therapeutic use, whilst maintaining stem cell and multipotency characteristics. As DPSCs have no reverse transcriptase, human telomerase catalytic subunit (hTERT) expression, this suggests that other mechanisms are responsible for variations in telomere lengths and replicative senescence susceptibilities between high and low proliferative DPSCs. As oxidative stress is a well-established mediator of cellular senescence, this study investigated whether DPSCs also differed in their susceptibilities to oxidative stress (0-200μM H_2O_2)-induced (telomere-independent) senescence and bimolecular damage; and whether differences in enzymic antioxidant profiles contribute to contrasting oxidative stress responses in high and low proliferative DPSC sub-populations.

Significant variations were shown in the susceptibilities of DPSC sub-populations to H_2O_2-induced senescence. Low proliferative DPSCs underwent accelerated senescence (<40PDs) with 0-200μM H_2O_2 treatments, accompanied by increased SA-β-galactosidase and senescence marker (p53, $p21^{waf1}$, $p16^{INK4a}$) detection. High proliferative DPSCs exhibited increased resistance to H_2O_2-induced senescence, reaching 80PDs (0-50μM H_2O_2) or 50-76PDs (100-200μM H_2O_2), prior to senescence marker detection. However, telomere lengths and stem cell marker expression were largely unaffected by increasing H_2O_2 treatment or culture expansion. Enhanced low proliferative DPSC susceptibility to H_2O_2-induced senescence was accompanied by increased oxidative stress-induced bimolecular damage at early PDs (2-10PDs), with elevated DNA (8-hydroxy-deoxy-guanosine), protein (carbonyl content) and lipid (peroxidation) detection, irrespective of H_2O_2 treatments. In contrast, high proliferative DPSCs exhibited limited oxidative biomolecular damage at early PDs (2-10PDs), only equivalent to low proliferative DPSCs at much later PDs (45-60PDs).

In line with their resistance to H_2O_2-induced senescence and biomolecular damage, significant increases in superoxide dismutase (SOD2) and glutathione S-transferase ζ (GSTZ1) expression, in addition to significantly increased total SOD activities, were identified in high proliferative DPSCs at early PDs (10-25PDs). However, SOD1/GSTZ1 expression and total SOD activities significantly declined during culture expansion (45-60PDs). In contrast, low proliferative DPSCs at early PDs (2-10PDs) mostly exhibited low/negligible SOD1, SOD2, SOD3, catalase, GSTZ1 and other glutathione-related antioxidant expression/activities overall, implying that antioxidant mechanisms are impaired in these sub-populations.

This study demonstrates that significant variations exist in the susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress-induced senescence and biomolecular damage, contributed to by inherent differences in SOD2 and GSTZ1 antioxidant profiles, which help maintain the proliferative, stemness and multipotency capabilities of high proliferative DPSCs during *ex vivo* expansion. Thus, the identification of such contrasting enzymic antioxidant profiles between high and low proliferative DPSCs enhances our understanding of DPSC biology and its inter-relationship with oxidative stress and cellular ageing overall.

Publications and Presentations

Publications

Alraies A, Alaidaroos NYA, Waddington RJ, Moseley R, Sloan AJ. Variations in Human Dental Pulp Stem Cell Ageing Profiles Reflect Contrasting Proliferative and Regenerative Capabilities. *BMC Molecular and Cell Biology* (2017), **18:**12-25.

Presentations

Alaidaroos N, Alraies A, Sloan AJ, Waddington RJ and Moseley R. Dental Pulp Stem Cells: Finding a Needle in a Dental Pulp Haystack. Speaking of Science Conference, Cardiff, UK, May 2015.

Alaidaroos N, Alraies A, Sloan AJ, Waddington RJ and Moseley R. Role of Oxidative Stress Responses in Clonal Variations in Dental Pulp Progenitor Cell Ageing and Regenerative Potential. 30th Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, UK, November 2015.

Alaidaroos N, Alraies A, Sloan AJ, Waddington RJ and Moseley R. Role of Oxidative Stress Responses in Clonal Variations in Dental Pulp Progenitor Cell Ageing and Regenerative Potential. 31st Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, UK, January 2017.

Alaidaroos N, Alraies A, Sloan AJ, Waddington RJ and Moseley R. Oxidative Stress Variations in Dental Pulp Progenitor Cell Ageing/Regenerative Potential. British Society for Oral and Dental Research (BSODR) Annual Meeting, Plymouth, UK, September 2017.

Alaidaroos N, Alraies A, Sloan AJ, Waddington RJ and Moseley R. Oxidative Stress Variations in Dental Pulp Progenitor Cell Ageing/Regenerative Potential. Cardiff Institute for Tissue Engineering and Repair (CITER) Annual Scientific Meeting, Cardiff, UK, September 2017.

Alaidaroos N, Waddington RJ, Sloan AJ and Moseley R. Role of Oxidative Stress Responses in Clonal Variations in Dental Pulp Progenitor Cell Ageing and Regenerative Potential. 32nd Annual School of Medicine and Dentistry Postgraduate Research Day, Cardiff, UK, January 2018 (**poster-prize awarded**).

Alaidaroos N, Waddington RJ, Sloan AJ and Moseley R. Role of Oxidative Stress Responses in Clonal Variations in Dental Pulp Progenitor Cell Ageing and Regenerative Potential. Annual School of Dentistry Research Day, Cardiff, UK, May 2018.

Alaidaroos N, Alraies A, Waddington RJ, Sloan AJ and Moseley R. Antioxidant Profiles Reflect Variations in Dental Pulp Progenitor Cell Proliferation/Regeneration. International Association for Dental Research (IADR/PER) Annual Meeting, London, UK, July 2018.

Alaidaroos N, Alraies A, Waddington RJ, Sloan AJ and Moseley R. Antioxidant Profiles Reflect Variations in Dental Pulp Progenitor Cell Proliferation/Regeneration. Cardiff Institute for Tissue Engineering and Repair (CITER) Annual Scientific Meeting, Hensol, UK, September 2018.

Glossary of Abbreviations and Nomenclature

3D	3 Dimensional
4-HNE	4-Hydroxynonenal
8-OHdG	8-Hydroxy-deoxy-guanosine
α-MEM	α-Modification minimal essential medium
AB-MSCs	Alveolar bone-derived, mesenchymal stem cells
AD-MSCs	Adipose-derived, mesenchymal stem cells
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ARE	Antioxidant responsive element
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BDNF	Brain derived neurotrophic factor
BM-MSCs	Bone marrow-derived, stem cells
BMP4	Bone morphogenic protein-4
BODIPY®	Boron dipyrromethene
BSA	Bovine serum albumin
CAT	Catalase
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CO_2	Carbon dioxide
CT	Cycle threshold
Ctrl	Control DNA sample
dCT	Delta cycle threshold/difference in cycle threshold
DDR	DNA damage response
DFPCs	Dental follicle progenitor stem cells
DIG	Digoxigenin
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DNPH	2,4-Dinitrophenylhydrazine
dNTPs	Deoxyribonucleotide triphosphates
DPSCs	Dental pulp stem cells
DTT	Dithiothreitol
E	Early PDs
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cell
EVs	Extracellular vesicles

FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GDNF	Glial derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GMSCs	Gingival mesenchymal stem cells
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSR	Glutathione reductase
GSS	Glutathione synthetase
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
GSTZ1	Glutathione S-transferase ζ
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
hTERT	Reverse transcriptase, human telomerase catalytic subunit
Klf4	Kruppel-like factor 4
ICC	Immunocytochemistry
IL	Interleukin
iPSCs	Induced pluripotent stem cells
L	Late PDs
LANGFR	Low affinity nerve growth factor receptor
LIF	Leukaemia inhibitory factor
LOOHs	Lipid hydroperoxides
MDA	Malondialdehyde
M-MLV	Moloney-murine leukaemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MVs	Microvesicles
MW	Molecular weight
NaCl	Sodium chloride
NRES	National Research Ethics Service
NADPH	Nicotinamide adenine dinucleotide phosphate
NGF	Nerve growth factor
Nrf2	Nf-E2 related factor 2

O_2	Oxygen
$\cdot O_2^-$	Superoxide radical
Oct4	Octamer-binding transcription factor 4
OD	Optical density
ODFR	Oxygen-derived free radical
·OH	Hydroxyl radical
PBS	Phosphate buffered saline
PDLSCs	Periodontal ligament stem cells
PDs	Population doublings
PMSF	Phenylmethylsulfonyl fluoride
PPARγ	Peroxisome proliferator-activated receptor-γ
pRb	Retinoblastoma protein
PRXs	Peroxiredoxins
PVDF	Polyvinylidene difluoride
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RQ	Relative quantification of gene expression
rRNA	Ribosomal RNA
RT	Reverse Transcription
RT-PCR	Reverse transcription-polymerase chain reaction
RT-QPCR	Real time quantitative polymerase chain reaction
SA-*β*-Gal	Senescence associated β-galactosidase
SASP	Senescence-associated secretory phenotype
SCAPs	Stem cells from dental papilla
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHEDs	Stem cells from human exfoliated deciduous teeth
SIPS	Stress-induced premature senescence
SOD	Superoxide dismutase
Sox2	Sex determining region Y-box 2
SSEA	Stage-specific embryonic antigen
TA	Transit amplifying
TAE	Tris acetate
TBE	Tris/borate/EDTA
TBS	Tris-buffered saline
TGF-β	Transforming growth factor-β

TGPCs	Tooth germ progenitor cells
TRF	Terminal restriction fragment
TRITC	Tetramethylrhodamine
TRX	Thioredoxin
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

Units of Measurement

%	Percentage
°C	Degrees Celsius
×g	Gravitational acceleration
AU	Arbitrary units
bp	Base pair
cm	Centimetre
µm	Micrometre
Nm	Nanometre
g	Gram
kb	Kilo base pair
M	Molar
mM	Millimolar
µM	Micromolar
mg	Milligram
µg	Microgram
ng	Nanogram
l	Litre
ml	Millilitre
µl	Microlitre
h	Hours
min	Minutes
S	Seconds
Rpm	Revolutions per minute
V	Volts
mW	Milliwatt

Table of Contents

Chapter 1

General Introduction

Chapter 1

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1.1 Overview

With the increase of aging populations worldwide, the development of new and successful treatment strategies to repair and regenerate traumatised tissue becomes a necessity to restore the form, vitality and function of the diseased organ. Many diseases may ultimately reach an advanced stage, causing total organ failure due to the limited available reparative treatment modalities. Tissue engineering utilising stem cell therapy is currently amongst the most promising therapeutic strategies that presents an area of particular interest to researchers and scientists across the world. With the remarkable progress being made in cellular therapy throughout the past decade, challenges still remain. Undoubtedly, the successful resolution of said issues will reflect positively on the quality of life, especially in the ever-increasing aged populations worldwide, through the regeneration of affected organs so providing cures for their late-stage disease.

Mesenchymal stem cells (MSCs) are considered an excellent resource for stem cell therapy and tissue repair, due to the special biological properties they possess. MSCs are privileged with self-renewal and multi-lineage differentiation potentials, in addition to immunomodulatory effects, which make them ideal for regenerative medicine applications. Dental pulp presents a unique source for MSCs due to their ease of obtainability, availability and the relative lack of ethical issues associated with their collection. However, the necessity for long-term culture expansion *in vitro* can lead to the onset of cellular senescence, which influences the biological properties of MSC populations, including dental pulp stem cells (DPSCs). Furthermore, as dental pulp is acknowledged to be composed of heterogeneous stem/progenitor cell populations with contrasting properties and behaviours, this presents another challenge due to the different biological characteristics each DPSC sub-population may possess.

This Thesis investigates one potential area behind the contrasting biological properties between DPSCs, by assessing the inter-relationships between DPSC

expansion capabilities, susceptibilities to cellular senescence, oxidative stress and the inherent enzymic antioxidant potentials of DPSC sub-populations. By identifying potential differences between DPSCs, this Thesis aims to enhance our understanding of the differences between DPSC sub-populations at a cellular and molecular level, whilst also being able to utilise the findings obtained for the selective screening, identification and isolation of more desirable DPSC sub-populations with superior proliferative and multipotency capabilities, for more efficient DPSC-based therapy development and regenerative medicine applications in future.

1.2 Stem Cells

Stem cells have the ability of indefinite self-replication through life. When certain signals are provided, these cells have the potential to self-regenerate and differentiate into various cell types, thereby forming the tissue required. Stem cells reside in quiescent state within various specialised niches located throughout the body (Gronthos et al., 2000). A niche is a highly organised micro-environment that provide a habitat for stem cells containing the elements necessary (such as growth factors and mediators of cell-cell/cell-extracellular matrix interaction), to enable stem cells self-renewal and the regulation of cell proliferation, differentiation and migration in response to injury (Gronthos et al., 2000; Discher, et al., 2009). Due to the essential roles that stem cells possess in the repair and regeneration of various tissues, there is a strongly held belief amongst scientists that stem cells offer highly promising potential as therapies for tissue engineering and regenerative medicine applications. Stem cells are essentially divided into two main types, embryonic stem cells and adult stem cells.

1.2.1 Embryonic Stem Cells

Embryonic stem cells (ESCs) are pluripotent stem cells that possess an ability to generate all cell types, due to their unlimited differentiation potential into the three germ tissue lineages: ectoderm, endoderm and mesoderm (Asatrian et al., 2015). These extraordinary abilities make ESCs amongst the most valuable stem cells source for the exploitation in regenerative medicine research. However, many ethical issues remain around their use, due to the unavoidable necessity to destruct their source, i.e. the embryo, to derive them. In addition, major obstacles exist due

the possibility of allogeneic rejection and teratoma formation that has been witnessed by several investigators (Grinnemo et al., 2006; Aldahmash et al., 2013). Thus, although ESC transplantation holds promising therapeutic potentials, safety issues remain a major limitation for their use in clinical tissue engineering (Asatrian et al., 2015).

Human ESCs are characterised by the expression of many cell surface markers and transcription factors, such as stage-specific embryonic antigen-3 (SSEA-3), stage-specific embryonic antigen-4 (SSEA-4), octamer-binding transcription factor-4 (Oct4), sex determining region Y-box 2 (Sox2) and Nanog (Rodda et al., 2005). Noteworthy, together with some specific growth factors, such as fibroblast growth factor (FGF, Pan and Thomson, 2007), Oct4 and Nanog are among the key transcription factors involved in maintaining the pluripotency and self-renewal ability of embryonic stem cells (Wang et al., 2006; Huang et al., 2014a). Oct4 is a transcription factor observed in cells of early embryos and in the undifferentiated embryonic stem cells, expressed in the embryonic neural crest (Guo et al., 2002). Bone morphogenic protein-4 (BMP4) and the leukaemia inhibitory factor (LIF) are also expressed in human embryonic cells to maintain pluripotency (Pan and Thomson, 2007).

1.2.2 Induced Pluripotent Stem Cells

The discovery of induced pluripotent stem cells (iPSCs) by Takahashi and Yamanaka (2006), is considered a major breakthrough in regenerative medicine. iPSCs are produced from somatic cells (such as human dermal fibroblasts), by their genetic reprogramming to a pluripotent, ESC-like state through the overexpression of key pluripotency genes, Oct4**,** Sox2, c-Myc and Kruppel-like factor 4 (Klf4) (Takahashi et al., 2007; Hackett and Fortier, 2011; Pennarossa et al., 2013). Transfections into reprogrammable cells are facilitated by methods, such as viral, plasmid, excisable constructs (Cre/LoxP system) or synthetic mRNA gene transfer (Yu et al., 2009; Yoshioka et al., 2013; Hu, 2014a, 2014b).

As iPSCs eliminate many of the ethical issues associated with human ESCs, these represent a viable alternative cellular source to ESCs for regenerative medicine-based therapies. Indeed, iPSCs are now being evaluated for a wide range of therapeutic applications in numerous biomedical fields, such as Neurology, Ophthalmology and Cardiology (Lee-Kubli and Lu, 2015; Hayashi et al., 2016;

Mazzola and Di Pasquale, 2020). However, despite such promise, many issues still remain as although iPSCs and ESCs exhibit similar cellular morphologies, gene expression profiles and teratoma forming capacities, studies have shown an increased risk of tumour formation with iPSCs (Gutierrez-Aranda et al., 2010). Therefore, ongoing research is aiming to overcome these issues, although iPSCs are also currently used as a multi-purpose tool in biomedical research, particularly in terms of the development and optimisation of iPSC-based models for drug development and disease modelling applications (Robinton and Daley, 2012; Chang et al., 2020; Nakao et al., 2020).

1.2.3 Adult Stem Cells

Adult stem cells have gained an ever-increasing growing popularity, due to their histocompatibility with patient tissue. In addition, these cells share certain characteristics to ESCs (Liao, 2005; Lin et al., 2008) and the expression of certain cell surface makers (Hoffman and Carpenter, 2005). Adult stem cells are defined as undifferentiated cells that reside within a specialised tissue. They are cells that have the potential for self-renewal, proliferation and differentiation towards multiple cell lineages (Polak and Bishop, 2006). Hence, adult stem cells give rise into all of the specialised cell types of their tissue of origin. This differentiation process eventually leads to changes in cell morphology, while expressing tissue specific proteins simultaneously (Weissman, 2002). The main function of adult stem cells is to maintain haemostasis, which is essential for tissue renewal and regeneration (Leblond, 1964; Gussoni et al., 1999). Adult stem cells have also been identified in the vast majority of body tissues and organs, such as brain, heart, dental pulp, blood vessels, bone marrow, the cornea and retina of the eye, pancreas, skin, liver, skeletal muscle, ovarian epithelium and testis (Bissels et al., 2013). Adult stem cells are classified according their location in the body. Bone marrow have at least two distinct types of stem cells populations: hematopoietic stem cells (HSCs) that form blood cell types within the bone marrow, and the stromal stem cells (also referred to as MSCs), which are capable of differentiating into different types of tissue, such as bone and cartilage (Otto and Wright, 2011).

1.2.3.1 Mesenchymal Stem Cells

MSCs have gained considerable interest in the regenerative medicine field, making them an excellent resource for stem cell therapy and tissue repair, due to the specialised characteristics they possess. MSCs can potentially present valuable therapeutic strategies for many diseases, as they are privileged with unique biologic properties (Sharma et al., 2014), including self-renewal and multipotent differentiation abilities (Figure 1.1), in addition to their paracrine effects on other cell types within the local stem cell niche or wound environments. Consequently, MSCs are considered ideal for regenerative medicine applications (Manuguerra-Gagné et al., 2013).

MSCs were first discovered in bone marrow by Friedenstein et al. (1968), who then discovered their ability to form fibroblastoid cells colonies (Friedenstein et al., 1970). Subsequent studies found that MSCs can be isolated from various tissues, such as adipose tissue (Eirin et al., 2012), umbilical cord blood (Martin-Rendon et al., 2008), placenta (Fukuchi et al., 2004), dental pulp (Huang et al., 2009), synovial membrane (Hermida-Gómez et al., 2011), peripheral blood (Tondreau et al., 2005), periodontal ligament (Park et al., 2011), and endometrium (Schwab et al., 2008). There is an evidence that MSCs are present in all vascularised tissues throughout the body (Crisan et al., 2008). However, the identity and the niche sources of MSCs *in vivo* are not entirely clear and under intense debate. Indeed, some scientists have proposed that MSCs are derived from the cells lining the capillaries i.e. the pericytes (Caplan, 2008), while others report that additional cell types can also give rise to MSCs (Corselli et al., 2011; Zhao et al., 2014).

1.2.3.2 Therapeutic Effects of Mesenchymal Stem Cells

MSCs can apply their therapeutic effects via several mechanisms. They can migrate to sites of inflammation, differentiate into various cell types and exert anti-inflammatory and immunomodulatory effects. In addition, MSCs can stimulate repair processes via the secretion of bioactive molecules (Otto and Wright, 2011; Wang et al., 2012). Many researchers have reported that MSC migration is influenced by a range of inflammatory cytokines and growth factors (Aggarwal and Pittenger, 2005; Yagi et al., 2010). Once MSCs reach sites of inflammation, they can release mediators that prevent immune cell proliferation and activate systemic anti-inflammatory responses. Furthermore, their secretion of specific cytokines and

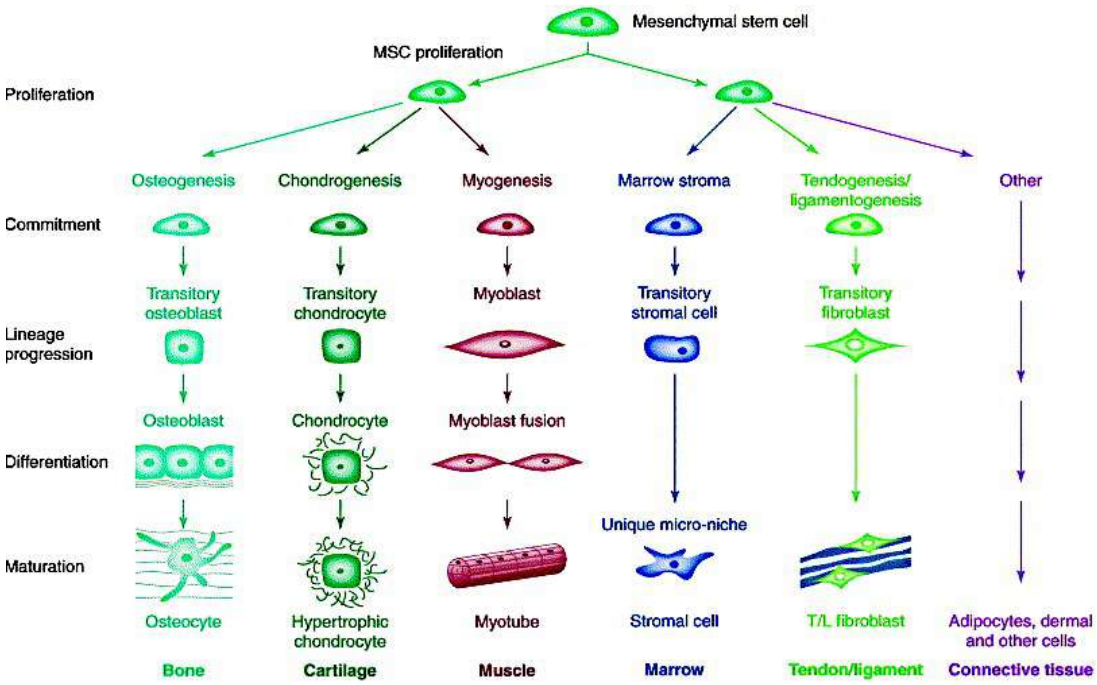


Figure 1.1. Multi-lineage differentiation of MSCs (Caplan and Bruder, 2001).

growth factors, such as transforming growth factor-ß (TGF-ß), interleukin-6 (IL-6) and IL-10, together with other trophic molecules that promote cell-cell interactions, positively influence repair mechanisms (Plotnikov et al., 2008; Ankrum and Karp, 2010). Moreover, MSCs have the potential to recruit autologous stem cells to sites of inflammation to promote tissue repair (Phinney and Prockop, 2007). Therefore, MSCs have unique immunomodulatory effects (Liechty et al., 2000; Popp et al., 2008). Although cell-cell contact and the release of immunosuppressive factors play major role in immunomodulatory effect of MSCs, the mechanism behind these effects is still not fully understood.

Although MSCs rely on various mechanisms to exert to their therapeutic effect, many clinical experiments have concluded that the therapeutic potential of MSCs is mainly attributable to the paracrine factors, the secretome, contained within released microvesicles (MVs), particularly the exosome fraction of extracellular vesicles (EVs). Indeed, ever-increasing evidence indicates a prominent role for such exosomes in mediating intracellular communications in various cell types, including MSCs (Spees et al., 2016). Thus, due to their ever-emerging immuno-modulatory and stimulatory properties on reparative processes, MSC-derived exosomes are increasingly be advocated and exploited as novel therapeutic candidates for regenerative medicine applications in a wide range of diseases and medical

conditions (Zhang et al., 2014; Colao et al., 2018; Nooshabadi et al., 2018; Zhao et al., 2019).

1.2.3.3 Challenges with Mesenchymal Stem Cells

With all the advantages of MSCs, some obstacles still remain. Although MSCs present low risk of cancer development compared to embryonic stem cells, tumour formation has been reported with MSCs (Fang et al., 2005). Obtaining MSCs can also be difficult, due to the inevitable invasive procedures needed to derive them from the donor. Another major obstacle hindering the successful use of MSCs in cell therapy and tissue engineering is the limited numbers of MSCs available to isolate. For instance, it is estimated that MSCs comprise only 0.01%-0.001% of the total number of cells within bone marrow tissues (Friedenstein et al., 1982). Therefore, extensive *in vitro* expansion is necessary to obtain sufficient cell numbers for successful tissue engineering therapy development and use. Indeed, it has been estimated that at least 10^8 total cells are ideally needed for a successful MSC transplant therapy (Ringdén et al., 2006).

Another obstacle is the limited differentiation potential with increasing donor age, which can present significant problems (Kern et al., 2006; Martens et al., 2012). In addition, as MSCs exhibit different lineage commitment relating to their *in vivo* niche environments and their tissue sources, this often results in heterogeneous population of MSCs being isolated (Russell et al., 2010; Lv et al., 2012). For example, the ectopic transplantation of bone marrow-derived MSCs (BM-MSCs) forms heterotopic bone tissue, while DPSC transplantation forms reparative dentine-like tissue (Batouli et al., 2003). By and large, MSCs are considered promising cellular sources for tissue engineering, due to their favourable biological properties, accessibility and diverse differentiation capabilities. However, a more comprehensive understanding of their behaviour could potentially minimise some of the issues currently surrounding their clinical development and applications as cell therapies, as described above.

1.2.3.4 Hierarchical Model of Mesenchymal Stem Cells

Although stem cells vary in their types and niche sources, they share common characteristics that make them distinctive from other mammalian cells, namely their self-renewal and multipotency properties. Stem cells possess a unique undifferentiated nature, in which they maintain their stemness through their self-renewal capabilities of generating identical daughter cells, as well as being multipotent through the generation of differentiated daughter cells capable of forming all cell types necessary for tissue formation or repair (Weissman, 2002; Riquelme et al., 2007). However, stem cells remain in a quiescent state, unless stimulated to enter the cell cycle and undergo cell division (Li and Clevers, 2010; Cheung and Rando, 2013). This process occurs in a very well executed hierarchy to minimise the risk of DNA replication mutation (Reya et al., 2001).

In order for MSCs to successfully accomplish their functions of self-renewal and differentiation, they go through a slow cell division process in which they first divide symmetrically producing identical daughter cells that both have equivalent low proliferative and multi-potential differentiation properties (Ho, 2005). Some of these adult stem cells continue this division process asymmetrically to produce cells with different cellular fates, giving rise to two distinct daughter cells, where one is an identical daughter cell copy of the original stem cell and the second cell is a progenitor cell known as transit amplifying (TA) cell, which has high proliferative and low differentiation capabilities. TA cells further divide to form large colonies to provide enough cell numbers, before achieving a more mature progeny in which they produce a smaller colony of progenitor cells that are more committed, yet with reduced proliferation abilities (Ho, 2005; Morrison and Kimble, 2006; Riquelme et al., 2007; Figure 1.2). In this context, although MSCs are capable of multi-lineage differentiation into adipogenic, osteogenic and chondrogenic lineages (Pittenger et al., 1999), this differentiation capacity is considered to be lineage restricted (Russell et al., 2010). It is also suggested that successful MSC differentiation potential *in vitro* depends on the protocol used and the cell donor source. However, studies have shown that 50% of MSC clones possess tri-potential, 30% have bi-potential and 10% exhibit uni-potential capabilities (Russell et al., 2010). In fact, while such variation in lineage potential renders MSC populations functionally heterogeneous that may influence their use for tissue regenerative purposes, it offers an opportunity to repair a wider range of tissues following cell screening and the identification of suitable

cell sub-populations for therapeutic use. For example, MSC populations screened for above-average chondrogenic potential demonstrated more robust cartilage repair properties, compared to unscreened populations (Jiang et al., 2014). Furthermore, another study showed that only approximately half of a subcutaneously-implanted MSC population demonstrated osteogenic potential in mice (Kuznetsov *et al.*, 1997).

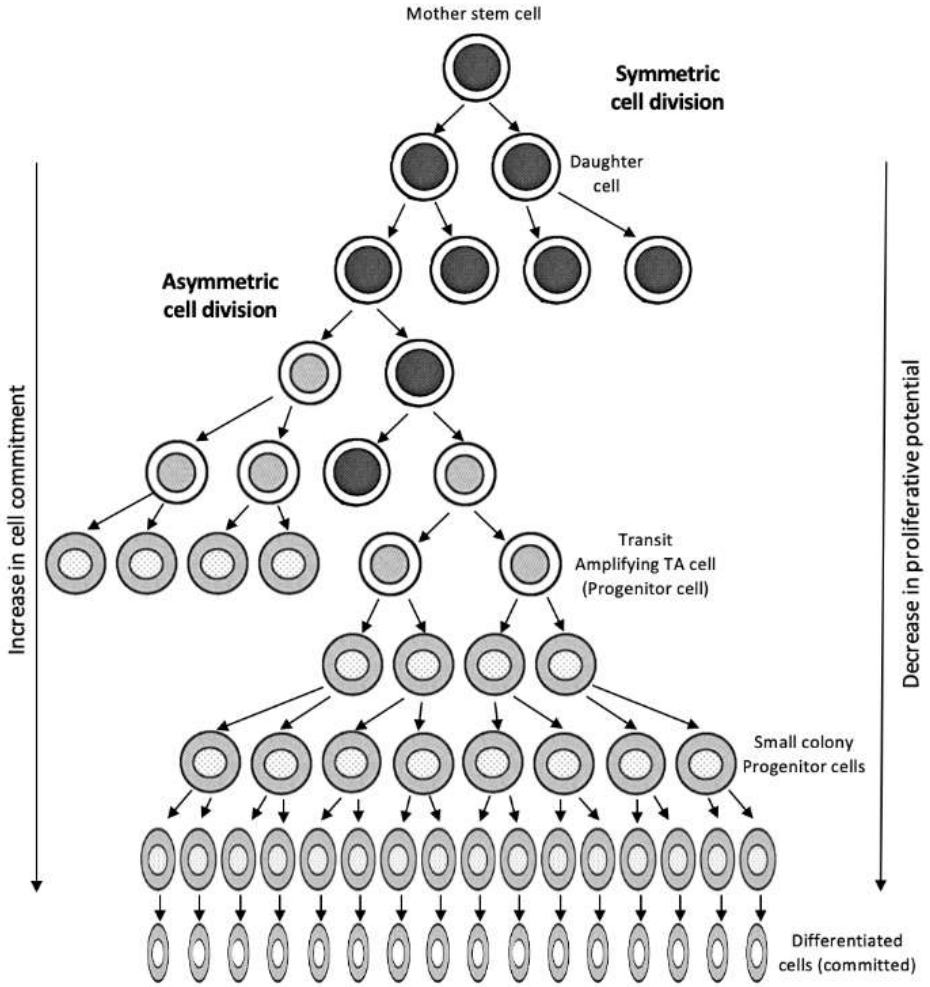


Figure 1.2. Hierarchy model of stem cells division (Chan et al., 2004).

1.2.3.5 Asymmetric Stem Cell Division

While the main goal of symmetric cell division goal is the proliferation and expansion of cell populations, usually in response to pathology or tissue injury (Morrison and Kimble, 2006), asymmetric cell division aims to generate cells of different properties while maintaining a constant stem cell population, thereby providing the cellular diversity found in all multicellular organisms (Berika et al., 2014; Figure 1.2). Although the exact mechanisms are unclear, asymmetric cell

division is controlled by a combination of complex biological pathways that include intrinsic and extrinsic cellular mechanisms within the stem cell niche (Yamashita, 2009). The main goal of cell division is to support self-renewal and maintain the balance between self-renewal and stem cell differentiation. This well-controlled balance is important for organ development and maintenance, but also critical for the repair and regeneration of tissues post-injury (Berika et al., 2014). For instance, during tissue repair, a temporary increase in the number of symmetric divisions may be required. On the other hand, chronic tissue injury may compromise stem cell abilities to adequately respond to repair the affected tissues. Indeed, it has been observed that over long periods in culture, one daughter cell can be less proficient as a stem cell than another, even when both daughter stem cells maintain their stem cell markers expression following a limited number of cell divisions (Inaba and Yamashita, 2012). This is an important finding and must be considered during the selection of stem cells for the use in stem cell therapy. Therefore, understanding the biological behavior of adult stem cells and the factors behind their properties is essential for *in vitro* cell expansion, maintenance of stem cell characteristics and their differentiation potential in culture, in order for stem cell populations to be of optimal quality for therapeutic purposes (Berika et al., 2014).

1.2.3.6 Other Types of Adult Stem Cells

Many other types of adult stem cells are present in different tissues for repair and regeneration, such as HSCs, epithelial stem cells, neural stem cells and endothelial stem cells. HSCs are adult stem cells that reside in the hematopoietic system (Stocum, 2001). Within this system, HSCs are the only cells that possess the potential for self-renewal and multipotency to generate all specialised blood cells, while maintaining sufficient pools of hematopoietic stem cells throughout the entire mammalian lifespan (Chotinantakul and Leeanansaksiri, 2012). Although the primary site for the maintenance of HSCs and haematopoiesis is the bone marrow, they can also be isolated from peripheral blood and umbilical cord blood, characterised based on their expression of cell surface markers, such as CD34, c-KIT and CD90 (Morrison and Scadden, 2014).

The remarkable potential that HSCs possess appears in their ability to differentiate into all mature blood cells involved in various biological activities, including the inflammatory response, haemostatic control and immune function

(Seita and Weissman, 2010). HSCs can also differentiate into other cells beyond the tissue where they reside, such as endothelial cells (Elkhafif et al., 2011), adipocytes (Sera et al., 2009), osteochondrocytes (Mehrotra et al., 2010), cardiomyocytes (Pozzobon et al., 2010), fibroblasts/myofibroblasts (Ebihara et al., 2006), liver cells (Sellamuthu et al., 2011) and pancreatic cells (Minamiguchi et al., 2008). For more than three decades, HSC transplantation has been widely used as the standard of care during the treatment of many diseases, such as leukemia and lymphoma (Hołowiecki, 2008). Nevertheless, HSC transplantation remains challenging and can still cause many serious complications to patients (Gratwohl et al., 2010).

Epithelial stem cells reside in the epidermis of skin and in the epithelial lining of all organs, such as the digestive tract (Barker et al., 2009; Van der Flier and Clevers, 2009; Kong et al., 2018), lungs (Rawlins and Hogan, 2006) and cornea (Ramos et al., 2015). Although epithelial stem cells have not been reported in human teeth, these have been located within the cervical loop regions of mouse root apices, where these generate ameloblasts that form enamel (Chavez et al., 2012). This could be related to the continuous need for enamel regeneration within the continually erupting mouse incisor, in contrast to human incisors.

Neural crest cells are initially formed during embryogenesis in the dorsal margins of neural folds, ultimately delaminating during epithelial-mesenchymal transition and migrate to several locations within the embryo, where they contribute to the formation of a wide variety of tissue types (Sauka-Spengler and Bronner-Fraser, 2008). Cranial neural crest gives rise to the neural cells that contribute mainly to the bone and cartilage of the orofacial region, in addition to the nerve ganglia, smooth muscles and connective tissues. As such, dental tissue-derived, MSCs are proposed to possess similar properties to those of neural crest cell progenitors, considering that they originate from the dental mesenchyme derived from neural crest tissue (Achilleos and Trainor, 2012).

Endothelial stem cells are located in the lining of blood vessels and are involved in the repair of endothelial tissues (Sen et al., 2011). Several studies have demonstrated that DPSCs exhibit endothelial-like phenotypes (d'Aquino et al., 2007). *In vivo* studies have demonstrated the ability of DPSCs to form capillary-like structures (Iohara et al., 2008; Marchionni et al., 2009) and increase blood vessel density (Aksel and Huang, 2017), suggesting differentiation properties similar to endothelial-derived, stem cells.

1.3 Dental Pulp

Dental pulp is a loose connective tissue located within the pulp chamber of the root canal. Through apical foramen, the dental pulp connects to the surrounding periodontium, allowing blood vessels, lymphatics and nerves to enter the pulp canal and provide nourishment, sensation and inflammatory reactions. The dental pulp originates from ectomesenchymal and mesenchymal origins that develop from the dental papilla. It is rich in vasculature and innervation, in addition to lymphatics, immune cells, fibroblasts, odontoblasts and water (Trowbridge, 2003). Dental pulp contains a distinct heterogeneous cell population existing in several niches within different zones of the pulp (Mitsiadis et al., 2003). Thus, it is established that DPSCs have contrasting proliferative capacities, which are attributed to these contrasting neural crest or mesodermal developmental origins that can be difficult to distinguish (Komada et al., 2012). Normally, odontoblasts are responsible for formation of primary dentine that surrounds the pulp (Smith et al., 1995), in addition to the secondary dentine formed throughout the life and in response to mild injury by reactionary dentinogenesis. However, during severe dental trauma or insult leading to odontoblast cell death, DPSCs induce dentine repair through their differentiation into odontoblast-like cells and secretion of tertiary reparative dentine (Smith et al., 1995; Duque et al., 2006).

1.3.1 Tooth Development

Human dental development is initiated prenatally and continues until the age of 18-25 years (Aiello and Dean, 1990). Embryonically, the oral cavity is lined by the ectoderm, which together with the endoderm and mesoderm, is one of the three germ layers that give rise to the organism. Tooth germs are characterised by their dual composition, ectoderm and mesoderm. While enamel is of ectodermal origin, all other dental tissues, including the dentine and dental pulp, originate from the associated mesenchyme. Specifically, dental pulp is formed by combination of ectodermal and mesodermal layers during the sixth week of embryogenesis (Thesleff and Sharpe, 1997; Sinanan et al., 2004).

Some studies suggested that odontoblasts, the dentine matrix and the majority of the pulpal tissue are all of cranial neural crest origin (Chai et al., 2000). The primary odontoblasts differentiate from dental papilla cells and ultimately secrete dentine. The very last division of dental papilla cells gives rise to two

daughter cells, one is fully differentiated into odontoblast while the other remains undifferentiated forming the sub-odontoblast population (Ruch et al., 1995; Smith and Lesot, 2001). As development continues, the dental pulp population grows further containing additional cells that are of non-neural crest origin. These cells are suggested to be derivatives of the first branchial arch mesenchyme (Chai et al., 2000). Thus, such events result in the existence of heterogeneous cellular populations that will be ultimately occupying the mature dental pulp.

1.3.2 Dental Pulp Stem Cells

Several dental tissue-derived, stem cell populations have been recognized within oral tissues. Not only have these been found to be appropriate for applications in oral and maxillofacial tissue engineering, but beyond. Indeed, these were described as MSCs according to the minimal criteria of the International Society for Cellular Therapy (Huang et al, 2009a). These include DPSCs (Gronthos et al., 2000), "stem cells from human exfoliated deciduous teeth" (SHEDs, Miura et al., 2003), "periodontal ligament stem cells" (PDLSCs, Seo et al., 2004), "dental follicle progenitor stem cells" (DFPCs, Morsczeck et al., 2005), "stem cells from dental papilla" (SCAPs, Sonoyama et al., 2006), "tooth germ progenitor cells" (TGPCs, (Ikeda et al., 2008), "gingival mesenchymal stem cells" (GMSCs, Zhang et al., 2009) and "alveolar bone-derived mesenchymal stem cells" (AB-MSCs, Matsubara et al., 2005; Figure 1.3).

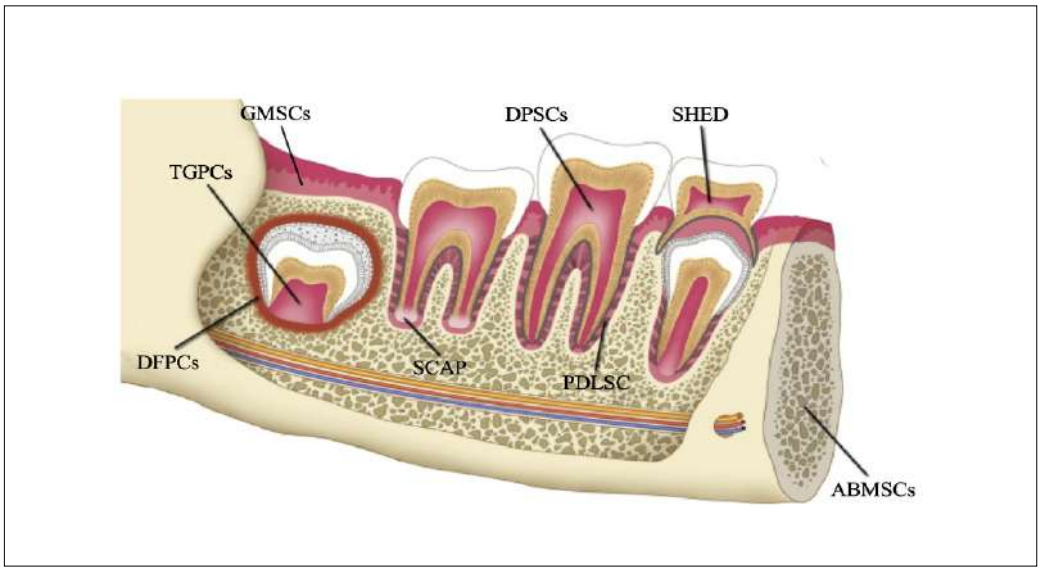


Figure 1.3. Main sources of human dental-derived stem cells (Egusa et al., 2012).

1.3.2.1 Origin and Sources of Dental Pulp Stem Cells

The dental pulp is considered a unique source of mesenchymal stem cells. DPSCs were first discovered by Gronthos et al. (2000), who isolated these cells from the dental pulp of impacted wisdom teeth of adult donors. Upon investigation, these cells were shown to exhibit some characteristics of both neural and epithelial stem cells. Various studies have since suggested that DPSCs are adult multipotent stem cells that arise from several stem cell niches. These niches are proposed to be located within three histologically distinguishable different zones in the dental pulp; namely the cell-rich zone, which is the innermost layer densely populated with cells including undifferentiated MSCs, fibroblasts, macrophages and capillaries, the pulp matrix zone in the central region of the pulp, which contain pulp capillaries, nerves and dental pulp cells and the outermost odontoblasts-containing layer which contain the odontoblasts responsible for dentine formation. Additionally, a fourth zone called zone of Weil is observed beneath the odontoblastic layer in the coronal region of the pulp. However, it is a cell-free zone suggested to be an area for odontoblasts replacement (Lizier et al., 2012; Figure 1.4). Morphologically, DPSCs are described as large spindle-shaped cells, containing a large centrally located nucleus and cytoplasmic extensions in culture, being morphologically identical to BM-MSCs (Gronthos et al., 2000).

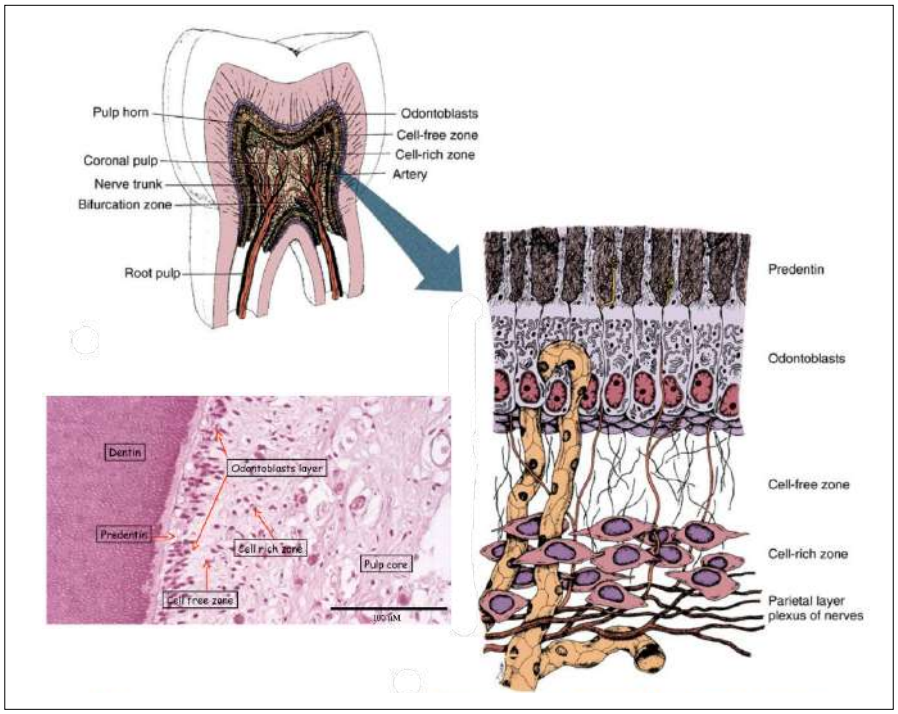


Figure 1.4. Architecture and zones within dental pulp (Orban and Bhaskar, 1972).

The origin of DPSCs is a subject of intense debate. Seo et al (2004) related DPSCs expression of early markers for both mesenchymal and neuro-ectodermal stem cells to their cranial neural crest origin. However, it was recently reported that a significant subpopulation of DPSCs are of glial origin. Based on this observation, it was suggested that each DPSC population can be defined based on its ability to regenerate diseased tissue and this will depend on their tissue of origin (Kaukua et al., 2014). DPSCs can be found in dental pulp tissue of primary and permanent human teeth and most commonly in wisdom teeth (d'Aquino et al., 2007). Interestingly, scientists have observed that third molar dental germ pulp distinctly contains some undifferentiated cells, located within the cell-rich zone of the dental pulp (Yalvaç et al., 2010). This may be due to the fact that the roots of wisdom teeth continue the growing process even by the age of eighteen years. Theoretically, wisdom teeth contain the most undifferentiated cells as they have the youngest pulp being the last teeth to erupt (Ponnaiyan and Jegadeesan, 2014).

Another source for DPSCs is the supernumerary teeth. Huang et al. isolated DPSCs from supernumerary tooth and noticed that DPSCs derived at the development stage of the crown are more proliferative than those derived at later stages (Huang et al., 2008a). The SHEDs from exfoliated human deciduous teeth are another source of DPSCs. These cells are immature and were observed to express a wide variety of neural stem cell markers, including nestin, β-III tubulin and glial fibrillary acidic protein (GFAP, Kerkis et al., 2006). In addition, they have demonstrated superior growth rates, compared to those derived from other regions. This can be related to their lower level of maturity, compared to other stem cells within the body and can provide a reasonable justification for their rapid growth rate (Kabir et al., 2014). Moreover, DPSCs can be isolated from inflamed pulps, such as pulp polyp tissue. This tissue have been reported to contain suitable amounts of dental pulp stem cells with multi-lineage differentiation potential analogous to those of normal pulp tissues (Attar et al., 2014). Likewise, Alongi et al. (2010) isolated DPSCs from inflamed pulps and although these lost some of their stem cell properties *in vitro,* they still demonstrated tissue regeneration capability *in vivo*. Nevertheless, inflamed pulps are still considered as an attractive source of DPSCs, particularly as inflamed pulps are always disposed of following pulpectomies.

1.3.2.2 Advantages of Dental Pulp Stem Cells

Dental pulp provides a remarkable source of MSCs, due to the many advantages they possess (Huang et al., 2006, Figure 1.5). Indeed, the MSC-like traits that DPSCs present render them potentially superior to other stem cell types described in the literature. Indeed, one of the most favourable characteristics of DPSCs is their multi-lineage differentiation potential (Huang et al., 2009a). DPSCs have an *in vitro* ability to differentiate into all classical mesodermal cell lineages, demonstrating osteogenic, chondrogenic and adipogenic capabilities, although their ability towards adipogenic differentiation appears to be less achievable (Struys et al., 2011; Hilkens et al., 2013). Moreover, many studies have reported that DPSCs have similar regenerative properties to BM-MSCs (Kawashima, 2012; Tatullo et al., 2015). Interestingly, it has been reported that human DPSCs have a higher proliferation potential, self-renewal capacity and a higher immunomodulatory ability *in vitro*, in comparison to human BM-MSCs (Gronthos et al., 2002). Another advantage of DPSCs is their relative ease of obtainability with minimal tissue interference. The reason behind this is that DPSCs are obtained from pulps of teeth that are to be disposed of, either because they are clinically un-restorable or due to orthodontic purposes (Woods et al., 2009), yet with a relatively lack of ethical and legal issues in their removal (Huang et al., 2009a). In addition, DPSCs are readily available due to the possibility of stem cell banking. Most studies have reported that DPSCs can be cryopreserved long-term, whilst maintaining their biological characteristics, including their viability, proliferative and differentiation capabilities, secretory profiles and functions upon thawing (Woods et al., 2009; Galipeau and Krampera, 2015; Wuchter et al., 2015). For example, osteoblasts derived from DPSC osteogenic differentiation were shown to form a woven bone-like tissue that successfully produced a 3-dimensional (3D) lamella bone tissue upon transplantation in immunocompromised rats, following long-term cryopreservation (Papaccio et al., 2006). Furthermore, DPSCs have been shown to preserve their stem cell properties after cell and whole dental tissue cryopreservation (Temmerman et al., 2010; Lee et al., 2012).

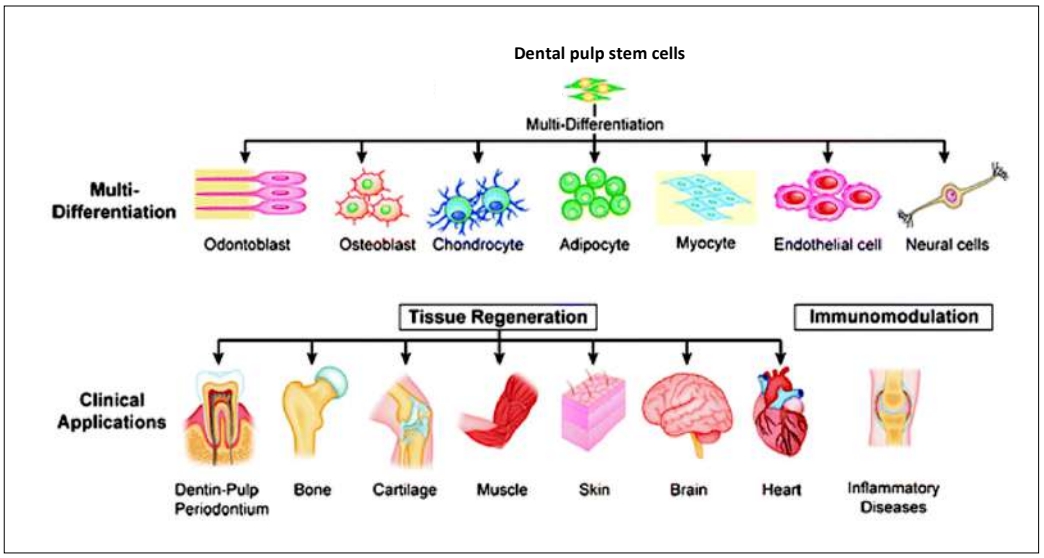


Figure 1.5. Multi-lineage differentiation capabilities of DPSCs and their potential uses in tissue regeneration (Lee et al., 2014).

Another important point to consider, prior to the clinical use of DPSCs is their genetic stability. Studies have demonstrated the presence of high percentages of aneuploid human MSCs during normal *in vitro* culture expansion, progressively increasing until senescence. Moreover, the more senescence prone sub-populations showed lower proliferation ability and higher incidence of aneuploidy, compared to the non-senescent sub-populations. Accordingly, aneuploidy must be taken in consideration as a significant factor in MSC senescence induction (Estrada et al., 2013). Therefore, although DPSCs can present an outstanding source for MSCs due to the favourable characteristics they possess, many factors should be taken into consideration to maintain those properties for safer and more effective clinical use in regenerative medicine.

1.3.2.3 Dental Pulp Stem Cells and Dental Tissue Repair

DPSCs are promising candidates in regenerative dental therapy. Many researchers have investigated the ability of DPSCs to form dentine-pulp tissue and proved that DPSCs hold the golden key for regenerative endodontics, that will help maintain the integrity of both hard and soft dental tissues (Na et al., 2013). Studies have demonstrated the ability of DPSCs to migrate and differentiate into odontoblasts, capable of mineralised nodules formation, promoting dentinogenesis and repairing damaged sites within teeth (Goldberg et al., 2006; About, 2011; Wang et al., 2014).

Consequently, several tissue engineering-based techniques have since been implemented to successfully regenerate dentine using DPSCs (Chiang et al., 2016; Zhang et al., 2016; Song et al., 2017; Loison-Robert et al., 2018). When comparing the *in vitro* mineralisation and dentinogenic capacities of DPSCs, BM-MSCs and adipose-derived MSCs (AD-MSCs), DPSCs showed the greatest capacity to form high volume of mineralised tissue (Davies et al., 2015). In addition, they showed a greater ability to regenerate the dentine-pulp complex, with good vascularity in a simulated perforation repair model (Alsanea et al., 2011) and in empty root canals using ectopic tooth transplantation models in immunocompromised mice (Huang et al., 2010). Furthermore, DPSCs transplanted subcutaneously using scaffolds into immunocompromised mice, resulted in soft connective tissue formation identical to dental pulp tissue (Nakashima and Iohara, 2011).

Many *in vivo* studies have been reported using scaffolds seeded with DPSCs to test their ability to develop tooth-like structures in an attempt to regenerate teeth of normal size and structure (Huang et al., 2009b). When bell-stage tooth bud stem cells were expanded *in vitro*, cultured in scaffold and re-implanted into the original alveolar socket of a swine model, dentine, root and periodontium formation were observed (Kuo et al., 2008). In a recent study, human tooth root canals filled with DPSC constructs and subcutaneously implanted into immuno-deficient mice, showed active self-organising ability forming odontoblast-like mineralising cells at sites in proximity to dentine and endothelial cells at the centre demonstrating a promising potential of DPSCs to form a blood vessel-rich pulp-like tissues and regenerate dental pulp tissue (Itoh et al., 2018).

1.3.2.4 Dental Pulp Stem Cells and Tissue Engineering

It is undoubted that DPSCs have a natural ability of responding well to tissue injury. Similarly, their distinctive differentiation potential towards different cell phenotypes is very promising (Lee et al., 2013). Indeed, their proliferative and regenerative potentials, availability and neurogenic and angiogenic capacities, have been shown to be greater than those for BM-MSCs (Alge et al., 2010; Khanna-Jain et al., 2012). These favourable characteristics make DPSCs a useful alternative stem cell source for cellular therapy and tissue engineering, even in non-dental tissues (Kim et al., 2012). However, although DPSCs have a stronger ability towards osteogenic lineage differentiation, they appear less able to undergo adipogenic differentiation than BM-

MSCs (Shi et al., 2001; Yamada et al., 2006). *In vivo* administration of human DPSCs to diseased animal models with myocardial infarction, cerebral stroke and neural disease, have demonstrated the remarkable therapeutic potential of DPSCs (Gandia et al., 2008; Leong et al., 2012), with no immune rejection or teratoma formation (Huang et al., 2008b). Additionally, their immunomodulatory properties in inducing T-cell apoptosis, while inhibiting the proliferation of both T-cell and peripheral blood mononuclear cells, were of great interest to researchers, especially as DPSCs exhibited superior immunosuppressive activities, compared to BM-MSCs (Tomic et al., 2010; Zhao et al., 2012).

DPSCs are also considered a potential cell source for cartilage repair (Poltavtseva et al., 2014) and bone defect regeneration (Bressan et al., 2012). Although some investigators reported a limited potential for *in vivo* bone regeneration using DPSCs (Annibali et al., 2013), several other studies have confirmed the osteogenic differentiation potential of DPSCs and ultimately, considered them as a potent cell source for 3D bone tissue structure fabrication, including around dental implant osseointegration (Ito et al., 2011; Kim et al., 2012). In a follow up clinical study, DPSCs were seeded into collagen scaffolds and loaded into the socket of an extracted wisdom tooth and compared to the opposite control socket, which received a cell-free scaffold. Although radiographical and histological analysis revealed significant less bone regenerated than controls 3 months post-surgery, follow up after three years showed that the regenerated bone was evenly vascularised, compact and of higher matrix density than control tissues (Giuliani et al., 2013). Other studies demonstrated that DPSCs differentiated into osteoblasts and added to scaffolds, resulted in enhanced bone mineral density in a rat calvarial critical bone defect model (Annibali et al., 2013; Maraldi et al., 2013). Interestingly, a pilot study showed that DPSCs isolated from inflamed dental pulps also exhibited similar positive results repairing periodontal bone defect (Li et al., 2016). Therefore, DPSC applications show strong promise as therapeutic approaches for the repair of craniofacial and alveolar bone (d'Aquino et al., 2009; Machado et al., 2012).

Various studies have documented the ability of DPSCs to differentiate into cardiac cells (Armiñán et al., 2009). Following injection of DPSCs into mice with myocardial infarctions, significant recovery was noticed compared to controls, albeit with no apparent cell differentiation; suggesting that DPSCs may promote cardiac repair by alternative mechanisms (Gandia et al., 2008). Many authors have

suggested that DPSCs present a great candidate for dentine-pulp regenerative purposes, due to their angiogenesis potential (Aksel and Huang, 2017). While DPSCs demonstrated a potent ability to enhance blood flow and improve vasculogenesis in animal models (Iohara et al., 2008; Aksel and Huang, 2017), they also showed an ability to form capillary-like structures when cultured with vascular endothelial growth factor (VEGF, Marchionni et al., 2009). In addition, DPSCs displayed a capacity to stimulate blood vessels formation and peripheral nerve tissue myelination of adult rats (Sasaki et al., 2008). Moreover, they also hold promising therapeutic potential for the treatment of muscular dystrophy, as DPSCs displayed significant engraftment upon treatment of muscular dystrophy in dogs (Yang et al., 2010). DPSCs have further been shown to differentiate into pancreatic insulin-producing cells (Annibali et al., 2013; Carnevale et al., 2013), with a higher differentiation potential towards insulin-producing cells than human PDLSCs (Sawangmake et al., 2014); suggesting that DPSCs may be used as a stem cell-based therapy for diabetes.

DPSCs manifested remarkable competency in promoting regeneration of nerve tissue (Young et al, 2013), due to their ability to differentiate into neuronal and oligodendrocyte-like cells, express neuronal cell markers and induce recovery post-neurological dysfunction (de Almeida et al., 2011; Sakai et al., 2012; Young et al., 2016). Indeed, human DPSCs demonstrate an ability to express neutrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF), in addition to low-affinity nerve growth factor receptor (LANGFR), VEGF and other proangiogenic growth factors (Nosrat et al., 2004; Mikami et al., 2010; Bronckaers et al., 2013). Expression of these factors aids the provision of the necessary trophic support to neuronal cells, thereby maintaining the reparative response and neurogenesis. Consequently, there is growing evidence that there is an active population of neural crest cells residing within dental pulp tissues responsible for regenerating odontoblasts and Schwann cells of the pulp (Kaukua et al., 2014; Aurrekoetxea et al., 2015). Following *in vivo* DPSCs transplantation into diseased rats with cerebral artery occlusion (Leong et al., 2012) and spinal injury (Sakai et al., 2012), marked neurogenesis associated with improved functional recovery were observed (Martens et al., 2014). Recently, human DPSCs successfully contributed to the generation of neovasculature in mouse brain tissue *in vivo*, after differentiation into endothelial

cells (Luzuriaga et al., 2019). Therefore, such properties make DPSCs promising candidates for neuronal tissue engineering.

DPSCs also exhibit an ability to differentiate into hepatocyte-like cells (Ishkitiev et al., 2012). Indeed, transplanted DPSCs into rat models prevented the progression of liver fibrosis and contributed to liver function repair (Ikeda et al., 2008; Cho et al., 2015). In addition, when unfractionated human DPSCs transplanted into surgically inactivated hair follicles, they have displayed potential to regenerate new end bulbs and to create multiple differentiated hair fibres (Reynolds and Jahoda, 2004). Therapeutic use of DPSCs extend to corneal reconstruction as well. In rabbits with corneal defects, transparency of cornea was enhanced after corneal epithelium reconstruction by transplantation of a human dental pulp stem cell sheet (Gomes et al., 2010).

1.3.2.5 Heterogeneity and Surface Markers in Dental Pulp Stem Cells

Human DPSCs are considered a promising source of multipotent MSCs analogous to MSCs from other sources. DPSCs exhibit a fibroblast-like morphology, plastic adherence and express MSC markers, CD73, CD90 and CD105, thus satisfy the minimal criteria for MSCs (Huang et al., 2009a; Kawashima, 2012). Indeed, DPSCs present a novel population of MSCs that uniquely express differentiation and lineage specific genes.

Researchers describe DPSCs as a heterogeneous population (Pisciotta et al., 2015). This presents a major challenge during the course of *in vitro* cell expansion, as the different sub-populations comprising the heterogeneous population as a whole manifest different biological behaviours, varied proliferation rates and specific MSC marker expression profiles (Wagner, et al., 2010; Kawashima, 2012). While DPSC heterogeneous populations exhibit multi-lineage differentiation potential into osteoblasts, chondrocytes, adipocytes, neurocytes and myocytes, it has been reported that there are occasions where adipogenic, chondrogenic and myogenic differentiation are impaired, potentially as a consequence of their stem cell niche of origin within dental pulp tissues (Huang et al., 2009a).

Studies on the properties of stem cell niches within human dental pulp tissues have shown that each niche possess cells expressing nestin, a neuro-epithelial marker known to be mostly expressed by neuronal stem cells (Park et al., 2010; Lizier et al., 2012). In addition, several embryonic stem cell markers have been reported to be

expressed in human DPSCs, such as SSEA-4, Oct4 and Nanog (Kawanabe et al., 2012). Notably, the multipotency properties of DPSCs have been reported to be related to Oct4 and Nanog expression, as these are transcriptional factors responsible for the maintenance of pluri-/multi-potency (Kerkis et al., 2006). Furthermore, Oct4 and Nanog overexpression was observed to be critical in promoting the induction of osteogenic, chondrogenic and adipogenic differentiation in DPSCs, whilst Oct4 and Nanog down-regulation reduced the MSCs properties of DPSCs (Huang et al., 2014b).

CD34 is a hematopoietic stem cell marker expressed by several cell types including MSCs (Lin et al., 2012), playing a role in cell adhesion, proliferation and differentiation (Scherberich et al., 2013). However, CD34 has also been found to be expressed by some subsets of human DPSCs (Hilkens et al., 2013). Other DPSC sub-populations are reported to express STRO-1 (Huang, et al., 2009a; Yang et al., 2009), which may have a role in clonogenicity, homing and angiogenesis in MSCs. In addition, CD146 and 3G5 are both considered as pericyte markers (Shi and Gronthos, 2003). Notably, CD146 expression has been observed in MSCs with multi-lineage, osteogenic, chondrogenic and adipogenic differentiation capabilities (Xu et al., 2008).

c-Kit (CD117) is a tyrosine-protein kinase receptor that is expressed by various types of stem cells, such as neural crest-derived cells (Laino et al., 2005), hematopoietic stem cells (Ashman, 1999), AD-MSCs (Blazquez-Martinez et al., 2014), BM-MSCs (Hatzistergos et al., 2010) and DPSCs (Pisciotta et al., 2015). Another marker that has a role in the differentiation of DPSCs is CD271, which is considered to be a marker for neural derived cells (Pisciotta et al., 2015). CD271 is a p75 neurotrophin receptor and nerve growth factor receptor that inhibits the differentiation of DPSCs into osteogenic, myogenic, chondrogenic or adipogenic lineages while promoting survival and differentiation to neuronal cells (Park et al., 2010). Consequently, CD271 can be considered an important marker to determine differentiation ability of cells (Mikami et al., 2010). Indeed, highly proliferative, multipotent DPSC sub-populations capable of >80 population doublings (PDs) during *in vitro* culture expansion, have been shown to exhibit no CD271 expression in contrast to low proliferative/unipotent DPSCs only capable of <40PDs in culture (Alraies et al., 2017). A study by Pisciotta et al. suggested the existence of more than one stem cell population within human dental pulp, those that are positive for

STRO-1, c-Kit and CD34, which showed nestin and CD271 marker expression and have stronger tendency towards neurogenic differentiation compared to the other DPSCs that are positive to STRO-1, c-Kit, but negative for CD34, nestin and CD271 (Pisciotta et al., 2015). However, both cell populations demonstrated no significant differences in differentiation potential towards osteogenic, adipogenic or myogenic lineages. This could be explained by their origin, which could be either the mesodermal or the neuro-ectodermal (Chai et al., 2000).

CD105 (endoglin, SH2) is a component of the TGF-β complex (Aslan et al., 2006). It is associated with cell migration and haematopoiesis (Conley et al., 2004) and identified to be expressed in DPSCs, although some studies reported relatively low levels (compared to BM-MSCs), that decreases towards osteogenic differentiation process (Ponnaiyan and Jegadeesan, 2014). However, other studies have observed high expression in MSCs capable of multi-lineage differentiation particularly osteogenesis (Maleki et al., 2014). Of note, upon comparison of DPSCs properties isolated from normal and inflamed dental pulp, STRO-1, CD90, CD105 and CD146 cell markers were expressed in higher levels in DPSCs derived from inflamed dental pulp tissues (Alongi et al., 2010; Pereira et al., 2012).

Therefore, DPSC and MSC heterogeneity in general, can have major implications for regenerative medicine, in terms of determining whether particular sub-populations provide viable therapeutic options or not. Generally, homogenous populations are more favourable for clinical use, due to their predetermined biological activity, unlike heterogeneous populations that may produce unwanted biological activities, such as teratoma formation or inefficient differentiation (Müller et al., 2010; Downes et al., 2011). Therefore, it is highly recommended that homogenous populations are selectively purified that impact positively on DPSCs biological behavior and subsequently through their regenerative potential and tissue engineering applications. However, the proposed limited numbers of MSCs within tissues (Friedenstein et al., 1982), necessitates extensive *in vitro* expansion in order to provide sufficient number to enable efficient clinical use (Wagner et al., 2010).

1.4 Mesenchymal Stem Cell Expansion and Cellular Senescence

The term “senescence” is derived from the Latin *“senex”*, meaning “growing old”. Cellular senescence is the limited ability of diploid cells to replicate when cultured *in vitro*, whilst limited cell proliferative lifespan have further been proposed as a

possible mechanism for human ageing *in vivo* (Hayflick and Moorhead, 1961). Consequently, while many advantages exist that DPSCs possess over MSCs from other sources, one barrier which can potentially have a negative impact on their development as therapies for clinical applications in regenerative medicine, is their finite culture expansion capabilities *in vitro* (Roobrouck et al., 2008). Indeed, MSCs gradually show less division and reduced proliferation rates during extended culture, until they enter a senescence state and stop proliferating, upon reaching their Hayflick limit (Hayflick, 1965; Bonab et al., 2006; Markert et al., 2009). Therefore, although the necessity to obtain adequate number of MSCs mandates extensive *in vitro* expansion, this eventually leads to proliferative decline and cellular senescence. Although senescent cells remain viable and metabolically active, the onset of senescence induces changes in cellular genotypes and phenotypes, coupled with an altered secretome associated with development of the senescence-associated secretory phenotype (SASP), loss of stem cell marker expression and impaired multipotent differentiation, immunomodulatory and regenerative potentials (D'Ippolito et al., 1999; Coppé et al., 2008; Wagner et al., 2010; de Jesus and Blasco, 2012; Malaquin et al., 2016; Alraies et al., 2017; McHugh and Gil, 2018). For example, it has been particularly shown that MSCs approaching the end of their proliferative life-spans tend to lose osteogenic potential in favour of adipogenic potential, a phenomenon known as the "osteogenic to adipogenic shift'' (Banfi et al., 2000; Stenderup et al., 2003).

Due to their altered or impaired genotypic and phenotypic characteristics, senescent MSC populations are not recommended for transplantation purposes, not only because this will decrease the chances for good results, but may even be harmful. Consequently, it is crucial to monitor the state of cellular senescence within MSC populations, throughout their proliferative lifespans. Although passage number can be an indicator of cellular senescence, as it indicates the number of times the cells have been sub-cultured and allowed to grow, this is not a precise method. Thus, a more accurate measure of cell growth/proliferation and the onset of senescence is to determine PDs (Larson et al., 2008), with senescence indicated once cells proliferate at <0.5PDs/week (Cristofalo et al., 1998). Therefore, it has been recommended to use MSCs at early PDs (no more than 4-7PDs) in preparations for therapeutic use due to their ability to maintain more primitive cell phenotype that is still have the ability to perform more cell divisions (Banfi et al., 2000; Walenda et

al., 2010).

PDs for different MSC populations vary significantly, depending on various factors, including the MSC tissue source and patient donor age (Bonab et al., 2006). For example, BM-MSCs have been reported to have a life-span that ranges from 20-40PDs (Banfi et al., 2000), with differences attributed to increasing patient donor age increasing cell susceptibility to premature cellular senescence (Stenderup et al., 2003; Mareschi et al., 2006; Hwang, 2014). Significant heterogeneity in DPSCs have also been reported, with heterogeneous DPSC populations being capable of >120PDs *in vitro*, although only 20% of purified DPSC sub-populations are capable of proliferating >20PDs (Gronthos et al., 2000, 2002; Huang et al., 2009a). Of these, only two-thirds were able to generate abundant ectopic dentine *in vivo*, this can indicate that DPSC populations have contrasting regenerative potential (Gronthos et al., 2002; Huang et al., 2009a). Furthermore, similar animal and human studies have established the effects of increasing donor age on the impairment of DPSC proliferative and regenerative capabilities (Ma et al., 2009; Bressan et al., 2012; Feng et al., 2013, 2014; Horibe et al., 2014).

Despite significant differences in the *ex vivo* expansion capabilities of individual DPSCs being recognized, only recently has work begun to address the impact of such variations in proliferative capabilities prior to senescence, on the multipotency of different DPSC sub-populations (Alraies et al., 2017). Although high proliferative DPSCs achieved >80PDs, low proliferating DPSCs only completed <40PDs before senescence, correlating with DPSCs with high proliferative capacities possessing longer telomeres (>18kb) than less proliferative populations (5-13kb). Low proliferative DPSC senescence was also associated with early loss of stem cell marker characteristics and impaired osteogenic and chondrogenic differentiation, in favour of adipogenesis. In contrast, high proliferative DPSCs retained multipotent differentiation capabilities, only demonstrating impaired differentiation following prolonged *in vitro* expansion (>60PDs, Alraies et al., 2017).

1.4.1 Telomere-Dependent Senescence

Cells appear to be able to undergo senescence via different pathways. As diploid cells only exhibit a finite ability to replicate, cells lose their ability to divide and are irreversibly arrested following a certain number of PDs (Hayflick and Moorhead

1961). In such circumstances, proliferative decline and subsequent cellular senescence is a consequence of replicative (telomere-dependent) senescence, characterised by progressive telomere shortening and the loss of telomeric TTAGGG repeats, due to repeated cell divisions (Campisi and d'Adda di Fagagna, 2007; Morsczeck et al., 2016). Indeed, due to this inherent limitation in the mechanics of DNA replication, telomeric shortening (erosion) occurs with each cell division (50-200bp per PD) (Harley et al., 1990). However, replicative senesce can also be a result of chronic activation of DNA damage response triggered by deformed telomeres (Pole et al., 2016).

Telomeres are specialised DNA-protein complexes at the end of eukaryotic chromosomes, which protect chromosomes against genomic instability-promoting events (Griffith et al., 1999; De Lange, 2005; Harbo *et al.*, 2012; Figure 1.6). Telomerase is a specialised ribonucleoprotein reverse transcriptase, with a role in maintaining telomere lengths through the complete replication of telomere ends, thereby protecting telomeres from progressive erosion, modification and degradation (Allsopp et al., 2003; Flores et al., 2005; Bernardo et al., 2007; Serakinci et al, 2008). In the absence of telomerase, when telomere shortening reaches a "critical length", cells are susceptible to chromosomal aberrations, such as end-to-end fusion and aneuploidy. In this state, the cells cease to divide, and replicative senescence ensues. Thus, by imposing a limit on the proliferative lifespan of MSCs and somatic cells, telomeric erosion represents an innate mechanism for tumour suppression (Faragher and Kipling 1998).

Replicative senescence has been described in numerous cell types, including MSCs (Wagner et al., 2010; Li et al., 2017). Upon reaching replicative senescence, cells become post-mitotic and under permanent growth arrest in the G_1 phase of the cell cycle, unable to undergo G_1-S phase transition. Increased expression of the tumour suppressor genes, p53 and retinoblastoma protein (pRb), mediate the induction of replicative senescence via the cyclin-dependant kinase inhibitors, $p21^{wafl}$ and $p16^{INK4a}$ (Campisi and d'Adda di Fagagna, 2007, Morsczeck et al., 2016; Figure 1.7). Other established markers of senescence include changes in cell morphology from bipolar spindle-shaped into enlarged, flattened, non-polar multinucleated cells with identifiable cytoskeletal stress fibres (Matsumura et al., 1980; Sethe et al., 2006; de Jesus and Blasco, 2012); and an increased detection of positive SA-β-galactosidase (SA-β-Gal) activity (Dimri et al., 1995).

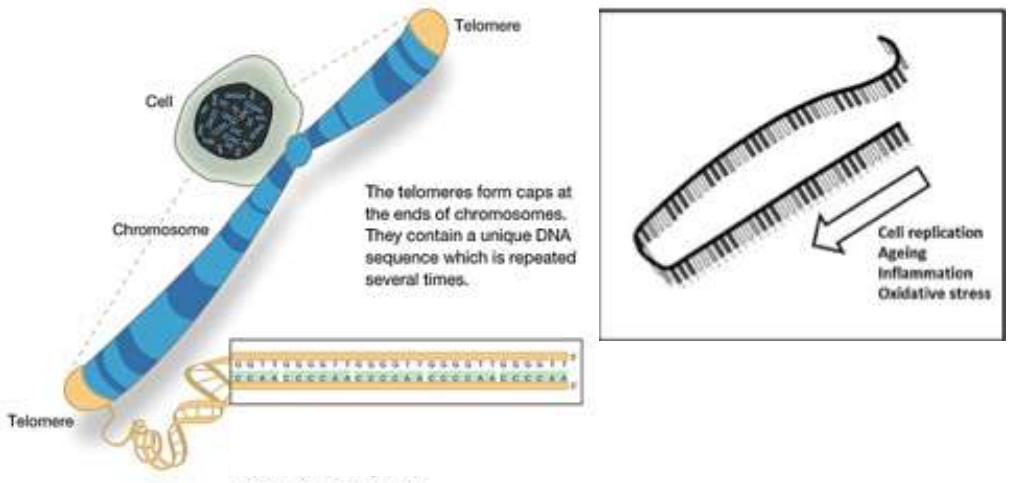


Fig 1.6. The structure of telomeres. Caps at the end of chromosome containing unique repeats of DNA sequence ("TTAGGG" in humans) that preserve genome integrity and prevents erosion (Harman, 2009).

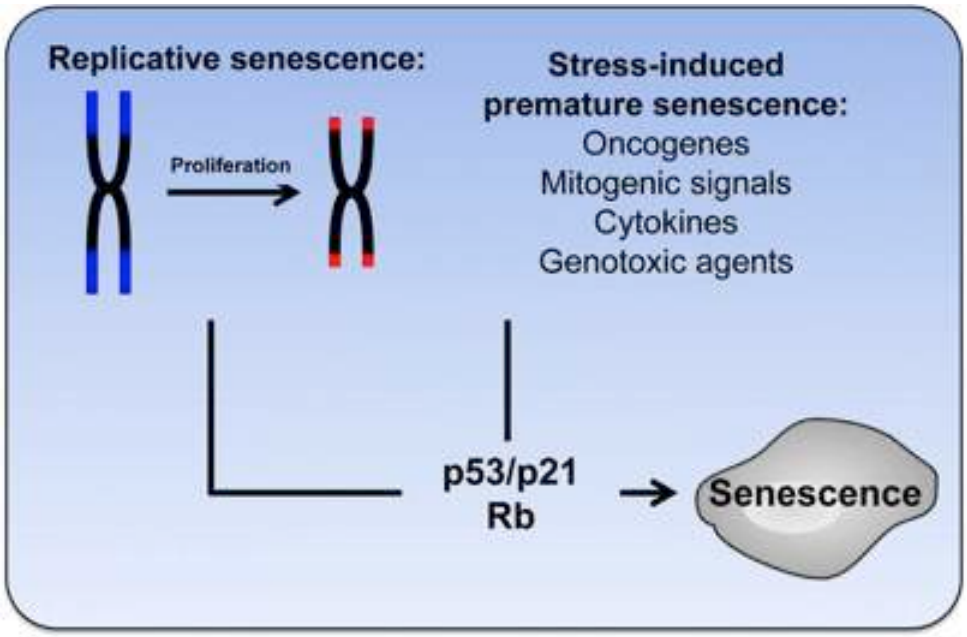


Figure 1.7. Summary of conditions causing cellular senescence. p53, pRb and $p21^{wafl}$ are common factors in both telomere-dependent (replicative) senescence and telomere-independent (stress-induced, premature) senescence (de Jesus and Blasco, 2012).

Replicative senescence has been proposed by many authors as the mechanism behind human ageing. Indeed, several studies provide evidence for the role of telomere dysfunction in cellular aging, demonstrating that telomere dysfunction results in decreased proliferation, acceleration of cellular ageing and impaired organ function (Saeed and Iqtedar, 2013; Birch et al., 2015). With the loss of proliferation, the ability of tissue to repair itself becomes limited and may account for many of the changes associated with senescent MSC and/or MSCs derived from more aged patient donors (D'Ippolito et al., 1999; Banfi et al., 2000; Stenderup et al., 2003; Mareschi et al., 2006; Coppé et al., 2008; Ma et al., 2009; Wagner et al., 2010; Bressan et al., 2012; de Jesus and Blasco, 2012; Feng et al., 2013, 2014; Horibe et al., 2014; Hwang, 2014; Malaquin et al., 2016; Alraies et al., 2017; McHugh and Gil, 2018). On the contrary, MSCs with longer telomeres demonstrate better repair capabilities, post-injury (Montgomery et al., 2011; Alraies et al., 2017).

Contrasting telomere lengths between MSC populations may reflect variations in telomerase activity among stem cell population from different developmental, tissue or niche origins. For example, the high level of telomerase present in ESCs renders these virtually immortal (Marion et al., 2009). Although this may be true, telomerase expression in adult stem cells is still under debate, as telomerase expression and activity have also been widely described in adult stem cells, albeit at much lower levels than ESCs (Bernardo et al., 2007). Expression of the reverse transcriptase, human telomerase catalytic subunit (hTERT) has also been reported in foetal cells, germ lines, stem cells and many tumour cells (Serakinci et al., 2008). However, although certain studies have reported positive hTERT expression in DPSCs (Flores and Blasco, 2010; Jeon et al., 2011; Hakki et al., 2015), most report no or negligible hTERT expression in DPSCs (Egbuniwe et al., 2011; Mehrazarin et al., 2011a; Murakami et al., 2013; Alraies et al., 2017). In addition, several studies have suggested that active telomerase activity is insufficient for the complete prevention of telomere loss (Mokry et al., 2010). Thus, it is unlikely that hTERT is responsible for maintaining telomere integrity and the superior proliferative/multipotency capabilities of high proliferative DPSC sub-populations (Alraies et al., 2017). In fact, many MSC populations, including DPSCs, are capable of maintaining proliferative lifespans during extended *in vitro* culture expansion, whilst maintaining their stemness, differentiation and regenerative properties, even in the absence of strong telomerase/hTERT expression capabilities (Mokry et al.,

2010; Saeed and Iqtedar, 2013; Fukada et al., 2014). Therefore, other intrinsic telomere protective mechanisms may account for the contrasting differences in telomere lengths, proliferation rates and differentiation capabilities between high and low proliferative DPSCs (Saretzki, 2009; Liu et al., 2011; Shyh-Chang et al., 2013; Li et al., 2017).

1.4.2 Telomere-Independent Senescence

In addition to telomere-dependent senescence, somatic cells can also undergo replicative arrest, independent of their telomere lengths (Campisi and d'Adda di Fagagna, 2007; Morsczeck et al., 2016). The main mechanisms purported to induce such telomere-independent senescence, include DNA damage mediated by p53, genotoxic agents, ionizing radiation, oxidative stress loss of heterochromatin and the expression of certain oncogenes, such as Ras or Raf (Shay and Wright, 2007; Alves et al., 2013; El Alami et al., 2014; Fukada, et al., 2014; Mas-Bargues et al., 2017).

Of these mechanisms, oxidative stress is a prominent and well-established mediator of telomere-independent senescence (also known as premature senescence or stress-induced premature senescence, SIPS). Telomere-independent senescence has been identified in various cell types, including somatic cells and MSCs (Liu et al., 2011; Shyh-Chang et al., 2013; Li et al., 2017). It is proposed that telomere-independent senescence induced by oxidative stress is promoted through the induction of DNA damage, resulting in single-strand breaks in telomeres without significant telomere shortening, unless exposed to high levels of oxidative stress over prolonged periods (Ben-Porath and Weinberg, 2004; Rübe et al., 2011). Indeed, recent evidence suggests that oxidative stress induces telomeric double stranded-breaks that are unrepairable, causing persistent DNA damage response signalling (DDR). Subsequently, cells undergo telomere-independent senescence, where cells cease replication irrespective of telomere lengths (Victorelli and Passos, 2017).

As with telomere-dependent (replicative) senescence, telomere-independent senescence is associated with increased cellular morphologies, positive SA-β-Gal activity and the activation of various signalling pathways, including those involving the tumour suppressor genes, p53 and pRb, via DNA damage-induced cyclin-dependant kinase inhibitor, $p21^{waf1}$, that commence growth arrest followed by increased $p16^{INK4a}$ expression that disrupts G_1-S phase transition and normal cell cycle progression (Itahana et al., 2001; Campisi and d'Adda di Fagagna, 2007;

Huang et al., 2011; Morsczeck et al., 2016; Figure 1.7). However, it appears that the main difference between replicative senescence and stress-induced senescence lies in the presence of telomere shortening and the time elapsed before proliferation ceases (Bielak-Zmijewska et al., 2018). Nonetheless, senescence mediated via both replicative and stress-induced mechanisms induce significant alterations in cellular genotype and phenotype, ultimately influencing their overall biological functions and mediating impaired repair, disease and organismal ageing (Sikora et al., 2011).

1.5 Oxidative Stress

Oxidative stress refers to the disturbance in the balance between the production of intracellular reactive oxygen species (ROS) and cellular antioxidant defence mechanisms. Although ROS are generated via a wide range of cellular mechanisms, mitochondria are considered the principle ROS source under normal physiological conditions (Balaban et al., 2005). Low ROS levels play important roles in the regulation of cellular functions, including metabolism, signal transduction, gene expression, cell proliferation and differentiation (Guzik and Harrison, 2006; Genestra, 2007; Storz, 2011; Coso et al., 2012). In particular, oxidative stress is capable of promoting indiscriminate biomolecular damage to cellular macromolecules, including DNA, proteins and lipids (Levine and Stadtman, 2001; Spiteller, 2001; Cooke et al., 2003; Evans et al., 2004; Chaudhari et al., 2014); in addition to accelerating telomere-independent senescence (Itahana et al., 2001; Ben-Porath and Weinberg, 2004; Campisi and d'Adda di Fagagna, 2007; Huang et al., 2011; Morsczeck et al., 2016). Consequently, oxidative stress induces significant alterations in cellular genotype and phenotype ultimately influencing their overall biological functions, resulting in age-related disease pathologies associated with impaired tissue function and repair processes (Dayem et al., 2010; Sikora et al., 2011; Atashi et al., 2015).

1.5.1 Reactive Oxygen Species

1.5.1.1 Physiological Role of ROS

ROS include oxygen-derived free radical (ODFR) species, such as superoxide ($O_2^{\cdot-}$) and hydroxyl ($^{\cdot}OH$) radicals, in addition to non-radical derivatives of oxygen, such as hydrogen peroxide (H_2O_2, Halliwell et al., 1992; Dröge, 2002; Bigarella et al., 2014; Figure 1.8). The radical species in particular, such as $O_2^{\cdot-}$ and $^{\cdot}OH$, are

extremely reactive due to the presence of one or more unpaired electrons in their outer orbitals.

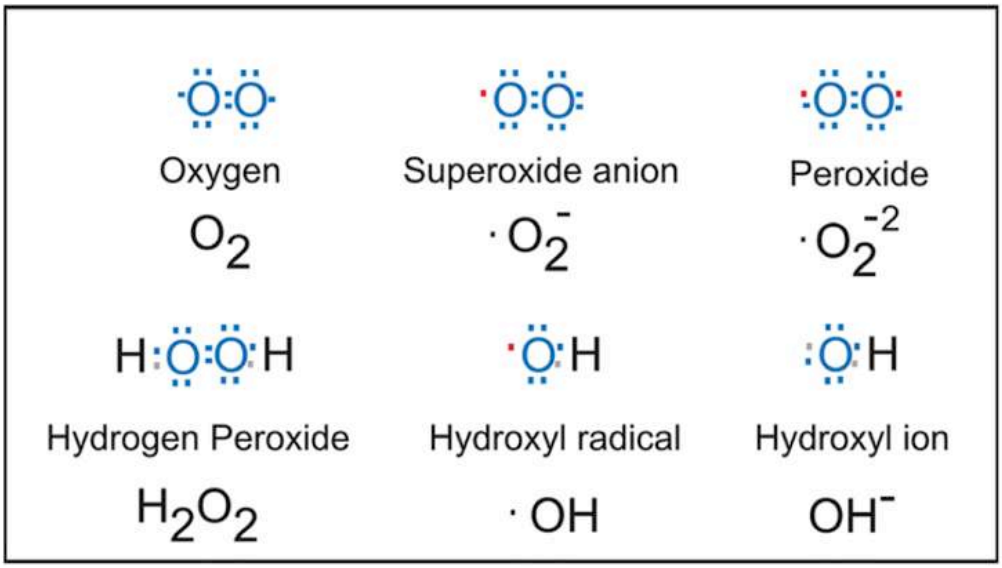


Figure 1.8. Electron structures of common ROS. The • in red indicates unpaired electrons (Halliwell et al., 1992).

ODFRs, especially $O_2^{\cdot-}$, are produced by a number of mechanisms in biological systems. Mitochondria are considered as the main source of ROS within the cell due to the presence of the electron transport chain within the inner mitochondrial membrane, ultimately resulting in production of adenosine triphosphate (ATP) during oxidative phosphorylation (Balaban et al., 2005). However, during high flux, electron leakage at complexes I and III causes the (Bigarella et al., 2014), causing reduction of O_2 and the generation of $O_2^{\cdot-}$ (Valko et al., 2007). In fact, it is suggested that ≅0.1-0.2% of O_2 consumed by mitochondria form ROS (Tahara et al., 2009), although the quantities of ROS produced by mitochondria varies depending on factors such as cell type and mitochondrial activity (Murphy, 2009). Other mechanisms responsible for cellular $O_2^{\cdot-}$ production include autoxidation reactions and numerous enzymic reactions, especially the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase present in the plasma membrane of cells, particularly phagocytes (Waddington et al., 2000). $O_2^{\cdot-}$ is also a precursor for the formation of other ROS. For instance, can undergo a dismutation reaction, which may be either spontaneous or catalysed by superoxide dismutases (SODs), to form H_2O_2 (Waddington et al., 2000; Atashi et al., 2015).

H_2O_2 is a physiological constituent of all living cells presumed to occur at intracellular levels of 1-700nM, depending on cell type (Gülden et al., 2010). It is normally involved in the cellular signalling process and is able to undergo transition metal ion-catalysed, Fenton or Haber-Weiss reactions to produce highly reactive $^{\cdot}OH$, which cause significant oxidative damage (Waddington et al., 2000). Consequently, any H_2O_2 concentration above 1μM is considered pathological and result in oxidative stress, eventually leading to cell senescence and/or cell death (Duan et al., 2005; Stone and Yang, 2006).

1.5.2 Consequences of Oxidative Damage to DNA, Proteins and Lipids

Although the physiologic upregulation of ROS production is essential for normal cellular functions, it is well-established that a chronically unbalanced redox status, as a consequence of excessive ROS production, affect cellular activities particularly at a biomolecular level. Cellular targets for oxidative modification by ROS include DNA, proteins and lipids, with the order of preference for modification depending on a number of factors, such as the location of ROS production, the relative ability for the molecule to be oxidized and the availability of metal ions (Evans et al., 2004; Lyublinskaya et al., 2015). Nonetheless, oxidative modification and/or degradation of such biomolecules can significantly alter cell fates and promote cellular senescence or even apoptosis (Kim et al., 2011; Mandal et al., 2011; Cummins et al., 2013). Indeed, several studies reported a direct link between oxidative stress and many pathological conditions, such as cancer (Dizdaroglu, 2015), diabetes (Muoio and Newgard, 2008), cardiovascular and other chronic inflammatory diseases (Waddington et al., 2000; Ho et al., 2013), neurodegenerative diseases (Halliwell and Whiteman, 2004; Agarwal et al., 2005; Kehrer and Klotz, 2015), and several age-related conditions (Jacob et al., 2013).

1.5.2.1 Effects of Oxidative Stress on DNA

Several pathological conditions can occur as a consequence of oxidative damage to DNA. For this reason, oxidative DNA damage has been widely studied in various cell lines. For instance, fibroblasts exhibit higher levels of 8-hydroxy-deoxy-guanosine (8-OHdG) in senescent cells than in young cells, indicating increased oxidative DNA damage (Chen et al., 1995). Notably, oxidative DNA damage results in different types of DNA lesions depending on the ROS involved, the duration of

exposure and the cellular reparative ability (Halliwell and Chirico, 1993). ROS interactions with DNA are mainly attributed to ·OH species causing DNA base modification, deoxyribose sugar damage, formation of DNA protein cross-links, single and double strand breaks, and the formation of apurinic lesions, many of which are mutagenic (Shibutani et al., 1991; Finkel and Holbrook, 2000). DNA bases are particularly vulnerable to oxidation by ROS, with guanine base having the lowest reduction/oxidation potential of the four bases (Neeley and Essigmann, 2006). Consequently, ·OH species induce DNA damage at the C-8 position of guanine base yielding 8-OHdG as an oxidation product, which has led to 8-OHdG becoming a well-established marker of in vitro and in vivo oxidative DNA damage (Toussaint et al., 2000; Tahara et al., 2001; David et al., 2007). Nonetheless, ROS can also attack other positions in the structures of guanine or other purine/pyrimidine DNA bases, yielding a wide range of oxidative DNA base damage products (Cooke et al., 2003; Evans et al., 2004). In contrast to ·OH species, less reactive species, such as $O_2^{\cdot -}$ and H_2O_2, cause indirect DNA damage by acting as a source for other ROS, such as OH species (Halliwell, 1999; Poulsen et al., 2000; Ames, 2001; Kasprzak, 2002). Indeed, DNA oxidation is a complex process and many studies have reported that ROS can promote site-specific damage (Enright et al., 1996). Accordingly, the cellular repair responses towards ROS-induced DNA lesions are also complex, as different DNA repair mechanisms may exist depending on the source and extent of oxidative damage induced (Giorgio et al., 2007; Hazane-Puch et al., 2010). Reports have also shown that increased mitochondrial ROS production can disrupt mitochondrial integrity causing mitochondrial DNA mutations, eventually resulting in a loop of further ROS generation and more DNA damage (Linnane et al., 1989), thereby accelerating cellular senescence (Madamanchi and Runge, 2007; Lee and Wei, 2007).

1.5.2.2 Effects of Oxidative Stress on Proteins

Previous research has recognized a strong association between protein oxidation and oxidative stress, senescence and aging, with numerous studies utilising oxidative protein damage as a marker of oxidative stress in research on many different pathological conditions (Flint Beal, 2002), such as diabetes (Almogbel and Rasheed, 2017), Alzheimer's disease (Greilberger et al., 2010) and arthritis (Pullaiah et al., 2018). Protein oxidation results from ROS accumulation (Berlett and Stadtman,

1997). Upon oxidation, protein damage is presented as modifications to amino acid structure leading to fragmentation to lower molecular weight products. ROS modification of amino acids results in the formation of numerous oxidized by-products, including oxo acids, aldehydes, hydroperoxides and hydroxylated derivatives, due to a range of chemical reactions, including the hydroxylation of amino acid side chains, decarboxylation and deamination reactions. Studies have highlighted a number of amino acids that are more susceptible to modification by ROS, including proline, histidine, lysine, arginine and tyrosine, resulting in formation of chemically stable moieties (Dean et al, 1997; Dalle-Donne et al., 2003). Additionally, carbonyl derivatives can be produced indirectly on the nucleophilic side chains of cysteine, histidine and lysine by secondary reaction with aldehydes produced during lipid peroxidation, or with reactive carbonyl derivatives generated during glycation and glycoxidation reactions (Berlett and Stadtman, 1997; Halliwell, 1999; Uchida, 2000). Indeed, the increased presence of protein carbonyl groups as a consequence of oxidative stress exposure and amino acid side chain modifications, is the most widely used biomarker to implicate oxidative protein damage in the aetiology and/or progression of various diseases and conditions (Dalle-Donne et al., 2003). Normally, oxidized proteins are degraded by proteasomes. However, proteasomal activity may not be sufficient to overcome chronic oxidative stress, resulting in oxidized protein accumulation during cellular senescence and ageing (Pole et al., 2016). Consequently, many studies have documented age-associated accumulation of oxidized proteins (Sitte et al., 2000; Grune et al, 2005; Torres and Perez, 2008; Baraibar et al., 2012; Rodríguez-Sureda et al., 2015). In addition to DNA damage, elevated mitochondrial ROS level can result in irreversible protein damage (Madamanchi and Runge, 2007).

1.5.2.3 Effects of Oxidative Stress on Lipids

Lipids constitute a major component of cell membrane rendering integrity. However, lipids are also liable to oxidative damage due to their high unsaturated fatty acid contents, resulting in lipid peroxidation (Halliwell, 1999; Spiteller, 2001; Kohen and Nyska, 2002). Although lipid peroxidation is a normal physiological process necessary for renovation of biological membranes, excessive activation is linked to many pathologies. Lipid peroxidation of membranes affects several aspects of cellular machinery, such as the loss of membrane fluidity and integrity leading to

impaired cellular function (Halliwell, 1999). ODFR particularly target carbon-carbon bonds of polyunsaturated fatty acids, forming lipid hydroperoxides (LOOHs) as primary products of lipid peroxidation, (Yin et al., 2011), in addition to secondary products, such as malondialdehyde (MDA) which has a high capability to react with multiple molecules including proteins and DNA resulting in adducts formation (Łuczaj and Skrzydlewska, 2003; Zarkovic et al., 2013) and 4-hydroxynonenal (4-HNE) (Halliwell, 1999; Spiteller, 2001; Kohen and Nyska, 2002). Therefore, end products formed, such as MDA, 4-HNE and the prostaglandin-like products, isoprostanes, are now commonly used as markers of oxidative lipid peroxidation (Halliwell and Chirico, 1993; Spiteller, 2001; Kohen and Nyska, 2002).

1.5.3 Antioxidants Defences Against Reactive Oxygen Species

The potential occurrence of excessive ROS-induced cell/tissue damage may be partly counteracted through ROS detoxification by a series of antioxidant defence mechanisms, thereby restoring cellular redox homeostasis. An antioxidant is defined as any substance that can inhibit or significantly delay the oxidation of an oxidisable substrate when present at lower concentrations than that substrate (Halliwell et al., 1992). These antioxidants act by several mechanisms in order to protect biological systems from oxidative damage, including via direct scavenging of ROS and by sequestration of free catalytic metal ions. In essence, antioxidants neutralise ROS by accepting their electrons, ultimately counteracting their effect by reducing ROS levels and by repairing the consequences of oxidative damage. Antioxidants vary from simple, low molecular weight compounds, such as ascorbate and α-tocopherol, to ROS detoxifying enzymes, such as SODs, catalase and glutathione-metabolising enzymes, including glutathione peroxidases (GPXs), transferases, reductases and synthetases (Waddington et al., 2000). Due to their paramount importance within cells, antioxidants are found in different locations within the cell corresponding to different functions. Their presence in the cytosol scavenge hydrophilic peroxide species, such as H_2O_2, whilst their presence in mitochondria allows the protection from $O_2^{\cdot-}$ and $^{\cdot}OH$ species.

Enzymic antioxidant defence mechanisms are mainly orchestrated by Nf-E2 related factor 2 (Nrf2), which activates the various antioxidant responsive element (ARE)-dependent genes that encode enzymic antioxidants, ultimately mitigating

oxidative stress (Kensler et al., 2007; Nguyen et al., 2009; Ma, 2013). SODs catalyse $O_2^{\cdot-}$dismutation into H_2O_2. Three SOD isoenzymes which have been identified in cells. SOD1 (known as copper zinc SOD or Cu/ZnSOD) occurs in the cytoplasm, the intermembrane space of mitochondria and in the nucleus. SOD2 (known as manganese SOD or MnSOD) is located to the mitochondria matrix and SOD3 (known as extracellular SOD or EC-SOD) is secreted into the extracellular matrix (Zelko et al., 2002; Fattman et al., 2003; Nozik-Grayck et al., 2005; Fukai and Ushio-Fukai, 2011). SODs catalyse the dismutation of O_2 into H_2O_2, a less reactive species, within their respective intracellular or extracellular locations (Waddington et al., 2000; Figure 1.9).

Most aerobic cells contain catalase, which catalyses the dismutation of H_2O_2 into H_2O and is mostly localised intracellularly in peroxisomes within the cytosol (Kirkman and Gaetani, 2007; Figure 1.9). However, considering that mitochondria is the primary source of ROS, many authors suggested that the catalase system may not be the most efficient due to absence of catalase expression in the mitochondria (Pervaiz et al., 2009). However, when catalase was upregulated and targeted specifically to the mitochondria in transgenic mice, less oxidative DNA damage was observed with 20% increases in organismal lifespan, suggesting an enhanced mitochondrial antioxidant defence mechanism. This indicate that mitochondrial ROS are considered important limiting factor of cell longevity and further emphasize the importance of mitochondria as ROS sources (Schriner et al., 2005).

GPXs provide another mechanism to remove excessive intracellular H_2O_2 levels, by catalysing the reduction of H_2O_2 to H_2O and LOOHs into their corresponding alcohols, using reduced glutathione (GSH) as a coenzyme which is then oxidized to glutathione disulphide (GSSG, Figure 1.9). GPXs are tetrameric proteins localised within the cytoplasm, mitochondria and nuclei, with each subunit containing an atom of selenium present at the active site as selenocysteine (Deponte, 2013, Lu, 2013). Reduced GSH is produced by the formation of γ-glutamylcysteine from glutamate and cysteine, followed by the formation of GSH from γ-glutamylcysteine, catalysed by γ-glutamylcysteine synthetase and glutathione synthetase (GSS), respectively (Lu, 2013). GSH is a non-enzymatic tripeptide thiol, synthesised by cells upon the need to initiate the defensive mechanism and detoxify ROS (Bigarella,et al., 2014). Although it exists abundantly in the cytosol in which it is exclusively synthesised, it is also distributed intracellularly in other cellular

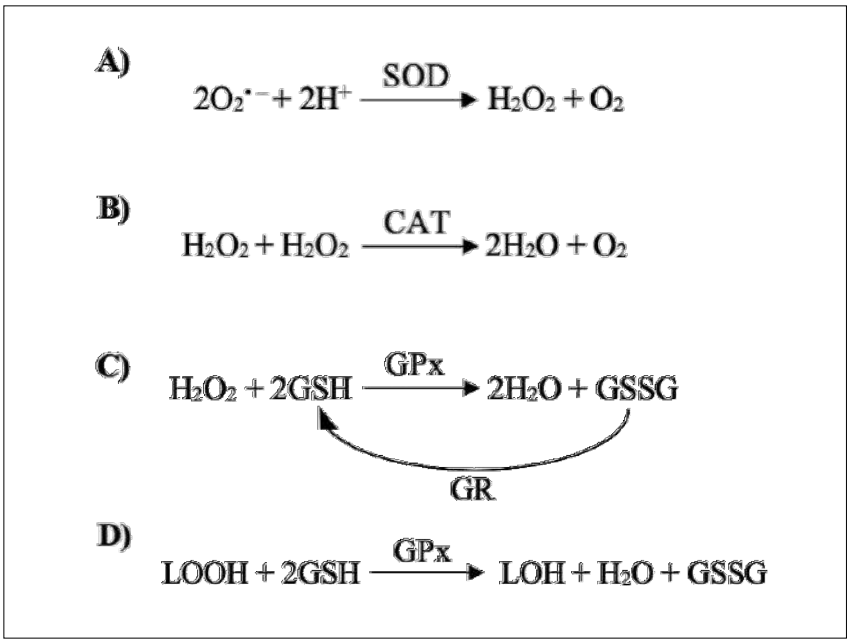


Figure 1.9. The main enzymatic antioxidant defence systems and their reactions. **A)** SOD catalyses the reduction of $O_2^{\cdot -}$ to H_2O_2 and O_2. **B)** Catalase catalyses the reduction of H_2O_2 to H_2O. **C-D)** GPXs catalyse the reduction of H_2O_2 to H_2O and LOOHs into their corresponding alcohols, using reduced GSH as a cofactor. GSR reduces the oxidized GSSG reacts with protein sulfhydryl group forming mixed disulfide. Therefore, the intracellular redox potential is largely determined by the GSH GSSG ratio.

compartments, including the nuclei, mitochondria and endoplasmic reticulum (Mari et al., 2009, 2010). Glutathione acts as a ·OH scavenger. Additionally, it acts as a cofactor for antioxidant enzymes, such as GPXs and certain Glutathione S-transferases (GSTs), to detoxify H_2O_2. In normal physiological state, glutathione exists in a thiol-reduced (GSH) state (90%), and disulphide-oxidized (GSSG) state (10%) within the cell (Pompella et al., 2003). During oxidation, disulphides are formed between thiol-groups of cysteine in glutathione GSH, resulting in a reaction between two reactive GSH forming glutathione disulfide (GSSG).

The GPX family consists of several distinct isozymes localised within specific tissues and cells, playing important defensive roles against oxidative stress (de Haan et al., 2003; Lubos et al., 2011). GPX1 was the first to be identified and it is present in mitochondria, nuclei and the cytosol. GPX2 is mainly gastrointestinal and found in the cytosol and nuclei. GPX3 is found mostly in extracellular fluids (Brigelius-Flohé, 1999) and cytosol (Lu, 2013). GPX4 has a key dual function as an

antioxidant and anti-apoptotic protein (Savaskan et al., 2007), found in the mitochondria, cytosol and nuclei (Schneider et al., 2009). GPx5 is expressed mainly into the epidydimal lumen (Lu, 2013). Although the GPXs family are potent ROS scavengers, very limited knowledge is available on their kinetics and activities.

GSTs are located within the cytoplasm, mitochondria and microsomal compartments of cells, where these catalyse the conjugation of GSH via sulfhydryl groups, to electrophilic centres on a wide variety of substrates, in order to detoxify compounds and make them more water-soluble (Wu and Dong, 2012; Board and Menon, 2013, Lu, 2013). Reduced GSH is regenerated by the activity of glutathione reductase (GSR), using NADPH as a reducing cofactor (Deponte, 2013). However, during persistent oxidative stress, it exceeds the cellular ability to regenerate GSH from GSSG. The GST superfamily also contains glutathione transferase ζ (GSTZ1, also known as maleylacetoacetate isomerase) amongst other isoforms, which catalyses the reaction of GSH with xenobiotics and endobiotics (Board and Anders, 2011). GSTZ1 is expressed in many tissues. However, although GSTZ1 is located in the cytosol, it is also localised within cellular mitochondria (Mannervik et al., 2005; Li et al., 2011). Some properties of cytosolic and mitochondrial GSTZ1 differ, these are not related to differences in amino acid sequence or post-translationally modified residues (Zhong et al., 2018). Furthermore, although GSTZ1 role in the oxidative defence mechanism is confirmed, and their deficiency results in generation of a constant level of oxidative stress (Blackburn et al., 2006), reports suggest that GSTZ1 function indirectly by modulating antioxidant response element (ARE)-regulated enzymes unlike other GSTs enzymes (Hayes et al., 2005).

1.5.4 Role of Oxidative Stress and Antioxidants in Mesenchymal Stem Cells

Despite possessing such an array of tightly-regulated, cellular antioxidant defence mechanisms, imbalance between ROS overproduction and cellular antioxidant capacity are strongly correlated with increased oxidative stress-induced biomolecular damage and the induction of telomere-independent senescence in a variety of cell types, particularly fibroblasts (Serra et al., 2000, 2003; Lorenz et al., 2001; Blander et al., 2003; Brown and Stuart, 2007; Richter and von Zglinicki 2007; Hammad et al., 2018). However, more recent studies into the effects of oxidative stress on embryonic stem cell (ESC) behaviour have confirmed that stem cells have contrasting responses to oxidative stress than somatic cells, due to the activation of

different antioxidant defence mechanisms (Barandalla et al., 2016). Furthermore, correlations between the susceptibilities of MSCs from various sources to cellular senescence, with their relative enzymic antioxidant expression and activity capabilities, have also been established (Valle-Prieto and Conget, 2010; Ko et al., 2012; Jeong and Cho, 2015; Yu et al., 2018; Chen et al., 2019). This is also the case, in terms of MSC susceptibilities to oxidative stress-induced biomolecular damage, with cellular resistance to oxidative damage being attributed to their relatively high enzymic antioxidant capacities (He et al., 2004; Ko et al., 2012; Yu et al., 2018; Chen et al., 2019). Thus, although the reasons behind such contrasting oxidative stress responses between different stem cell populations is still under debate, the majority of theories are increasingly focussed on the roles of antioxidants as being major influences in providing stem cell resistance to oxidative stress, via their possession of superior enzymic antioxidant induction capabilities (Liu et al., 2011; Shyh-Chang et al., 2013; Urao and Ushio-Fukai, 2013; Benameur et al., 2015; Sheshadri and Kumar, 2016).

1.6 Aims of Thesis

Based on previous findings that hTERT plays a minimal role in facilitating variations in proliferative life-span and the onset of replicative (telomere-dependent) senescence between high and low proliferative DPSC sub-populations (Alraies et al., 2017), this implies that other intrinsic telomere protective mechanisms may account for the contrasting differences in telomere lengths, proliferation rates and differentiation capabilities between high and low proliferative DPSCs. Therefore, as oxidative stress and its counteraction by enzymic antioxidants are well-established concepts in the relative susceptibilities of somatic and MSCs to cellular senescence, this Thesis evaluated whether similar variation existed in the relative susceptibilities high proliferative/multipotent and low proliferative/unipotent DPSCs to oxidative stress-induced biomolecular damage and telomere-independent senescence and whether such differences were related to contrasting enzymic antioxidant profiles between these DPSC sub-populations. In summary, the specific objectives of this Thesis are:

1. To investigate the effect of oxidative stress (H_2O_2)-induced (telomere-independent), cellular senescence in DPSC sub-populations via the analysis of

various parameters associated with the establishment of the senescence phenotype, such as PDs, morphological changes, telomere lengths, SA-β-gal staining and stem cell marker (p53, $p21^{waf1}$ and $p16^{INK4a}$) expression.

2. To investigate whether in DPSC sub-populations also differed in their respective susceptibilities to oxidative stress-induced, biomarker formation via the analysis of established oxidative stress marker levels of DNA (8-OHdG), protein (carbonyl content) and lipid (peroxidation) damage.

3. To investigate whether differences in specific enzymic antioxidant capabilities further contributed to the contrasting susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress-induced telomere-independent senescence and biomolecular damage.

By confirming such differences in the relative susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress-induced, senescence and biomolecular damage, as a consequence of superior enzymic antioxidant capabilities of high proliferative DPSCs, would significantly enhance our understanding of DPSC biology and its inter-relationship with oxidative stress and cellular senescence.

Chapter 2

Assessment of Dental Pulp Stem Cell Susceptibility to Oxidative Stress-Induced Cellular Senescence

Chapter 2

Assessment of Dental Pulp Stem Cell Susceptibility to Oxidative Stress-Induced Cellular Senescence

2.1 Introduction

Dental pulp stem cells (DPSCs) are increasingly being characterised and evaluated as alternative stem cell sources, for the development of more effective stem cell-based regenerative therapies for clinical use (Huang et al., 2009; Sloan and Waddington, 2009; Ledesma-Martínez et al., 2016; Nuti et al., 2016; Chalisserry et al., 2017; Anitua et al., 2018). Such developments are based on DPSCs being recognized as a viable stem cell source, due to their accessibility, multi-lineage (e.g. osteogenic, chondrogenic, adipogenic, myogenic, neurogenic) differentiation capabilities and similarities to bone marrow-derived stem cells (Gronthos et al., 2002; Shoi et al., 2014). Indeed, DPSCs have already been shown to be beneficial to repair following transplantation into various animal model defects *in vivo*, related to diseases and traumas within clinical fields such as Dentistry, Orthopaedics, Neurology, Ophthalmology and Cardiology (Ledesma-Martínez et al., 2016; Nuti et al., 2016; Chalisserry et al., 2017; Anitua et al., 2018).

Distinct DPSC subsets/niches are proposed to exist within dental pulp (i.e. the sub-odontoblast layer near existing post-mitotic odontoblasts, associated with the pulpal vasculature and within the central pulpal stroma itself), with contrasting proliferative and regenerative capabilities (Sloan and Waddington, 2009). This is an important consideration for the exploitation of DPSCs, as a significant limitation of stem cell therapy is the extensive *in vitro* expansion necessary to produce sufficient cell numbers for clinical use, which leads to proliferative decline and cellular senescence, accompanied by altered cellular differentiation and regenerative capabilities (Campisi and d'Adda di Fagagna, 2007; Wagner et al., 2010; Li et al., 2017). Thus, DPSCs have a distinctive highly heterogeneous nature with multiple progenitor cell populations residing within different niches of the pulp (Gronthos et al., 2000, 2002; Shi and Gronthos, 2003; Løvschall et al., 2005; Lizier et al., 2012; Kaukua et al., 2014). This is supported by studies demonstrating that despite

heterogeneous DPSC populations being capable of >120 population doublings (PDs) *in vitro* before reaching senescence, only 20% of purified DPSCs undergo >20PDs, suggesting that DPSC have sub-populations that differ in their proliferative capacities and developmental potential (Huang et al., 2009).

It has been recognized for some time that there are significant differences in the relative *ex vivo* expansion capabilities of individual DPSCs isolated from dental pulp tissues, with their heterogeneous nature potentially posing challenges during *in vitro* expansion due to the diverse proliferative and regenerative potential of the different subsets ultimately affecting their stemness (Huang et al., 2009). However, only recently has work begun to address the reasons behind these differences and the subsequent impact of such variations in proliferative capabilities on differentiation and regenerative potential. Previous findings have confirmed major variations in proliferative capacity and senescence within different DPSC sub-populations (Alraies et al., 2017). Whilst highly proliferative DPSCs reached >80PDs before senescence, other low proliferative DPSCs only achieved <40PDs, correlating with DPSCs with high proliferative capacities possessing longer telomeres (18.9kb) than less proliferative populations (5-13kb). High proliferative capacity DPSCs exhibited prolonged stem cell marker expression, but lack CD271. Early-onset senescence, stem cell marker loss and positive CD271 expression in DPSCs with low proliferative capacities were associated with impaired osteogenic and chondrogenic differentiation, favouring adipogenesis. DPSCs with high proliferative capacities only demonstrated impaired differentiation following prolonged expansion (>60PDs). Therefore, we have previously shown that DPSC proliferative and regenerative heterogeneity is related to contrasting telomere lengths and CD271 expression between DPSC populations.

Although certain studies have reported positive human DPSC expression for the reverse transcriptase, human telomerase catalytic subunit (hTERT), capable of the complete replication of telomere ends and counteracting telomeric erosion (Flores and Blasco, 2010; Jeon et al., 2011; Hakki et al., 2015), most report no or negligible hTERT expression in DPSCs (Egbuniwe et al., 2011; Mehrazarin et al., 2011a; Murakami et al., 2013; Alraies et al., 2017). Therefore, hTERT expression and activity appears not to be responsible for the maintenance of telomere lengths in high proliferative DPSCs or the variations in proliferative capabilities. Therefore, other intrinsic telomere protective mechanisms may be responsible for these

contrasting differences in telomere length, proliferation rates and regenerative potential between high and low proliferative DPSCs, such as differential oxidative stress responses and antioxidant profiles between DPSC populations. For most cell types, *in vitro* expansion and subsequent cellular senescence is a consequence of replicative (telomere-dependent) senescence, characterised by progressive telomere shortening and the loss of telomeric TTAGGG repeats, due to repeated cell divisions (Campisi and d'Adda di Fagagna, 2007).

Oxidative stress is another mechanism well-established to be capable of promoting cellular senescence (also known as telomere-independent senescence, premature senescence or stress-induced premature senescence, SIPS), in various cell types, including somatic cells and mesenchymal stem cells (Liu et al., 2011; Shyh-Chang et al., 2013; Li et al., 2017). Oxidative stress refers to the disturbance in the balance between the production of intracellular reactive oxygen species (ROS), such as superoxide and hydroxyl radical species ($O_2^{\cdot -}$ and $^{\cdot}OH$, respectively) and non-radical species, such as hydrogen peroxide (H_2O_2) and cellular antioxidant defence mechanisms. ROS are generated via a wide range of cellular mechanisms; however, under normal physiological conditions, mitochondria is the primary source for ROS production (Balaban et al., 2005). Although low ROS levels play important roles in the regulation of cell signalling and functions (Genestra, 2007), elevated ROS levels at the expense of antioxidant levels and subsequent oxidative stress, is capable of accelerating cellular senescence *in vitro* and *in vivo*, through the induction of DNA damage without significant telomere shortening, unless exposed to high levels of oxidative stress over prolonged periods (Ben-Porath and Weinberg, 2004). Indeed, recent evidence suggests that oxidative stress induces telomeric double stranded-breaks that are unrepairable, causing persistent DNA damage response signalling (DDR). Subsequently, cells undergo telomere-independent senescence, where cells cease replication irrespective of telomere lengths (Victorelli and Passos, 2017).

Both replicative and stress-induced senescence are associated with increased cellular morphologies, positive senescence associated β-galactosidase (SA-*β*-Gal) staining and the activation of various signalling pathways, including those involving the tumour suppressor genes, p53 and retinoblastoma protein (pRb), via the cyclin-dependant kinase inhibitors, $p21^{waf1}$ and $p16^{INK4a}$, respectively (Campisi and d'Adda di Fagagna, 2007; Morsczeck et al., 2016). This eventually blocks cell transfer from G1 to S phase causing cell cycle arrest. However, it appears that the main difference

between replicative senescence and the other forms of cell senescence lie in the presence of telomere shortening and the time elapsed before proliferation ceases (Bielak-Zmijewska et al., 2018). Although senescent cells are under a permanent state of cellular growth arrest, in which cells cease dividing without undergoing apoptosis, they remain metabolically active and secrete various pro-inflammatory factors, such as proteases, chemokines and cytokines (commonly known as the senescence-associated secretory phenotype, SASP), which influences cellular behaviour within the surrounding tissue microenvironments (Malaquin et al., 2016; McHugh and Gil, 2018). Consequently, senescence mediated via both replicative and stress-induced mechanisms induce significant alterations in cellular genotype and phenotype, ultimately influencing their overall biological functions and mediating impaired repair, disease and organismal ageing (Sikora et al., 2011).

2.2 Chapter Aims

In light of the minimal role that hTERT plays in facilitating variations in proliferative life-span and the onset of replicative (telomere-dependent) senescence between high and low proliferative DPSCs (Alraies et al., 2017), this Chapter aimed to identify whether DPSCs also differed in their respective susceptibilities to oxidative stress-induced (telomere-independent), cellular senescence via the analysis of various parameters associated with the establishment of the senescence phenotype, such as PDs, morphological changes, telomere lengths, SA-β-gal staining and stem cell marker (p53, $p21^{waf1}$ and $p16^{INK4a}$) expression. By confirming such differences between high and low proliferative DPSCs, this would potentially provide additional evidence to support high proliferative DPSCs being more resistant to SIPS, compared to low proliferative DPSCs, thereby enhancing our understanding of DPSC biology and its inter-relationship with cellular ageing.

2.3 Materials and Methods

2.3.1 Isolation of Dental Pulp Stem Cells

Human DPSCs were isolated from third molar teeth collected from adult patients (Patients A, C and D, all female, age range 18-30 years), undergoing orthodontic extractions at the School of Dentistry, Cardiff University, UK; as previously described (Alraies et al., 2017). Teeth were collected with informed patient consent and ethical approval from the South East Wales Research Ethics Committee of the

National Research Ethics Service (NRES), UK. To disinfect the teeth, the tooth outer surfaces were sterilised with 70% ethanol and soft tissue removed using a paper towel. Teeth were then grooved in the mesial, distal and occlusal regions with a slow speed rotary bone saw and halved on a dissection board using a chisel and hammer. Pulps were removed using a forceps and immediately placed in culture medium to maintain hydration, consisting of α-modified Minimum Essential Medium (αMEM) containing ribonucleosides, deoxyribonucleosides, 4mM L-glutamine, 100U/ml penicillin G sodium, 0.1µg/ml streptomycin sulphate, 0.25µg/ml amphotericin; and 20% foetal calf serum (FCS; all ThermoFisher Scientific, Paisley, UK), in addition to 100µM L-ascorbate 2-phosphate (Sigma, Poole, UK). Pulp tissues were subsequently minced on a glass slide and placed in 5ml bijoux tubes containing 4µg/µl collagenase/dispase (1ml, Roche, Welwyn Garden City, UK), for 1h at 37ºC, with gentle agitation every 20min. Pulpal tissues from individual patients were prepared separately. Pulpal digests were filtered through 70µm nylon cell strainers, eluted using αMEM medium (10ml). The eluents were collected in 50ml Falcon tube (BD Biosciences, Wokingham, UK), to obtain single cell suspensions. Cells were centrifuged (1,800rpm, 5min, Labofuge 400, ThermoFisher Scientific) and further re-suspended in αMEM medium (1ml). Cell counts were calculated using 0.4% Trypan Blue vitality stain (Sigma). As viable cells with an intact cell membrane do not allow the Trypan Blue stain to pass through, viable cell counts were determined through counting the non-stained cells. Aliquots (10µl) of each DPSC suspension were mixed well with Trypan Blue (10µl) and cells counted using a Neubauer Improved Haemocytometer (ThermoFisher Scientific) by light microscopy at x100 magnification (Nikon Eclipse TS100 Microscope, Kingston upon Thames, UK).

2.3.1.1 Fibronectin Adhesion Assay for Dental Pulp Stem Cell Isolation

DPSCs were preferentially selected and isolated via a fibronectin adhesion assay as previously described, based on their expression of high β_1-integrin levels on stem cell surfaces (Jones and Watt, 1993; Waddington et al., 2009; Harrington et al., 2014; Alraies et al., 2017). Following the calculation of viable cell counts, cells were seeded onto 6-well plates (Sarstedt, Leicester, UK), previously coated overnight with fibronectin (10µg/ml, 1ml/well, derived from human plasma, Sigma), reconstituted in 0.1M phosphate buffered saline (PBS), pH 7.4. Cell were seeded onto fibronectin-

coated wells at 4,000 cells/cm^2. Plates were maintained at 37ºC/5% CO_2 for 20min, to allow cell adherence (Adherent 1). Following adhesion, culture medium and non-adherent cells were removed and stored in new sterile Universal tubes. The 6-well plates containing adherent cells were replenished with fresh αMEM medium (2ml). The collected non-adherent cells were then seeded onto fresh fibronectin-coated 6 well-plates and allowed to adhere at 37ºC/5% CO_2 for 40min (Adherent 2). Following adhesion, the culture medium containing non-adherent cells was removed and stored and the 6-well plates containing adherent cells replenished, as described above. The collected non-adherent cells were subsequently seeded onto fresh fibronectin-coated 6 well-plates and again allowed to adhere at 37ºC/5% CO_2 for 40min (Adherent 3). All adherence cells isolated from procedures Adherent 1, 2 and 3 above, were maintained at 37ºC/5% CO_2 in αMEM medium (2ml), with culture medium changed every 2 days.

Isolated DPSCs were monitored daily throughout culture, using an inverted light microscopy (Nikon Eclipse TS100) at x10 magnification and the number of cells per colony was recorded. DPSC colonies were allowed to form up to 12 days in culture, with a minimum of 32 cells considered as a colony (Jones and Watt, 1993), although rapidly forming colonies were isolated sooner to prevent colony merger. Identified colonies of ≥32 cells were harvested within cloning rings, using pre-warmed, StemPro® Accutase® (100µl, ThermoFisher Scientific) and re-seeded into 1 well of a 96-well plate (Sarstedt), with fresh αMEM medium (100µl). Isolated colonies were subsequently expanded at 37ºC/5% CO_2, until the numbers were sufficient for seeding into T-75 flasks (VWR International, Lutterworth, UK). Culture medium was changed every 2-3 days.

All DPSCs utilised in this study were isolated according to the protocols described above. However, certain DPSC sub-populations were isolated previously by Dr Amr Alraies, School of Dentistry, Cardiff University (Alraies et al., 2017), whereas other DPSCs were isolated by myself, as above.

2.3.2 General Cell Culture

To ensure sterility of the working environment, safety cabinets and equipment were sprayed with 70% ethanol prior to carrying out any cell culture. In addition, the incubators, safety cabinets and water baths were cleaned on a weekly basis. All

consumables, glassware, some plastics, PBS and double-distilled water, were sterilised through autoclaving (123°C, 15lb/m^2, 15 min) and any equipment required remained in the tissue culture laboratory.

All procedures involving cell culture were performed under sterile conditions in aseptic Class II Tissue Culture Hoods (Astec Microflow, Andover, UK). 70% ethanol was used to disinfect work areas and any item to be placed in the hoods. Cells were maintained in sterile cell culture plates and flasks within a 5% CO_2 humidified, 37°C incubators (Sarstedt).

T-75 flasks containing DPSCs were maintained at 37ºC/5% CO_2 in αMEM medium. Upon reaching 80-90% confluence, DPSCs were washed with 0.1M PBS, before treating with pre-warmed, StemPro® Accutase® (2ml). Flasks were incubated at 37ºC/5% CO_2 for 5-10min, to ensure cell detachment. The StemPro® Accutase® was subsequently inactivated by the addition of αMEM medium (10ml) and centrifuged (1800rpm, 5min). The resulting pellets were re-suspended in αMEM medium, before cell counting (as above) and reseeding.

2.3.2.1 Cryopreservation and Cell Retrieval

To cryopreserve cells, DPSCs were centrifuged (1,800rpm, 5min), counted using Trypan Blue and re-suspended in freezing mix (1ml), containing 10% dimethyl sulfoxide (DMSO, Sigma) and 90% FCS. Freezing mix was added to cryovials and placed in Mr Frosty freezing pots at -80ºC. After 24h, cryovials were transferred to liquid nitrogen for long term storage. When cells were required, DPSCs were rapidly thawed by transferring to a 37ºC water bath. Cells were immediately re-suspended in 5ml αMEM medium and centrifuged, as above. Cells were then rewashed with αMEM medium and expanded in culture.

2.3.2.2 Screening of Mycoplasma Contamination

Routine screening was performed on DPSCs on a monthly basis through their proliferative life-spans in culture, to determine whether mycoplasma contamination was present. Samples of culture media (100µl) were obtained when DPSCs reached 90-100% confluence. Samples were stored at -20°C until required for assessment, then thawed on ice before incubated at 95°C for 5min, then centrifuged to pellet cell debris. Mycoplasma detection was confirmed using a VenorGeM Mycoplasma Detection Kit for Conventional PCR (Cambio, Cambridge, UK), according to

manufacturer's protocol. Media supernatant aliquots (2µl) were used to establish polymerase chain reaction (PCR) Master Mix reactions (total volume, 25µl), containing nuclease-free water, GoTaq Green buffer, magnesium chloride, primer/nucleotide mix, internal control, GoTaq polymerase (all from Promega Ltd., Southampton, UK). A negative control of nuclease-free water only and a positive control DNA, were used to determine whether mycoplasma contamination was present. RNA-free water was used for negative control, while DNA template as positive control. PCR cycles was performed as follows: single cycle at 94°C for 2min, followed by 39 cycles at 94°C for 30s, 55°C for 30s and 72°C for 30s then held at 4°C. The samples were run on 2% agarose gels (ThermoFisher Scientific), at 100V for 20min, using a Mini-Horizontal Electrophoresis Unit (Jencons, Leighton Buzzard, UK). Each gel was viewed on a GelDoc™ Scanner using Doc™ EZ Imaging System with Image Lab™ Version 5 for taking images (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). A positive mycoplasma contamination was shown by the presence of a strong band present at 267bp. An internal control always produces a band at 191bp, to show the reaction had worked, although the internal control band at 191bp was not visible if strong mycoplasma contamination was present.

If DPSCs were found to be mycoplasma positive, they were subsequently treated with BM Cyclin (Roche, Burgess Hill, UK) for 3 weeks, consisting of 3 cycles of treatment. Contaminated culture medium was removed, with fresh αMEM medium containing BM Cyclin 1 (4µl of stock solution/ml, final concentration 10µg/ml) added. DPSCs were cultured as previously described for three days and passaged when necessary. Culture medium containing BM Cyclin 1 was subsequently removed and replaced with fresh αMEM medium containing BM Cyclin 2 (4µl of stock solution/ml, final concentration 5µg/ml). Cells were cultured as previously described for 4 days and passaged when necessary. This 7-day treatment cycle was performed 3 times, before fresh αMEM medium was reassessed to confirm eradication of mycoplasma contamination.

2.3.3 Dental Pulp Stem Cell Expansion Under Oxidative Stress Conditions

DPSCs were seeded at 5,000 cells/cm^2 in T-75 flasks in and maintained at 37ºC/5% CO_2 in αMEM medium, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, ThermoFisher Scientific). Culture

medium was changed every two days. Cells were grown to 80-90% confluence and sub-cultured, as described above. DPSCs were subsequently expanded in culture throughout their proliferative life-spans to senescence.

2.3.4 Population Doubling Analysis

PD analysis is an established and widely used method to detect and quantify cellular senescence. It is defined as the number of times cell number is doubled and based on the observation that proliferative potential decreases during cellular aging (Ben-Porath and Weinberg, 2005). PDs for all DPSCs cultured in the absence and presence of H_2O_2 (0, 50μM, 100μM or 200μM), were monitored throughout their proliferative life-spans to senescence. Upon reaching 80-90% confluence, DPSCs were sub-cultured as described above, from which the total numbers of viable cell counts at each sub-culture were obtained using a haemocytometer and the number of cells reseeded was recorded. PDs were calculated based on the original number of DPSCs seeded and the final cell number, using the formula:

$$\frac{\log10\ (\text{total cell counts obtained}) - \log10\ (\text{total cell counts re-seeded})}{\log10\ (2)}$$

Cumulative PDs were subsequently plotted against time in culture, with the onset of cellular senescence confirmed when DPSCs underwent <0.5PDs/week (Cristofalo et al., 1998; Alraies et al., 2017).

2.3.5 Senescence-Associated β-Galactosidase Staining

The detection of positive SA-β-Gal activity is one of the most widely used markers to identify senescent cells. It is a lysosomal hydrolase that is only active in senescent cells at pH 6, unlike non-senescent cells, where it is active at pH 4 (Dimri et al., 1995; Krishnamurthy et al., 2004). SA-β-Gal activity as a marker of senescence was assessed in DPSC populations once they exhibited <0.5PDs/week, using Senescence Cell Histochemical Kits (Sigma), according to manufacturer's instructions. DPSCs at selected PDs were seeded in 6 well-plates at 5,000 cells/cm^2 and maintained at 37ºC/5% CO_2 in αMEM medium for 24h, in the absence and presence of H_2O_2 (0, 50μM, 100μM or 200μM). The culture medium was subsequently aspirated and the DPSCs washed (x2) with PBS (in Kit) diluted 1:10 in ultrapure water. Fixation

Buffer (1:10 diluted in ultrapure water, 1.5ml/well) was added for 7min at room temperature. Meanwhile, 10ml of Staining Mixture was prepared by mixing pre-warmed Staining Solution (1ml/well, in Kit), Reagent B (0.125ml, in Kit), Reagent C (0.125ml, in Kit) and X-gal solution (0.25ml, in Kit), with ultrapure water (8.5ml). Following incubation with Fixation Buffer, cells were rinsed (x3) with PBS (1ml/well). Staining Mixture (1ml) was added to each well and incubated at 37ºC/5% CO_2 overnight, with the plate sealed with Parafilm to prevent drying. DPSCs were subsequently observed by light microscopy (Nikon Eclipse TS100 Microscope) and the mean % positively blue-stained cells calculated in triplicate, from 20 random regions within the fields of view (30μm^2). Finally, the percentage of cells expressing β-galactosidase activity were calculated.

2.3.6 Gene Expression Analysis

2.3.6.1 mRNA Extraction

DPSCs at selected PDs were seeded in 6 well-plates at 5,000 cells/cm^2 and maintained at 37ºC/5% CO_2 in αMEM medium, in the absence and presence of H_2O_2 (0, 50μM, 100μM or 200μM), until they reached 80-90% confluence. The culture medium was subsequently aspirated and mRNA extraction performed using RNeasy Mini Kit (Qiagen, Manchester, UK), following manufacturer's protocols. DPSCs in each well were lysed with RLT Lysis Buffer (300μl, Qiagen), supplemented with 10μl/ml β-mercaptoethanol (Sigma). Lysates (in 600μl aliquots) were transferred to QiaShredder columns (Qiagen) and centrifuged (13,500g, 2min, Spectrafuge 24D, Jencons), to shear cellular components and separate cellular debris. Lysates were subsequently treated with 1:1 volumes of 70% molecular-grade ethanol (Sigma), followed by centrifugation (10,000g, 15s), using RNeasy Mini Kit columns (Qiagen). After discarding the flow-through, columns were washed with 350μL of Buffer RW1 (350μl, Qiagen) and centrifuged (10,000g, 15s). Resultant flow-though was discarded and a mixture of 10μL of DNase I stock (10μl) and Buffer RDD (70μl, both Qiagen) added. To remove any contaminant DNA, columns were incubated for 15min at room temperature. Subsequently, Buffer RW1 (350μl) was added and columns centrifuged (10,000g, 15s), followed by addition of Buffer RPE (500μl) and further centrifugation (10,000g, 1min) and the flow-through discarded. New 1.5ml Eppendorf tubes (ThermoFisher Scientific) were used for the column inserts, DNase-free water (40μl, Promega) was added and the columns centrifuged (10,000g, 1min).

To maximise RNA yields, elutes were transferred back into the columns and re-centrifuged. Finally, RNA collected in each Eppendorf tube was stored at -80ºC, until required. For RNA quantification, NanoVue (GE Healthcare, Little Chalfont, UK) was used. Initially, the Nanovue was standardised with DNase-free water (2µl) and RNA samples (2µl) were subsequently quantified. RNA purity was determined using the ratio between absorbance values at 260 and 280nm (260/280 ratio), with purities over 1.7 considered sufficient for downstream applications.

2.3.6.2 Reserve Transcription (RT)-PCR

cDNA was synthesised by adding 0.5µg random primers (Promega) to total RNA (1µg), followed by the addition of followed by DNase-free water to a final volume of 15µl. Mixtures were incubated in a G-Storm™ GS1 Thermal Cycler (Genetic Research Instrumentation, Braintree, UK), at 70°C for 5 min. Master mixes consisting of 5X Moloney murine leukaemia virus (M-MLV) buffer (5µl), Random Primers (0.5µg), RNasin (0.6µl), deoxynucleotide triphosphates (dNTPs; 10mM, 1.25µl) and M-MLV reverse transcriptase (1µl), were then added immediately to the cooled product followed by addition of DNase-free water, to a final volume of 25µl. Reaction reaction mixtures minus mRNA were also prepared as negative controls. All reactions were performed on a G-Storm™ GS1 Thermal Cycler (Genetic Research Instrumentation, Braintree, UK), at 37ºC for 1h, followed by 95ºC for 5min. Resultant cDNA samples were stored at -20°C, until required.

All PCR reactions were established by the addition of cDNA (1µl), 5x Green GoTaq™ Flexi Buffer (5µl), 25mM Magnesium Chloride Solution (1µl), 10mM PCR Nucleotide Mix (0.5µl), 5U/µl GoTaq™ DNA Polymerase (0.25µl, all Promega), 10µM forward and reverse primers (both 1.25µl). PCR were performed using the primer sequences described in Table 2.1, with β-actin serving as the housekeeping gene. All reactions were established to 25µl with nuclease-free water. Reactions were run on a G-Storm™ GS1 Thermal Cycler, with an initial denaturing step of 95°C (5min), followed by 35 cycles at a denaturing step temperature of 95ºC (1min), 1 cycle at primer-specific annealing temperatures of 52-62ºC (1min), 1 cycle at 72°C (1min) and 1 cycle at extension step temperature of 72°C (5min). Total human RNA (ThermoFisher Scientific) was used as a positive control for all genes analysed. Primer replacement with nuclease-free water served as negative controls.

Table 2.1: Details of the forward and reverse primers used for PCR amplification.

Gene Marker	Primer Sequence	Annealing Temp. (°C)	Cycles	NCBI Reference Sequence
CD73	F:5'-GTCGCGAACTTGCGCCTGGCCGCCAAG-3' R:5'-TGCAGCGGCTGGCGTTGACGCACTTGC-3'	65	35	NM_001204813.1
CD90	F:5'- ATGAACCTGGCCATCAGCATCG-3' R:5'- CACGAGGTGTTCTGAGCCAGCA-3'	55	35	NM_006288.3
CD105	F:5'-GAAACAGTCCATTGTGACCTTCAG-3' R: 5'-GATGGCAGCTCTGTGGTGTTGACC-3'	65	35	NM_001114753.2
CD271	F:5'- CTGCAAGCAGAACAAGCAAG-3' R:5'- GGCCTCATGGGTAAAGGAGT-3'	55	35	NM_002507.3
CD45	F:5'-GTGACCCCTTACCTACTCACACCACTG-3' R:5'-TAAGGTAGGCATCTGAGGTGTTCGCTG-3'	65	35	NM_002838.4
p53	F:5'- AGACCGGCGCACAGAGGAAG-3' R:5'- CTTTTTGGACTTCAGGTGGC-3'	55	35	NM_001126118.1
$p21^{waf1}$	F:5'- GGATGTCCGTCAGAACCCAT-3' R:5'- CCCTCCAGTGGTGTCTCGGTG-3'	60	35	NM_001291549.1
$p16^{INK4A}$	F:5'- CTTCCTGGACACGCTGGT-3' R:5'- GCATGGTTACTGCCTCTGGT-3'	55	35	NM_001195132.1
β-actin	F:5'- AGGGCAGTGATCTCCTTCTGCATCCT-3' R:5'- CCACACTGTGCCCATCTACGAGGGGT-3'	65	35	NM_001101.3
BMI1	F:5'- CTGGTTGCCCATTGACAGCG -3' R:5'- AAATCCCGGAAAGAGCAGCC-3'	59	35	NM_005180.8
CD166	F:5'- TCA TAC CTT GCC GAG TTG ACG-3' R:5'- TCT GGT ACT GGC CAT CAA TCC-3'	57	35	NM_001243283.1
P29	F:5'- ATG TGT CG ACC TGC CTT GG-3' R:5'- CCA TGA CCT CGT TGT TCC CA-3'	57	35	NM_002211.3
CD117	F:5'- AAATCCATCCCCACACCCTG -3' R:5'- CTTTTGTCGGCCTTGGTTGG -3'	59	35	XM_005265742.1
Nanog	F:5'- TGCCTCACACGAGACTGTC-3' R:5'- TGCTATTCTTCGGCCAGTTG-3'	59	35	XM_011520851.1
SSEA4	F:5'- CAAAGAGGGGGACCCCTAGA-3' R:5'- GACGGGGGAAATGTTACCGT-3'	59	35	NM_006927.3
hTERT	F:5'- CGGAAGAGTGTCTGGAGCAA-3' R:5'- GGATGAAGCGGAGTCTGG-3'	59	35	XM_011514106.1
Slug	F:5'- GAGCATACAGCCCCATCACT-3' R:5'- CTCCCCCGTGTGAGTTCTAA-3'	59	35	NM_003068.4
CD146	F:5'- ACAAGACCAAGATCCACAGCGAGT-3' R:5'- ATGCACACAATCACAGCCACGATG-3'	59	35	NM_006500.2
Oct4	F:5'- AGGAGTCGGGTGGAGAG-3' R:5'- CGTTTGGCTGAATACCTTCC-3'	59	35	NM_002701.5

2.3.6.3 Agarose Gel Electrophoresis

Agarose gels were prepared by adding 1.4g of agarose powder to 0.5x Tris/borate/EDTA (TBE) buffer (70ml), followed by microwave heating for 1min with swirling every 20s. When solutions became clear, ethidium bromide (7µl, 10mg/ml, Promega) was added and gels poured into casting trays with a comb mounted and left for 25min to set at room temperature. When gel set, the combs and borders were removed and the trays containing the gels transferred to the electrophoresis tank containing sufficient volumes of 0.5xTBE buffer. A 100 base pair (bp) DNA ladders (10µl, Promega) was loaded into the first and last wells of each gel, with PCR samples (10µl) loaded into the wells between. PCR products and 100bp DNA ladders were separated on 2% agarose gels (80mV, 45min), in 1x TBE running buffer. Gel images were captured under UV light (312nm) and PCR products visualised and analysed, using a Gel Doc 3000 Scanner and Image Analysis Software (Bio-Rad Laboratories).

2.3.7 Telomere Length Analysis

2.3.7.1 DNA Extraction

DPSCs at various PDs throughout their proliferative lifespans, were seeded in T-75 flasks at 5,000 cells/cm^2 and grown to 80-90% confluence. DPSCs were detached using StemPro® Accutase® and centrifuged at 1800rpm for 5min. Supernatants were discarded and the pellets re-suspended in PBS (200µl), mixed with proteinase K (20µl) and incubated at 56°C for 10min, followed by centrifugation (8,000g, 1min). 100% ethanol (200µl) was added and briefly centrifuged (8,000g, 15s). Genomic DNA was isolated from the resulting mixtures using QIAmp® DNA Mini Kits (Qiagen), according to manufacturer's instructions. Once mixtures were placed in QIAamp® Mini Spin Columns, these were centrifuged (8,000g, 1min) and collected in 2ml collection tubes. Buffer AW1 (500µl) was added to each column and centrifuged (8,000rpm, 1min). New collection tubes were then used and Buffer AW2 (500µl) added, followed by centrifugation (14,000rpm, 3min). Columns were then transferred to 1.5ml microcentrifuge tubes, Buffer AE (200µl) added and left at room temperature for 1min, followed by centrifugation (8,000rpm, 1min). DNA yields were subsequently quantified at 260nm (NanoVue) and samples stored at -20°C, until required.

2.3.7.2 Terminal Restriction Fragment Assay and Southern Blot Analysis

DPSC telomere length analyses were performed using the TeloTAGGG Telomere Restriction Fragment Length (TRF) Assay Kit (Roche), according to manufacturer's instructions. DNA samples (1µg) and positive DIG-labelled, Control DNA samples (in Kit) were digested with 2µl of x10 digestion buffer and 1µl of Hinf1/Rsa1 (both 40U/µl, in Kit), following incubation at 37°C for 2h. After 2h, reactions were stopped using 4µl of loading buffer. 0.8% agarose gels were prepared using highly pure, nucleic acid grade agarose (Agarose MP, Geneflow, Lichfield, UK), containing 0.2µg/ml ethidium bromide. When hardened, combs were removed, and gels were covered with 1x TAE buffer. DNA samples (20µl), a digoxigenin (DIG)-labelled, Molecular Weight Marker (20µl, in Kit) and positive DIG-labelled, Control DNA samples (20µl), were separated by agarose gel electrophoresis (80V, 5min and 15V, overnight), in 1x TAE buffer. Gels were subsequently treated with 0.25M hydrochloric acid solution (ThermoFisher Scientific) for 10min and rinsed (x2) with double-distilled water, before treatment (2x15min) with Denaturation Solution (0.5M sodium hydroxide, 1.5M sodium chloride solution). This was followed by rinsing (x2) with double-distilled water, and treatment with Neutralisation Solution (0.5M Tris-HCl buffer, pH 7.5, containing 3M sodium chloride, 2x15min).

Separated products were transferred onto positively charged nylon membranes (Roche) by Southern blotting using 20x standard sodium citrate (SSC) buffer (3M sodium chloride/0.3M sodium citrate solution, pH 7.0). Nylon membranes were fixed by UV-crosslinking for 2x10s (Stratalinker, Agilent Technologies, Stockport, UK) and washed with SSC (x2), prior to hybridisation using a hybridisation oven (VWR International), with DIG Easy Hyb (25ml, in Kit), at 42ºC for 1h under gentle rotation on a rotisserie. This was replaced with Hybridisation Solution (10ml) mixed with Telomere Probe (2µl, in Kit) and incubated at 42ºC for 3h, under gentle rotation. Membranes were washed (x2) with Stringent Wash Buffer I (25ml), at room temperature for 5min, under gentle rotation. This solution was replaced with Stringent Wash Buffer II (25ml) and the membranes washed (x2) at 50°C for 20min under gentle rotation. Stringent Wash Buffer II was replaced by incubation in Blocking Solution (25ml, in Kit), at room temperature for 30min, followed by incubation in Anti-DIG-AP Working Solution (25ml, in Kit), at room temperature for 30min, both under gentle rotation. Membranes were subsequently washed in Washing Buffer (2x25ml, in Kit), at room temperature for

15min each under gentle rotation followed by treatment with Detection Buffer (25ml, in Kit). Finally, membranes were incubated in Chemiluminescent Substrate Solution (in Kit), at room temperature for 5min under gentle rotation, before being exposed (1min) to High Performance Chemiluminescence Film (Hyperfilm®, GE Healthcare) and developed using a CURIX 60 X-Ray Film Processor (AGFA, Brentford, UK).

Average DPSC telomere lengths at each PD analysed were calculated from the Southern blot images, using ImageJ® Software (*http://rsb.info.nih.gov/ij/*), to quantify the densitometric intensity profile of the entire length of the separated products within each particular lane. In line with TRF Assay Kit instructions, each lane was overlaid along its entire length with a grid consisting of 30 equal squares, where telomere-specific signal was detectable. The background intensity of each lane was calculated by selecting squares within each lane containing no telomere-specific signal and by subtracting these averaged values from the squares containing telomeric signal. For each square containing detectable telomeric signal, the total signal density within each square (ODi) and the corresponding molecular weight at the mid-point of each square (Li) (based on the DIG-labelled, Molecular Weight Marker bands, kb), were calculated. Southern blots were performed in triplicate and average telomere lengths calculated using the following formula, where OD_i is the chemiluminescent signal and L_i is the length of the telomeres at position i:

$$\frac{\Sigma\,(OD_i)}{\Sigma\,(OD_i / L_i)}$$

2.4 Statistical Analysis

Statistical analyses were performed via use of GraphPad Prism Software (GraphPad Software Inc., La Jolla, USA). Graphical data for each experimental condition/sample group are exhibited as average±standard error of the mean (SEM). For SA-β-Gal data, statistical significance was determined using Analysis of Variance (ANOVA), with post-hoc Tukey's multiple comparisons tests. Significance was considered at $p<0.05$.

2.5 Results

2.5.1 Dental Pulp Stem Cell Population Doublings Under Oxidative Stress

A number of different DPSC sub-populations were successfully isolated and characterised from 3 individual patient donors (Patients A, C and D), using the procedures described above. However, in order to assess the relative susceptibilities of DPSC sub-populations to oxidative stress-induced senescence, the effects of increasing H_2O_2 concentrations (0, 50μM, 100μM, 200μM) on PDs were assessed throughout the proliferative life-spans to senescence, for 4 separate DPSC sub-populations (A1, A2, C3 and D4), from patients A, C and D (Figures 2.1-2.4, respectively). Overall, PDs and proliferative capacity showed marked variations in the susceptibilities of DPSCs to oxidative stress-induced senescence, with each demonstrating differences in PDs irrespective of whether DPSCs were derived from the same or different patients.

DPSC population (A1, Patient A), exhibited the highest achievable PDs overall and consequently, a high degree of resistance to oxidative stress-induced senescence (Figure 2.1). Over 160 days in culture, untreated controls DPSCs (A1) were determined to achieve >80PDs. A1 DPSCs treated with increasing H_2O_2 concentrations (50μM, 100μM, 200μM), exhibited slower PDs after 10 days in culture in a dose-dependent manner and the earlier onset of stress-induced senescence, compared to control DPSCs. However, DPSCs exposed to oxidative stress were still capable of undergoing considerable PDs, prior to senescence being reached (i.e. <0.5PDs/week). Indeed, A1 DPSCs achieved 76PDs over 160 days, 62PDs over 145 days and 58PDs over 145 days, in 50μM, 100μM and 200μM H_2O_2, respectively.

In contrast to A1, DPSC population (A2, Patient A) was capable of much reduced PDs, as untreated controls only exhibited 34PDs over 120 days in culture and therefore, demonstrated low proliferative capacity overall (Figure 2.2). As such, although A2 DPSCs exposed to 50μM H_2O_2 had limited effect on PDs (32PDs over 120 days) and therefore, exhibited some resistance to oxidative stress-induced senescence at this concentration; increasing exposure to H_2O_2 concentrations at 100μM and 200μM induced significantly slower PDs after 8 days in culture in a dose-dependent manner and the earlier onset of stress-induced senescence, compared to control DPSCs and those treated with 50μM H_2O_2.Indeed, A2 DPSCs only achieved 30PDs over 110 days and 27PDs over 98 days, in 100μM and 200μM

Figure 2.1. Characterisation of DPSC (A1, Patient A) population doublings (PDs) during extended culture to senescence, under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A1 demonstrated high proliferative capacity, with untreated controls achieving >80PDs over 160 days in culture. DPSCs exposed to increasing H_2O_2 concentrations exhibited slower PDs after 10 days in culture, prior to senescence: 50µM H_2O_2 (76PDs over 160 days), 100µM H_2O_2 (62PDs over 145 days) and 200µM H_2O_2 (58PDs over 145 days).

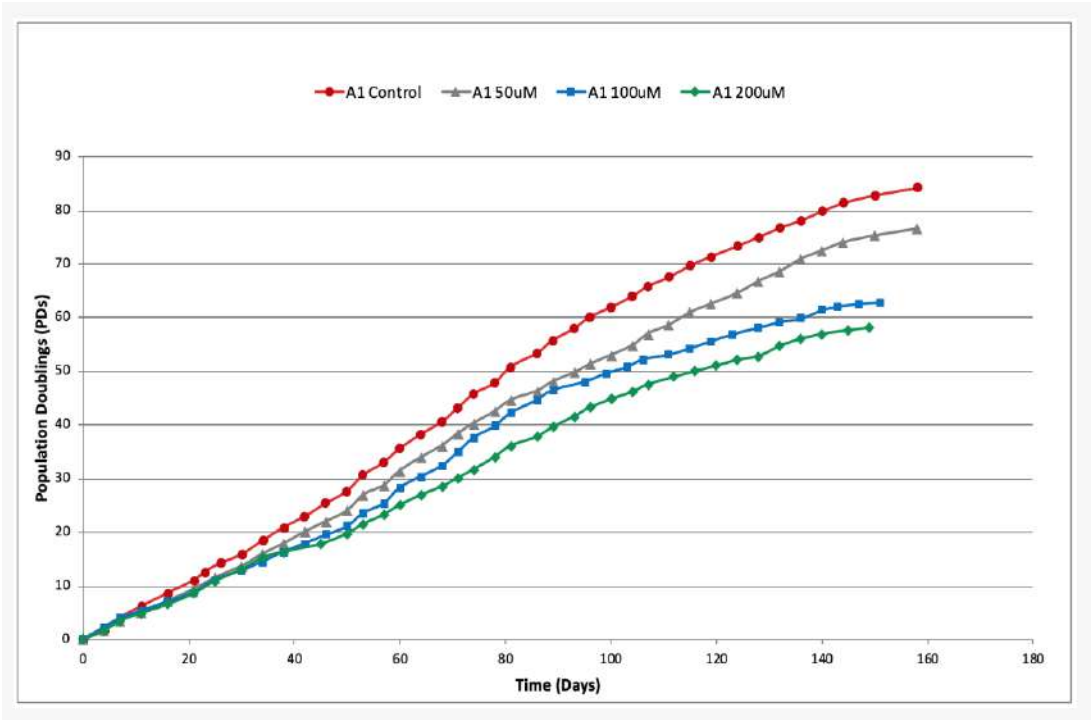
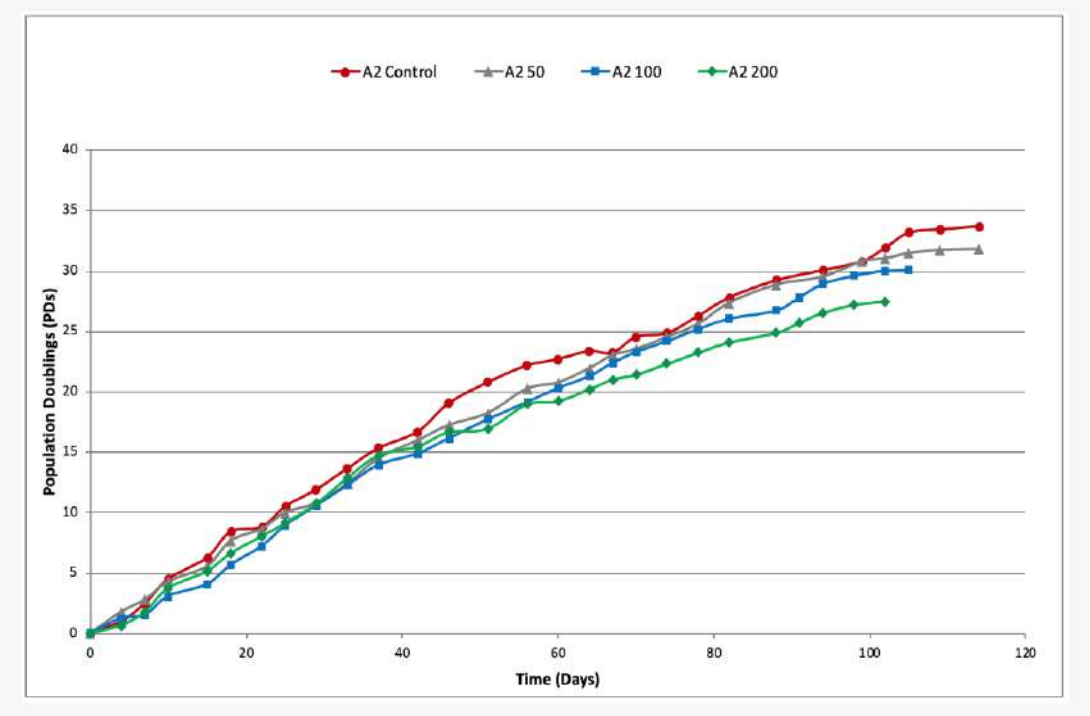


Figure 2.2. Characterisation of DPSC (A2, Patient A) population doublings (PDs) during extended culture to senescence, under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A2 demonstrated low proliferative capacity, with untreated controls achieving 34PDs over 120 days in culture. Although DPSCs exposed to 50µM H_2O_2 had limited effect on PDs (32PDs over 120 days), increasing H_2O_2 concentrations exhibited slower PDs after 8 days in culture, prior to senescence: 100µM H_2O_2 (30PDs over 110 days) and 200µM H_2O_2 (27PDs over 98 days).



H_2O_2, respectively.

DPSC populations (C3, Patient C) and (D4, Patient D) were also deemed to be low proliferative capacity DPSCs overall, based on the much reduced PDs these were capable of achieving overall (Figures 2.3 and 2.4, respectively). For C3, this was confirmed as untreated controls only exhibited 20PDs over 43 days in culture (Figure 2.3). Although C3 DPSCs exposed to 50μM H_2O_2 had limited effect on PDs (>18PDs over 43 days), C3 exhibited lower resistance to oxidative stress-induced senescence with increasing exposure to H_2O_2 concentrations at 100μM (15PDs over 29 days) and 200μM (12PDs over 25 days) after 13 days in culture and the earlier onset of stress-induced senescence, compared to control DPSCs and those treated with 50μM H_2O_2. Similar PD profiles were obtained with D4 DPSCs (Figure 2.4), with untreated control DPSCs only capable of 11PDs over 40 days in culture. Consequently, D4 DPSCs exhibited low resistance to oxidative stress-induced senescence, evident by D4 achieving dose-dependently reduced PDs with increasing exposure to H_2O_2 concentrations at 50μM (7PDs over 40 days), 100μM (6PDs over 35 days) and 200μM (4PDs over 30 days) after 2 days in culture and the earlier onset of stress- induced senescence, compared to control DPSCs.

Therefore, based on comparing all 4 DPSC sub-populations, distinct differences in oxidative stress-induced senescence were identified between individual DPSC sub-populations overall. It was evident that DPSC population (A1, Patient A) was highly proliferative and extremely resistant to oxidative stress-induced senescence, compared to the other DPSC populations analysed, A2 (Patient A), C3 (Patient C) and D4 (Patient D), which underwent early/accelerated senescence. However, in all 4 populations, reduction in PDs induced by oxidative stress were largely manifested in a dose-dependent manner, with the highest H_2O_2 concentrations (100μM and 200μM) inducing the most significant decreases in PDs.

Figure 2.3. Characterisation of DPSC (C3, Patient C) population doublings (PDs) during extended culture to senescence, under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. C3 demonstrated low proliferative capacity, with untreated controls achieving 20PDs over 43 days in culture. Although DPSCs exposed to 50µM H_2O_2 had limited effect on PDs (>18PDs over 43 days), increasing H_2O_2 concentrations exhibited significantly decreased PDs after 13 days in culture, prior to senescence: 100µM H_2O_2 (15PDs over 29 days) and 200µM H_2O_2 (12PDs over 25 days).

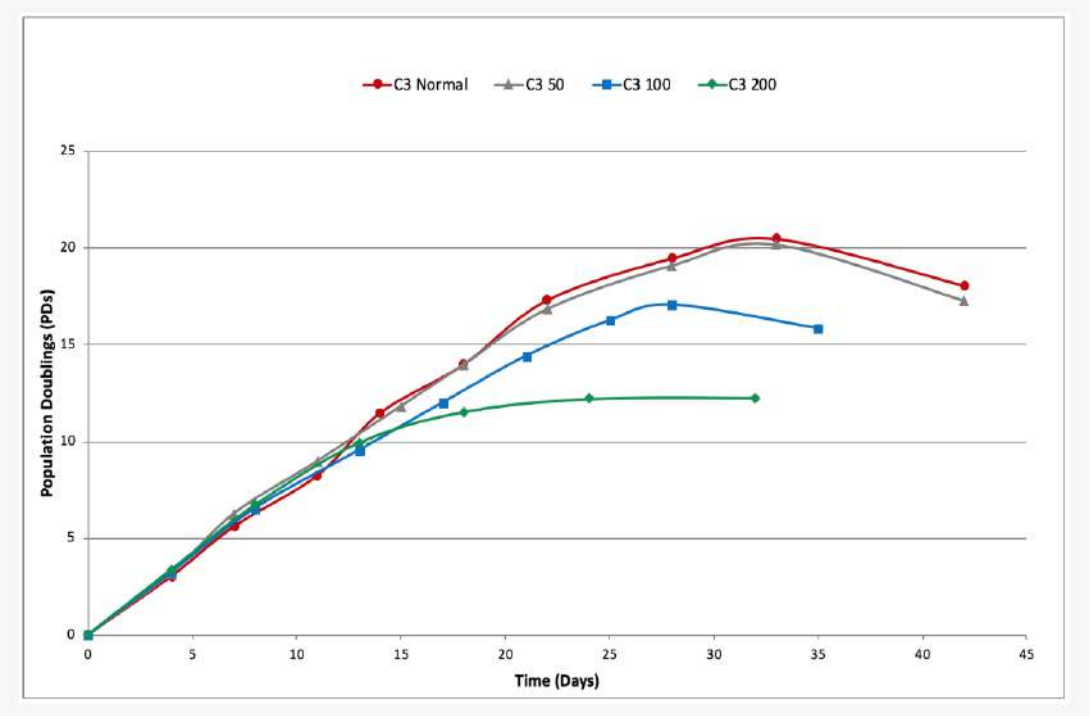
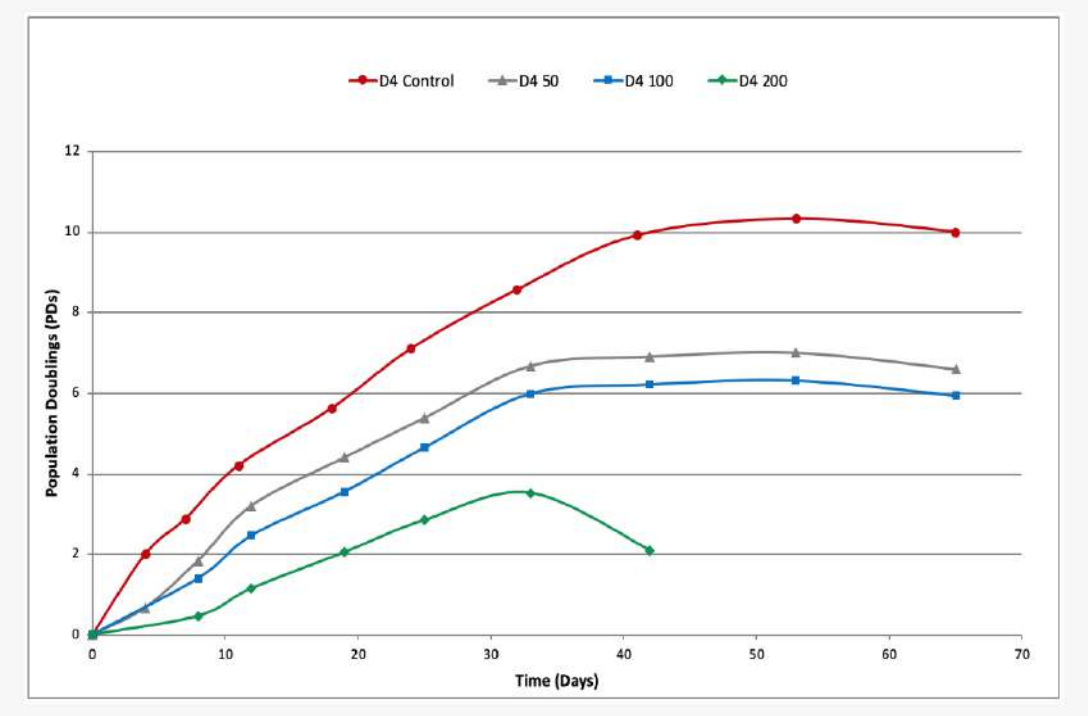


Figure 2.4. Characterisation of DPSC (D4, Patient D) population doublings (PDs) during extended culture to senescence, under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. D4 demonstrated low proliferative capacity, with untreated controls achieving 11PDs over 40 days in culture. DPSCs exposed to increasing H_2O_2 concentrations exhibited significantly slower proliferation PDs after 2 days in culture, prior to senescence: 50µM H_2O_2 (7PDs over 40 days), 100µM H_2O_2 (6PDs over 35 days) and 200µM H_2O_2 (4PDs over 30 days).



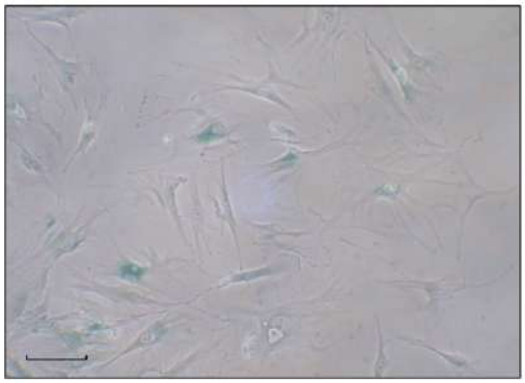
2.5.2 Senescence-Associated β-Galactosidase Staining

To confirm cellular senescence, SA-β-Gal staining was performed when PDs were <0.5/week. Once DPSCs from the four treatment groups had reached senescence, SA-β-Gal staining was performed on all experimental groups simultaneously for comparison purposes. Senescent DPSCs became positively stained due to X-gal being converted to blue stain at pH 6 by the β-galactosidase enzyme (Dimri et al., 1995; Krishnamurthy et al., 2004). Calculation of the % positive blue stained DPSCs by light microscopy.

As described above (Section 2.5.1), A1 DPSCs exhibited the highest PDs resistance to oxidative stress-induced senescence overall. Consequently, high % positive SA-β-Gal staining was only particularly evident in A1 DPSCs upon confirmation of the induction of senescence (<0.5PDs/week) after reaching 58PDs in culture treated with 200 μM H_2O_2 (89% positivity, Figure 2.5). Similarly, A1 DPSCs treated with 100μM H_2O_2 demonstrated high % positive SA-β-Gal staining (87%), after reaching 62PDs. However, less positive SA-β-Gal staining was determined for A1 DPSCs treated with 50μM H_2O_2 (39% at 73PDs), although significantly less SA-β-Gal positivity was detectable in untreated A1 controls, even following 80PDs in culture (25%, $p<0.001$). In contrast to A1, low proliferative DPSC populations, A2, C3 and D4, demonstrated high levels of positive SA-β-Gal staining at much earlier PDs, especially with increasing H_2O_2 exposure in line with these being identified as low proliferative DPSC sub-populations overall. Despite untreated DPSC sub-population, A2, demonstrating 30% positive SA-β-Gal staining at 30PDs (Figure 2.6), these increased further with 50μM H_2O_2 at 29PDs (35%, $p<0.001$), 100μM H_2O_2 at 28PDs (86%, $p<0.001$) and 200μM H_2O_2 at 26PDs (91%, $p<0.001$). Such increases in SA-β-Gal staining were equally prominent at earlier PDs with DPSC populations, C3 and D4 (Figures 2.7 and 2.8, respectively). Although untreated sub-population, C3, only exhibited 18% SA-β-Gal positivity at 19PDs (Figure 2.7), detectable staining significantly increased with 50μM H_2O_2 at 19PDs (20%, $p<0.05$), 100μM H_2O_2 at 16PDs (69%, $p<0.001$) and 200μM H_2O_2 at 12PDs (89%, $p<0.001$). This was also the case with D4 DPSCs, with both untreated and 50μM H_2O_2 treated D4 displaying 95% positive SA-β-Gal staining at 7PDs and 10PDs, respectively ($p>0.05$) and 100% positive staining at 100μM and 200μM H_2O_2 concentrations (at 6PDs and 2PDs, respectively; $p<0.001$ versus untreated controls).

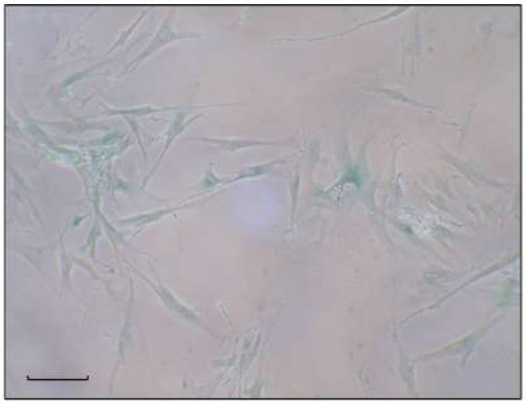
Figure 2.5. Comparison of % positive SA-β-Gal staining for DPSC (A1, Patient A) at 144 days, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. Scale bar 100µm, ×10 magnification. N=3, average±SEM, ***p<0.001.

Untreated Controls (25% at 80PDs)

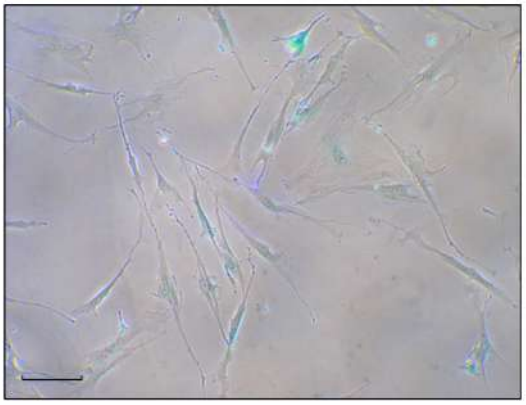


50µM H_2O_2 (39% at 73PDs)

100µM H_2O_2 (87% at 62PDs)



200µM H_2O_2 (89% at 58PDs)



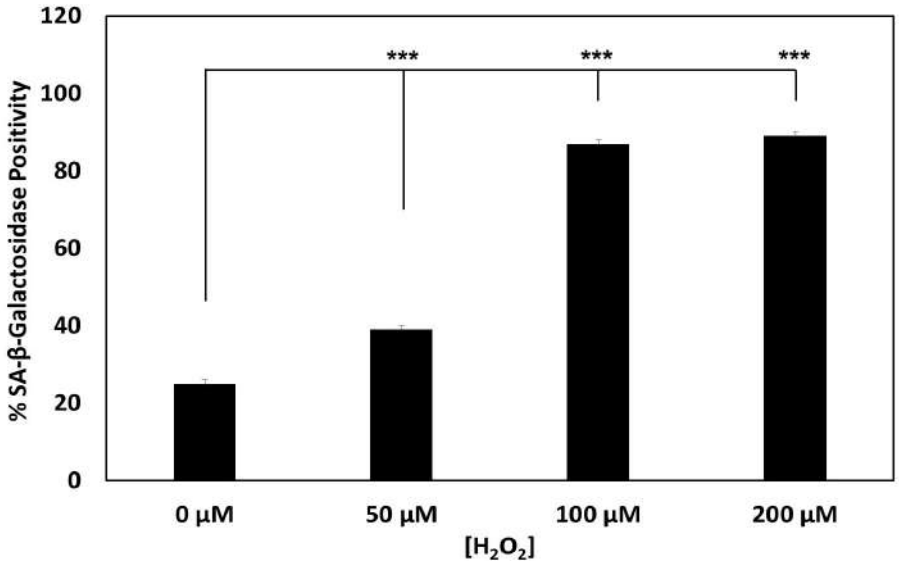
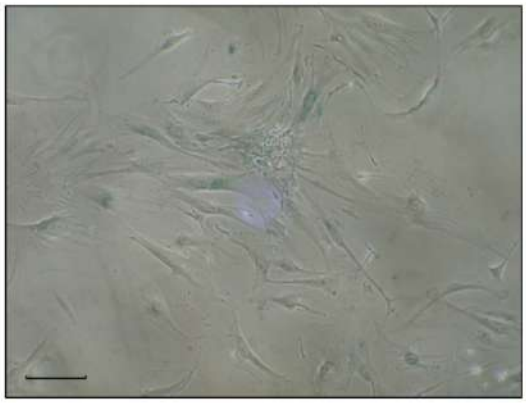
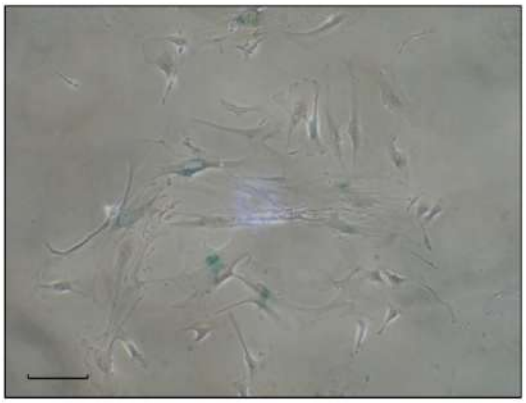


Figure 2.6. Comparison of % positive SA-β-Gal staining for DPSC (A2, Patient A) at 95 days, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. Scale bar 100µm, ×10 magnification. N=3, average±SEM, ***p<0.001.

Untreated Controls (30% at 30PDs)

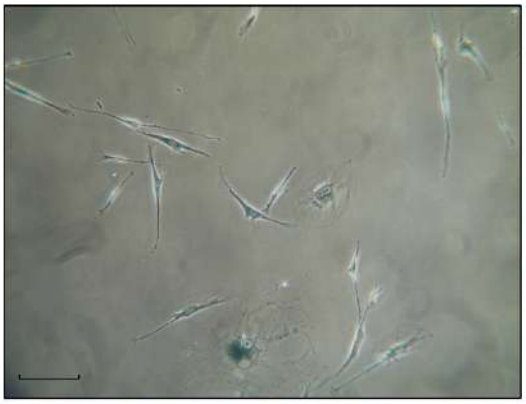


50µM H_2O_2 (35% at 29PDs)



100µM H_2O_2 (86% at 28PDs)

200µM H_2O_2 (91% at 26PDs)



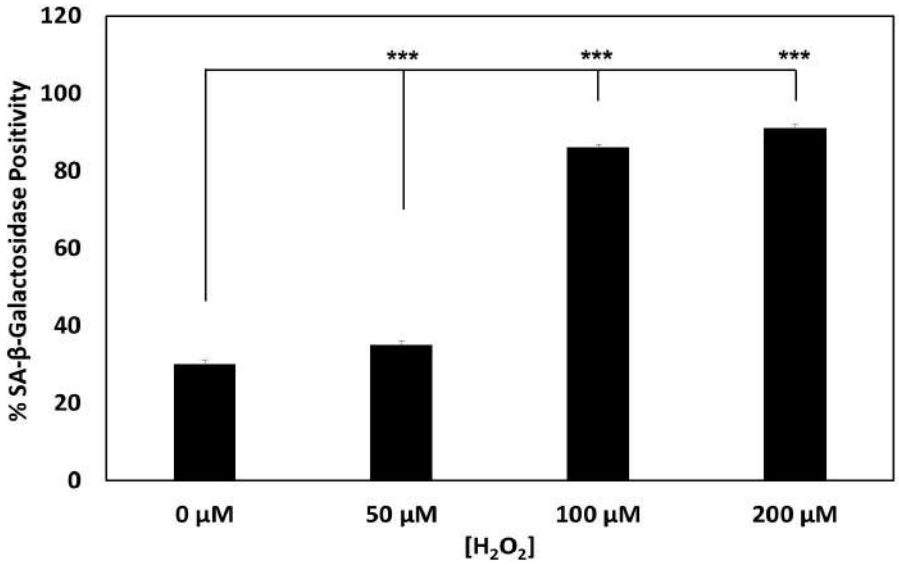
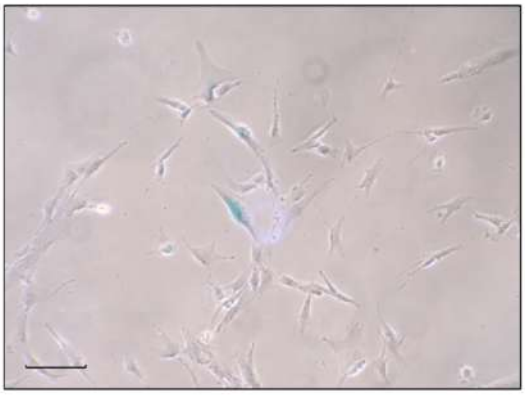
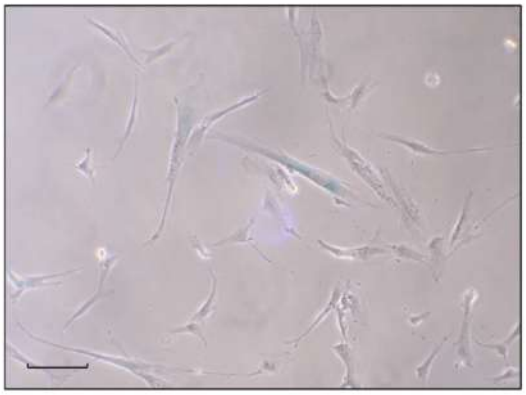


Figure 2.7. Comparison of % positive SA-β-Gal staining for DPSC (C3, Patient C) at 25 days, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. Scale bar 100µm, ×10 magnification. N=3, average±SEM, *p<0.05 and ***p<0.001.

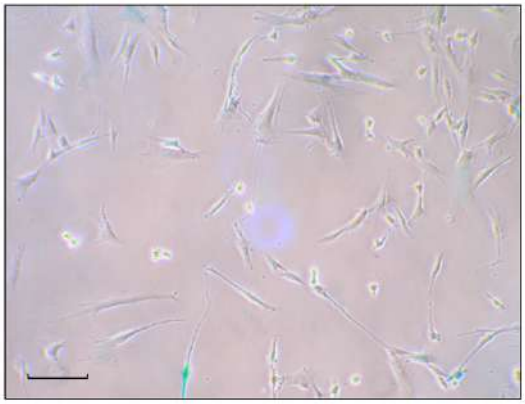
Untreated Controls (18% at 19PDs)



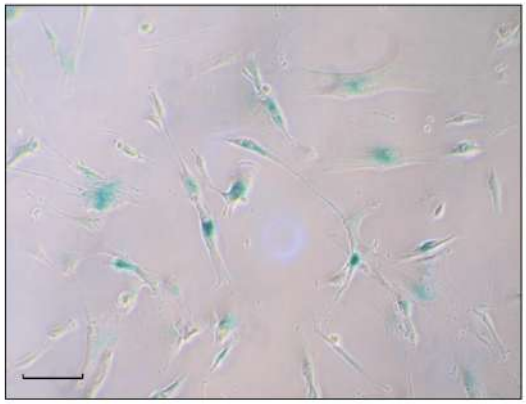
50µM H_2O_2 (20% at 19PDs)



100µM H_2O_2 (69% at 16PDs)



200µM H_2O_2 (89% at 12PDs)



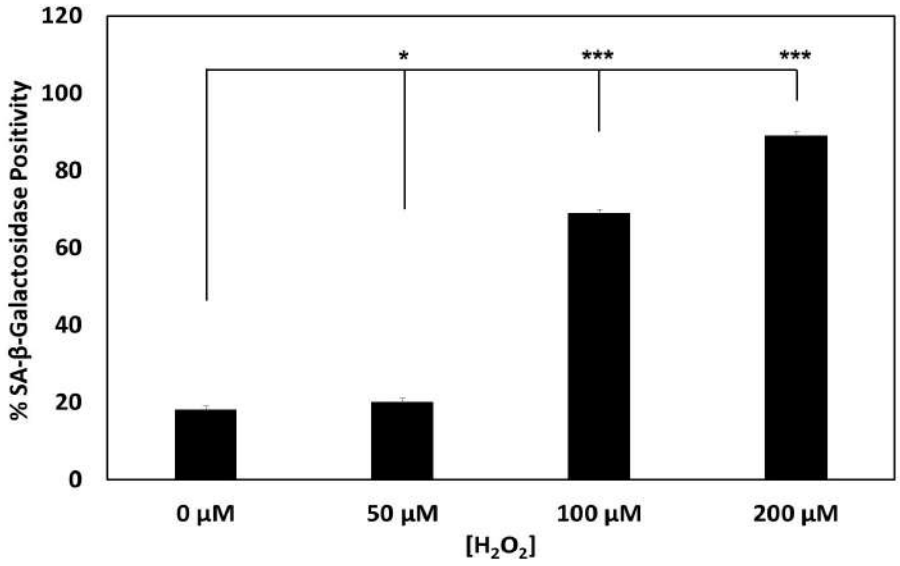
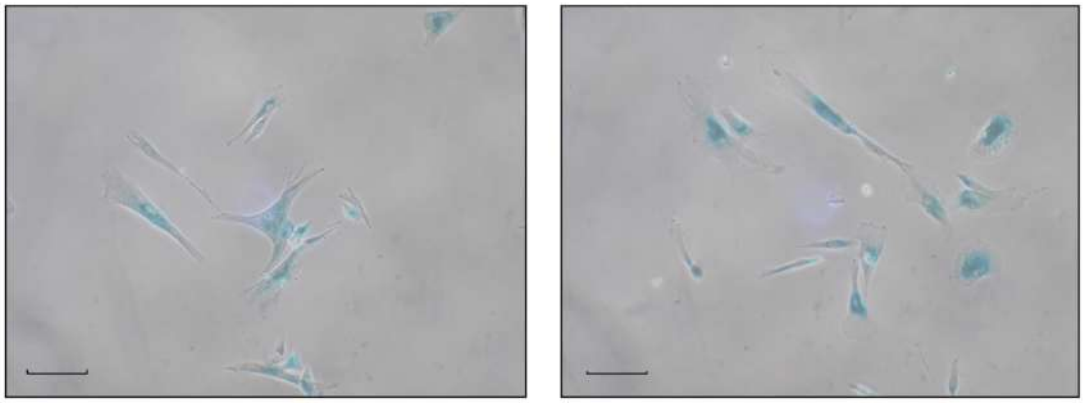
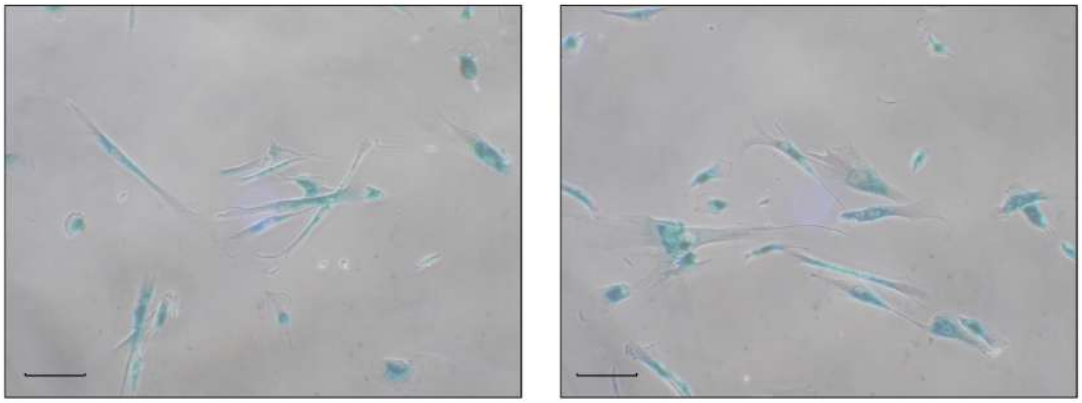


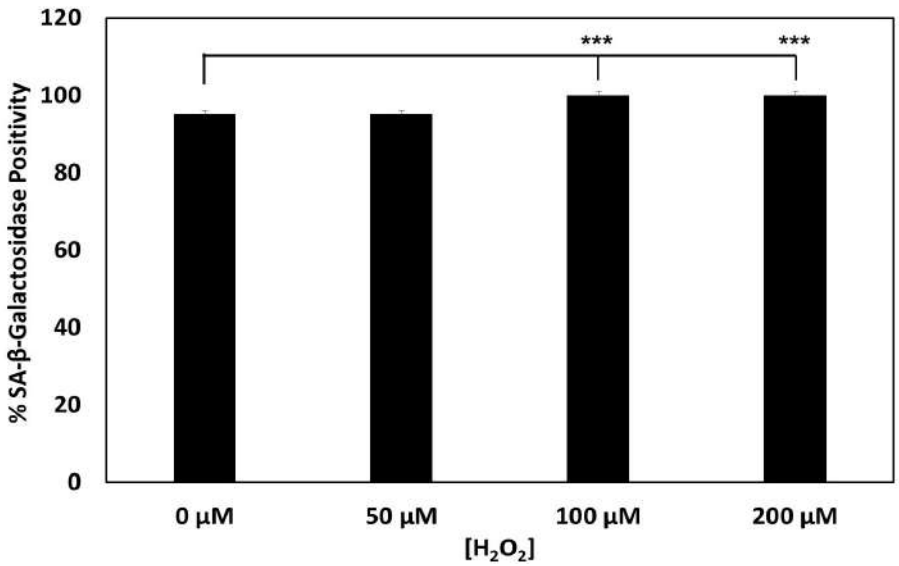
Figure 2.8. Comparison of % positive SA-β-Gal staining for DPSC (D4, Patient D) at 42 days, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. Scale bar 100µm, ×10 magnification. N=3, average±SEM, ***p<0.001.

Untreated Controls (95% at 7PDs) **50µM H_2O_2 (95% at 10PDs)**



100µM H_2O_2 (100% at 6PDs) **200µM H_2O_2 (100% at 2PDs)**





2.5.3 Senescence Marker Gene Expression

Analysis of cellular senescence-related genes (p53, $p21^{waf1}$, $p16^{INK4a}$ and hTERT) expression for DPSCs sub-populations, A1, A2, C3 and D4, demonstrated that all DPSCs assessed were negative for hTERT expression at all PDs analysed (Figure 2.9). Highly proliferative DPSC sub-population, A1, displayed undetectable p53 and $p16^{INK4a}$ expression at early PDs (10-25PDs), irrespective of H_2O_2 treatment. However, low level $p21^{waf1}$ expression was apparent for A1, both at early (10-25PDs) and later (45-60PDs) PDs, independent of H_2O_2 treatment. Low level $p16^{INK4a}$ expression was also detectable for A1, but only at later (45-60PDs) PDs. Similar to A1, low proliferative DPSC population, A2, demonstrated low levels of $p21^{waf1}$ and $p16^{INK4a}$ expression at early PDs (2-10PDs), irrespective of H_2O_2 treatment, although p53 expression was undetectable at early PDs (2-10PDs). However, both low proliferative DPSC populations, C3 and D4, demonstrated high levels of detectable p53, $p21^{waf1}$ and $p16^{INK4a}$ expression at early PDs (2-10PDs), again independent of H_2O_2 treatment.

2.5.4 Telomere Length Analysis

Average telomere lengths of DPSCs sub-populations, A2, C3 and D4, in the absence and presence of H_2O_2 treatment (50μM, 100μM, 200μM), were measured at early PDs (2-10PDs), with additional quantification of telomere lengths in DPSCs, A1, at early (10-25PDs) and later PDs (45-60PDs). Telomere lengths were determined using TRF length analysis and Southern blotting. Band densities and lengths varied considerably between the different DPSC sub-populations, with untreated highly proliferative DPSC sub-population, A1, possessing longer average telomere lengths (12.8kb) at early PDs (10-25PDs), compared with low proliferative DPSCs, A2 (9.8kb), C3 (9.6kb) and D4 (7.5kb), at comparable PDs (2-10PDs, Figures 2.10-2.13, for DPSCs, A1, A2, C3 and D4, respectively). Untreated high proliferative DPSC sub-population, A1, possessed an average telomere length of 12.8kb, although reductions in telomere lengths (2-5kb) were calculated with H_2O_2 treatment at early PDs (10-25PDs, Figure 2.10). At later PDs (45-60PDs), untreated A1 DPSCs demonstrated minor reductions (1-2kb) in telomere lengths (11.2kb), versus early PDs, with further reductions (3-4kb) with H_2O_2 treatment. However, low proliferative DPSCs, A2, C3, and D4, demonstrated less obvious decreases in telomere lengths (≤1kb), with H_2O_2 treatment (Figures 2.11-2.13, respectively).

Figure 2.9. Comparison of senescence marker (p53, $p21^{waf1}$, $p16^{INK4a}$ and hTERT) gene expression by DPSC sub-populations, A1, A2, C3 and D4, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. β-actin was used as the housekeeping gene. Total human RNA was used as a positive control for all genes analysed. Water and RT-negative experimental controls had no expression.

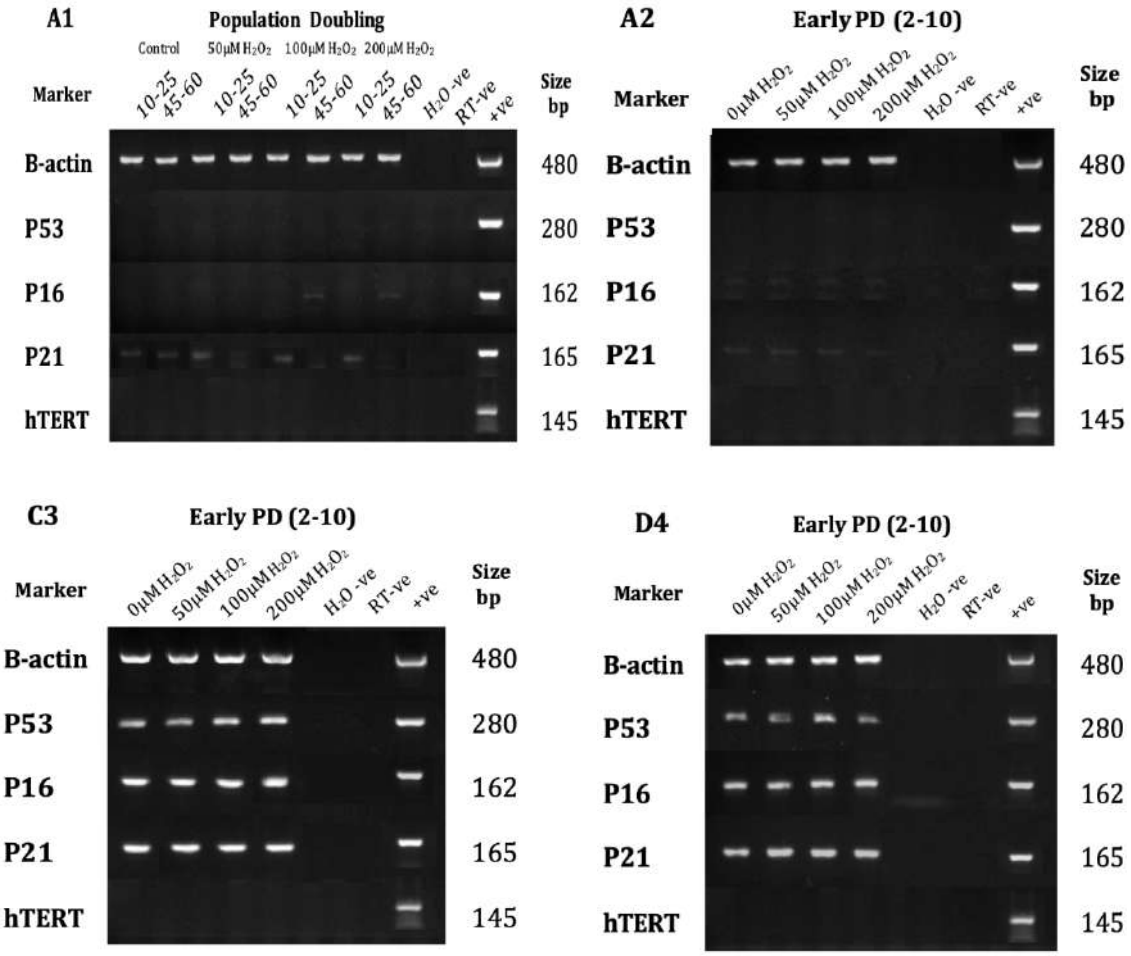
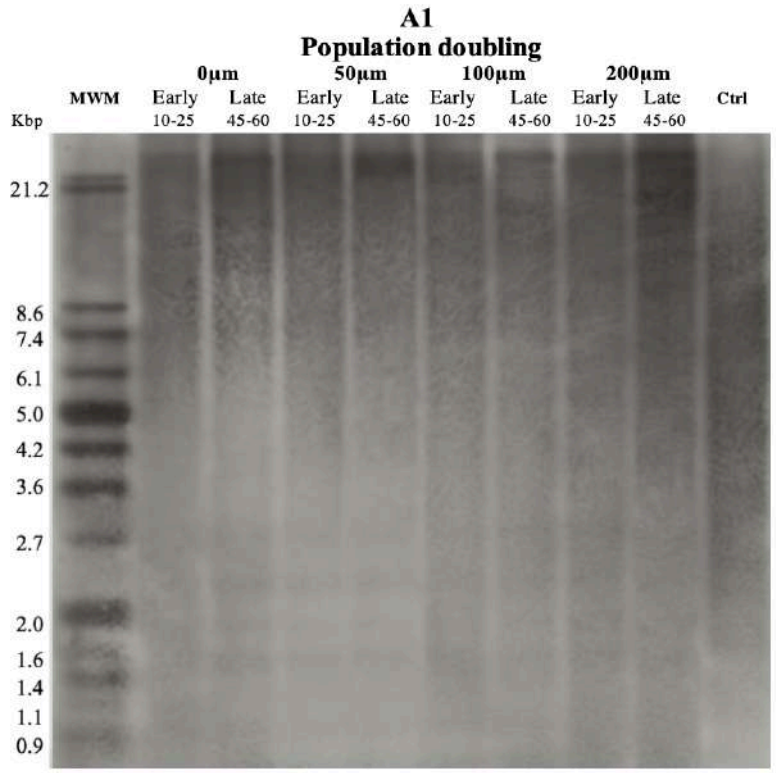
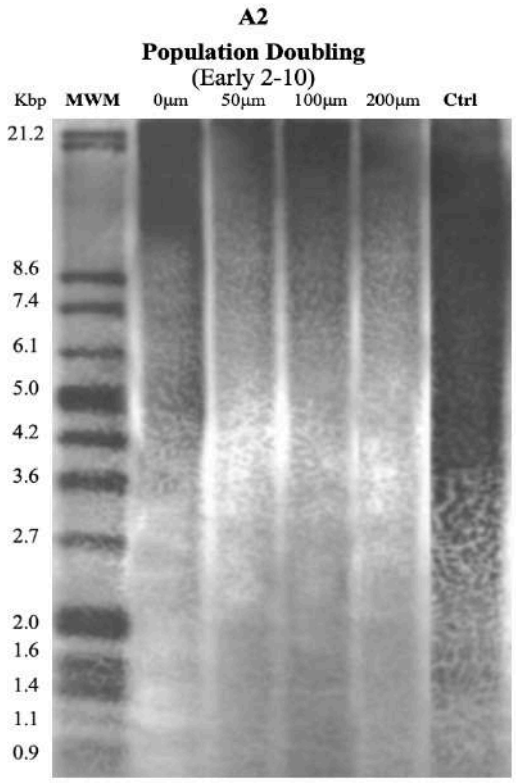


Figure 2.10. Southern blot analysis of average telomere lengths in DPSC sub-population, A1, at early (10-25PDs) and later (40-65PDs) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A digoxigenin (DIG)-labelled, Molecular Weight Marker (Ladder) and a positive DIG-labelled, Control DNA sample (Ctrl), were also included.



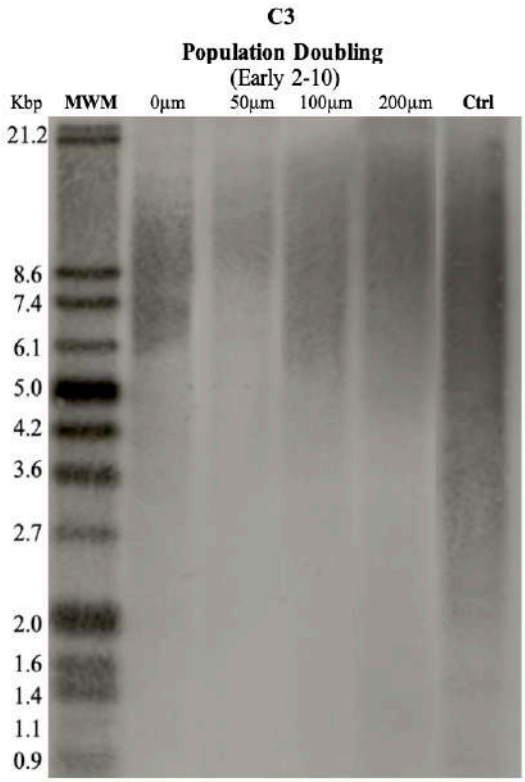
A1		Mean TRF (kb) ± SE			
		Control	**50µM H_2O_2**	**100µM H_2O_2**	**200µM H_2O_2**
PD	**Early**	12.8±0.4	9.9±0.3	7.9±0.1	8.4±0.4
	Late	11.2±0.2	7.8±0.2	7.4±0.3	7.5±0.3

Figure 2.11. Southern blot analysis of average telomere lengths in DPSC sub-population, A2, at early (2-10PDs) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A digoxigenin (DIG)-labelled, Molecular Weight Marker (Ladder) and a positive DIG-labelled, Control DNA sample (Ctrl), were also included.



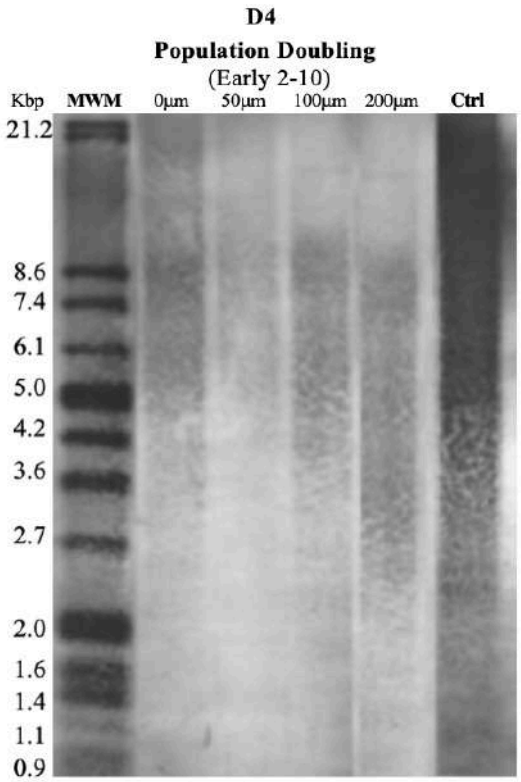
A2		Mean TRF (kb) ± SE			
		Control	**50µM H_2O_2**	**100µM H_2O_2**	**200µM H_2O_2**
PD	**Early**	9.8±0.1	9.1±0.1	8.7±0.3	8.8±0.1

Figure 2.12. Southern blot analysis of average telomere lengths in DPSC sub-population, C3, at early (2-10PDs) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A digoxigenin (DIG)-labelled, Molecular Weight Marker (Ladder) and a positive DIG-labelled, Control DNA sample (Ctrl), were also included.



C3		Mean TRF (Kb) ± SE			
		Control	50µM H_2O_2	100µM H_2O_2	200µM H_2O_2
PD	Early	9.6±0.3	9.0±0.5	8.6±0.2	8.6±0.4

Figure 2.13. Southern blot analysis of average telomere lengths in DPSC sub-population, D4, at early (2-10PDs) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A digoxigenin (DIG)-labelled, Molecular Weight Marker (Ladder) and a positive DIG-labelled, Control DNA sample (Ctrl), were also included.



D4		Mean TRF (kb) ± SE			
		Control	**50µM H_2O_2**	**100µM H_2O_2**	**200µM H_2O_2**
PD	**Early**	7.5±0.4	7.2±0.4	6.8±0.3	7.0±0.4

2.5.5 Stem Cell Marker Gene Expression

RT-PCR analysis was performed to confirm that sub-populations, A1, A2, C3 and D4, were DPSCs and to elucidate whether increasing H_2O_2 treatment (50μM, 100μM, 200μM) and oxidative stress-induced senescence accelerated the loss of stem cell characteristics in DPSCs. All DPSC sub-populations examined showed varying degrees of positive gene expression for MSC markers, CD73 (ecto-5-nucleotidase), CD90 (Thy-1) and CD105 (endoglin), at early PDs (Figure 2.14). In contrast, hematopoietic stem cell marker, CD45 (hematopoietic surface antigens), was undetectable in all DPSCs assessed and at all PDs analysed. However, MSC marker, CD29 (integrin β_1), expression was evident to varying degrees in low proliferative DPSCs, A2, C3 and D4, only. CD90 expression was largely retained in high (A1) and low (A2, C3 and D4) proliferative DPSCs through culture expansion and irrespective of H_2O_2 treatment, CD73 and CD105 expression showed declined detection at later PDs (40-65PDs), shown to be dependent and independent of H_2O_2 treatment, respectively. CD29 expression in low proliferative DPSCs, A2, C3 and D4, were also unaffected by H_2O_2 treatment. However, reductions in CD73 and CD105 expression were less evident in low proliferative DPSCs, A2, C3 and D4.

RT-PCR analysis was also performed to assess the expression of various other stem cell markers, including those representative of embryonic and neural crest lineages, in DPSC sub-populations, A1, A2, C3 and D4 and whether increasing H_2O_2 treatment (50μM, 100μM, 200μM) and oxidative stress-induced senescence, accelerated the loss of these stem cell markers. All DPSC sub-populations examined showed strong positive expression for self-renewal and multipotent adult stem cell marker, BMI-1, at early PDs (Figure 2.15). Although MSC marker (CD146), multipotent stem cell marker (CD271) and stem cell differentiation regulator (CD166), were absent or negligibly expressed in high proliferative DPSCs, all low proliferative DPSCs exhibited strong CD146 and CD271 expression, as did DPSCs, C3 and D4 in terms of CD166 expression. Embryonic stem marker, Oct4, was absent in high proliferative DPSCs, but expressed by all low proliferative DPSCs analysed. Other embryonic stem cell markers, SSEA4 and Slug, were positively expressed in high proliferative DPSCs and low proliferative DPSCs, C3 and D4, but both absent from low proliferative DPSC, A2. Negligible expression of the pluripotent (Nanog) or neural crest (CD117, c-kit), stem cell markers were found in all DPSCs examined.

Figure 2.14. Comparison of mesenchymal and hematopoietic stem cell marker gene expression by DPSC sub-populations, A1, A2, C3 and D4, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. β-actin was used as the housekeeping gene. Total human RNA was used as a positive control for all genes analysed. Water and RT-negative experimental controls had no expression.

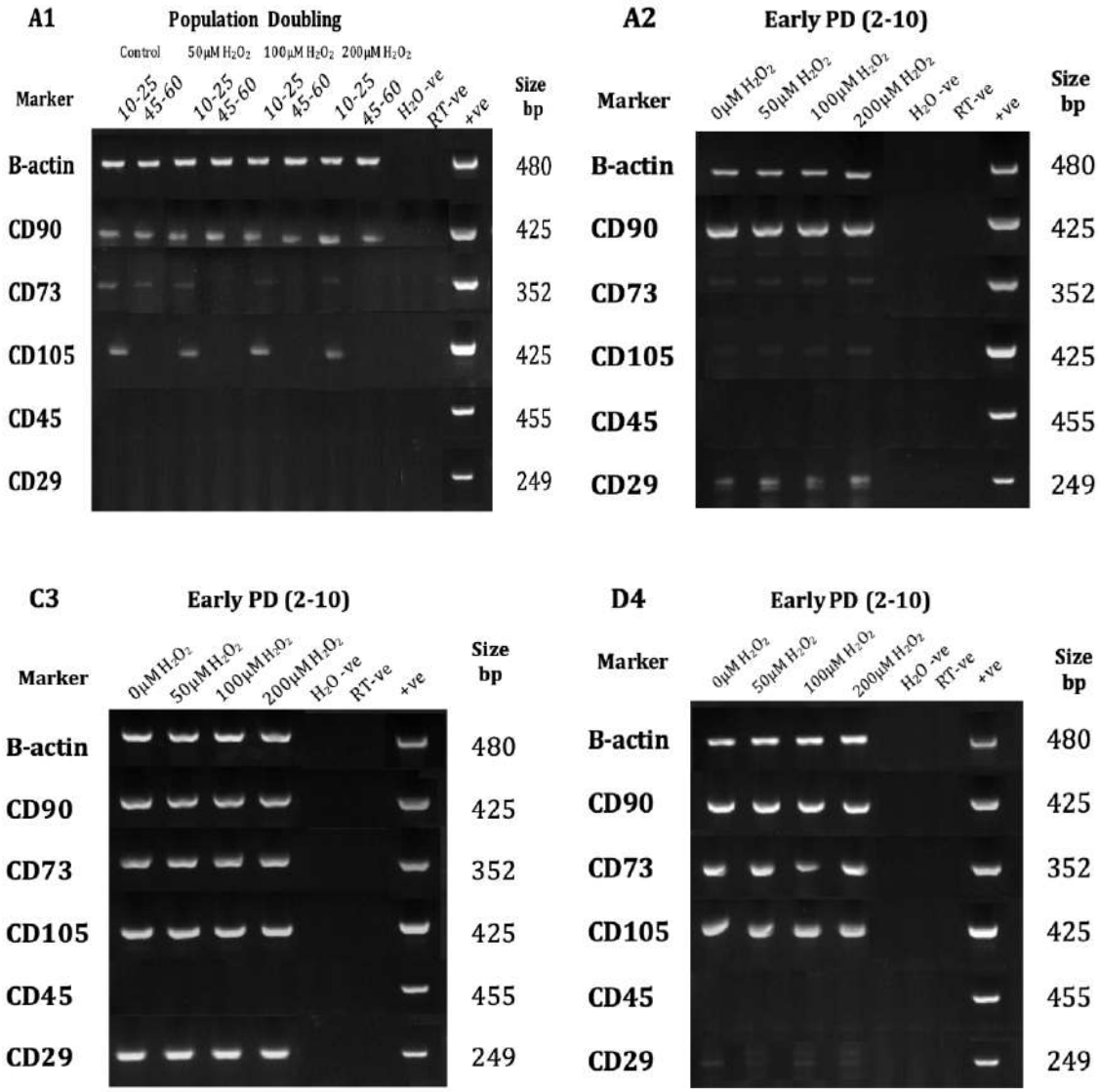
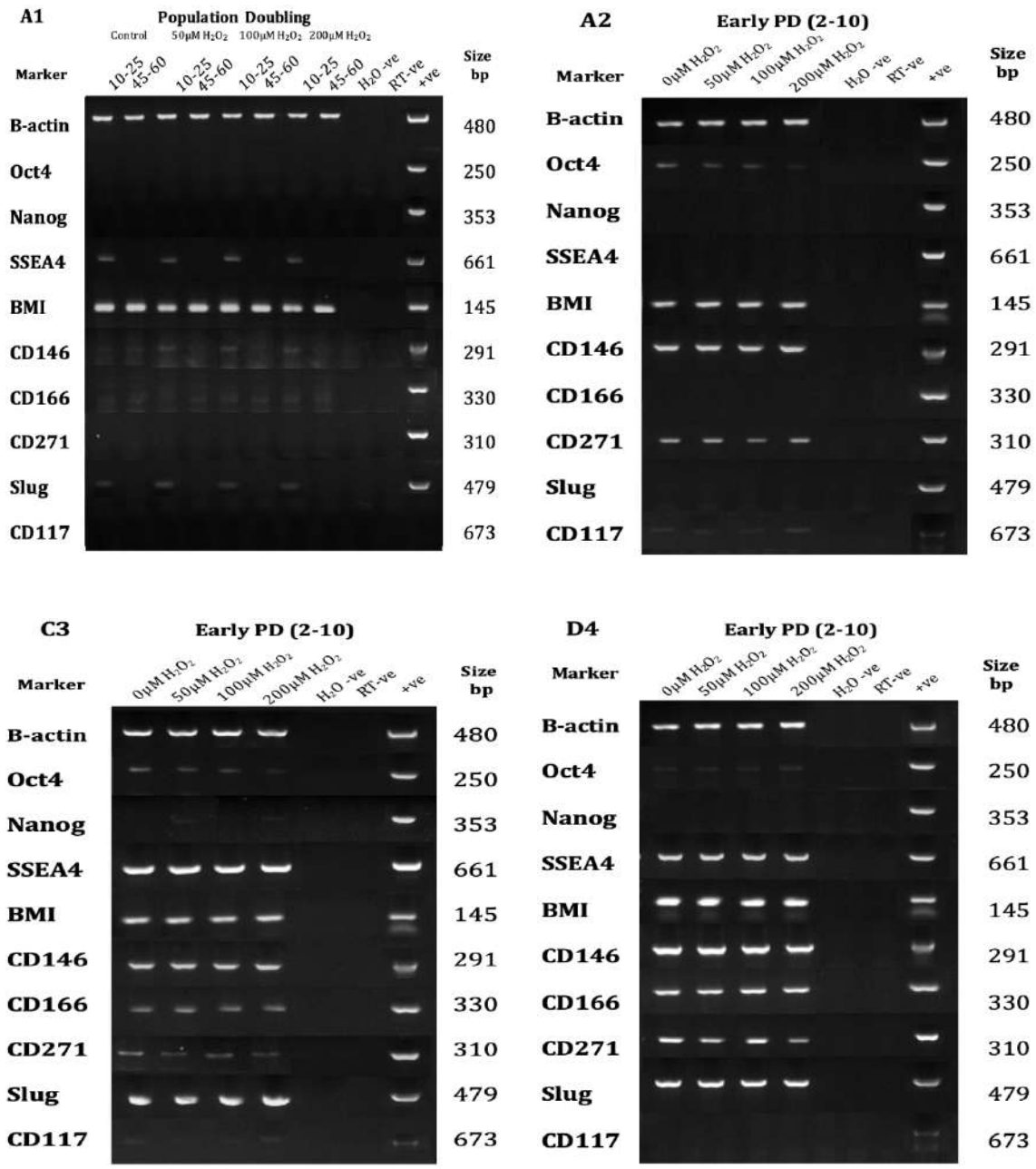


Figure 2.15. Comparison of embryonic and neural crest stem cell marker gene expression by DPSC sub-populations, A1, A2, C3 and D4, following extended culture to senescence under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. β-actin was used as the housekeeping gene. Total human RNA was used as a positive control for all genes analysed. Water and RT-negative experimental controls had no expression.



Overall, expression of these stem cell markers was largely unaffected by increasing H_2O_2 treatment or culture expansion. However, SSEA4 and Slug expression declined at later PDs (45-60PDs) in high proliferative DPSCs, whilst increasing H_2O_2 treatment reduced the expression of Oct4 and CD271 in low proliferative DPSCs (A2 and C3 and D4, respectively).

2.6 Discussion

Previous findings have confirmed major variations in proliferative capacity and replicative (telomere-dependent) senescence within different DPSC sub-populations, as a consequence of inherent differences in average telomere lengths between high and low proliferative DPSCs (Alraies et al., 2017). Therefore, this Chapter examined whether DPSCs also differed in their respective susceptibilities to oxidative stress-induced (telomere-independent) senescence, via the analysis of various parameters associated with the establishment of the senescence phenotype, following continual H_2O_2 treatment in culture.

Animal and human studies have established the effects of increasing patient donor age on the impairment of DPSC proliferative and regenerative capabilities (Ma et al., 2009; Bressan et al., 2012; Feng et al., 2013, 2014; Horibe et al., 2014; Iezzi et al., 2019). However, consistent with only 20% of purified DPSCs undergo >20PDs, compared with heterogeneous DPSC populations being capable of >120PDs, highlighting the presence of DPSC sub-populations in dental pulp tissues with contrasting proliferative and differentiation capabilities (Gronthos et al., 2000, 2002; Huang et al., 2009), recent studies have reported key differences in telomere lengths of individual DPSC sub-populations within a young donor age group (18-30 years) and their correlations to the distinct differentiation capabilities of each population even when DPSCs are isolated from the same pulpal tissue sample (Alraies et al., 2017). Whilst highly proliferative DPSCs reached >80PDs before undergoing telomere-dependent senescence, other low proliferative DPSCs only achieved <40PDs, correlating with DPSCs with high proliferative capacities possessing longer telomeres than those from low proliferative DPSCs. Unlike low proliferative DPSCs, these highly proliferative DPSCs exhibited prolonged stem cell marker expression and retained multipotent differentiation capabilities, which only became impaired at much later PDs (>60PDs). Therefore, it has been proposed that such high proliferative DPSCs are responsible for the extensive expansion potential

of heterogeneous populations (>120PDs) *in vitro* (Gronthos et al., 2000, 2002; Huang et al., 2009), as DPSCs with less proliferative potential are selectively lost from the mixed population during extended sub-culture (Waddington et al., 2009; Harrington et al., 2014; Alraies et al., 2017).

In addition to significant variations in the susceptibilities of DPSC sub-populations to telomere-dependent senescence (Alraies et al., 2017), the data presented in this Chapter confirmed that similar variations also exist in the relative susceptibilities of DPSCs to oxidative stress-induced (telomere-independent) senescence. All DPSC populations examined exhibited accelerated susceptibilities to oxidative stress-induced senescence in a H_2O_2 dose-dependent manner (Xiao et al., 2014), especially at 100µM and 200µM concentrations. However, as with telomere-dependent senescence, highly proliferative DPSCs, such as A1, exhibited the highest resistance to H_2O_2-induced senescence, compared to untreated controls (>80PDs); being capable of achieving 50-76PDs even following continual treatment with 50µM, 100µM and 200µM H_2O_2 over 145-162 days in culture. In contrast, low proliferative DPSCs, such as A2, C3 and D4, showed greater acceleration and the earlier induction of oxidative stress-induced senescence, even in untreated controls. Indeed, DPSC population, A2, was only capable of achieving 27-32PDs with 50-200µM H_2O_2 treatments, compared to untreated controls (34PDs), whilst populations, C3 and D4, only accomplished 12-19PDs and 4-7PDs with 50-200µM H_2O_2 treatments, respectively, versus their untreated counterparts (20PDs and 11PDs, respectively).

Chen and Ames (1994), provided the first experimental evidence to demonstrate the onset of oxidative stress-induced cellular senescence in cells, using sub-lethal doses of H_2O_2. Since then, many studies have confirmed the varying negative effects of H_2O_2 treatments on the viability, proliferation, senescence induction and differentiation capacities of MSCs derived from various sources (Brandl et al., 2011; Denu et al., 2016; Vono et al., 2018). However, it has now been established that MSCs are more resistant to H_2O_2-induced senescence, compared to non-somatic cell types, such as fibroblasts (Brandl et al., 2011; Choo et al., 2014). Consequently, much higher non-lethal H_2O_2 concentrations are routinely utilised to promote oxidative stress-induced senescence in various MSC populations without affecting telomere length, as evident herein (50-200µM); due to its impact on the cell cycle, such as by increasing $p16^{INK4A}$ levels (Chen et al., 2001, de Magalhães et

al., 2002). However, it should be noted that cells may have accelerated responses towards H_2O_2 *in vitro*, due to contrasting effects of endogenously-generated, intracellular H_2O_2 compared to that externally supplemented. When produced intracellularly, it is found to be a constant rate production resulting in a steadily increase in H_2O_2 level, whereas H_2O_2 added to culture medium in a single dose will be rapidly consumed by cellular- and medium-based, antioxidants (Liochev, 2013).

In support of these contrasting PD findings, DPSC sub-population, A1, was further shown to possess fewer SA-β-Gal positive cells and lacked the expression of p53 and $p16^{INK4a}$, at PDs where low proliferative DPSCs, especially C3 and D4, demonstrated overall increases in the detection of these and other senescence-associated markers, such as $p21^{waf1}$, particularly with increasing H_2O_2 concentrations. As with telomere-dependent senescence, senescence mediators, such as p53 and $p16^{INK4a}$, are also associated with oxidative stress-induced, telomere-independent senescence (Campisi and d'Adda di Fagagna, 2007). MSC senescence is acknowledged as a multi-step process driven by p53, which promotes growth arrest by inducing $p21^{waf1}$ expression, thereby inhibiting G_1-S phase progression. p53 and $p21^{waf1}$ act as tumor suppressors to regulate cell proliferation to rates that provide genomic stability during MSC division (Macloud et al., 1995). Therefore, p53 and $p21^{waf1}$ expression regulate MSC expansion in an undifferentiated state. MSC senescence can also initiate $p16^{INK4a}$ checkpoints, suppressing proliferation and triggering senescence. Consequently, as both p53 and $p16^{INK4a}$ are both regarded as the principal mediators of senescence in MSCs (Shibata et al., 2007; Muthna et al., 2010; Mehrazarin et al., 2011; Feng et al., 2014); and as $p21^{waf1}$ also has a role in maintaining stem cell renewal due to its positive effects on cell cycle progression (Kippin et al., 2005; Ju et al., 2007); this may explain the presence of early $p21^{waf1}$ expression in all DPSC sub-populations analysed. Nonetheless, the contrasting p53 and $p16^{INK4a}$ expression between high (A1) and low (A2, C2 and D4) proliferative capacity DPSCs, further confirms the earlier onset of oxidative stress-induced senescence with low proliferative DPSC sub-populations, versus high proliferative DPSCs at equivalent PDs.

The principle reason previously identified to be responsible for the contrasting proliferative responses and susceptibilities to telomere-dependent senescence in DPSC sub-populations, were the respective average telomere lengths between these populations (>18kb versus 5-13kb for high and low proliferative

DPSCs respectively, Alraies et al., 2017) with such superior telomere dynamic characteristics in high proliferative DPSCs sub-populations permitting extended PDs in culture, whilst protecting these DPSC from the onset of replicative senescence. However, in line with previous findings that oxidative stress-induced senescence can occur irrespective to telomere length and without telomere shortening (Fumagalli et al., 2012; Bielak-Zmijewska et al., 2014, 2018), all DPSCs analysed generally retained their average telomere length profiles during extended culture, both in the absence and presence of oxidative stress. Intriguingly, the DPSC sub-population whose telomere lengths were mostly influenced by extended culture and H_2O_2 treatment, was the high proliferative DPSC, A1. As such, this may imply that A1 may have also undergone telomere-dependent senescence mechanisms to some extent during extended culture. Alternatively, it has been proposed that prolonged MSC culture under low chronic stress or mild/short-term sub-lethal H_2O_2 doses, can promote the gradual shortening of telomeres due to telomeric oxidative damage and single strand breaks leading to accelerated telomere shortening (von Zglinicki et al., 1995, 2000, 2002, Duan et al., 2005; Brandl et al., 2011). Although we can only speculate as to the extent to which telomere-dependent and telomere-independent mechanisms contributed to senescence induction in DPSC sub-population, A1, based on the overall telomere erosion identified for A1 was limited to 2-5kb over 75-100 days in culture (i.e. between 10-25PDs and 45-60PDs), oxidative stress-induced, telomere-independent senescence is likely to be the principle senescence mechanism involved during A1 culture.

Although cellular senescence can be telomere-initiated reflecting DNA damage, resulting in shorter and dysfunctional telomeres (di Fagagna et al., 2003; Brandl et al., 2011), many *in vitro* studies are now challenging this concept, instead reporting that although telomere shortening may be absent, random DNA damage occurs throughout each chromosome, which initiates the senescence response. Although this damage is repairable throughout most of the chromosomal region, telomeric regions damage are difficult to repair (Fumagalli et al., 2012). Consequently, although it was suggested that telomere-independent senescence was directly mediated by oxidative damage (Parrinello et al., 2003), more recent studies argue that telomeric DNA damage/telomere-associated foci are more responsible, as these have been shown to increase with age regardless of telomere length, eventually causing senescence (Hewitt et al., 2012, Jurk et al., 2014).

In agreement with the majority of previous studies (Egbuniwe et al., 2011; Mehrazarin et al., 2011a; Murakami et al., 2013; Alraies et al., 2017), no or negligible hTERT expression was identified in the high and low proliferative DPSC sub-populations assessed herein. While hTERT expression is often an indication of the cell pluri-/multi-potency, down-regulation result in progressive telomere shortening and cellular senescence (Zimmermann et al., 2003, Flores and Blasco, 2010). Such findings imply that hTERT expression and activity appears not to be responsible for the maintenance of telomere lengths in high proliferative DPSCs or the variations in proliferative capabilities. Therefore, other intrinsic telomere protective mechanisms may be responsible for these contrasting differences in telomere length, proliferation rates and regenerative potential between high and low proliferative DPSCs, such as differential oxidative stress responses and antioxidant profiles between DPSC populations (Liu et al., 2011; Shyh-Chang et al., 2013; Li et al., 2017).

This Chapter also evaluated the expression of various mesenchymal, hematopoietic, embryonic and neural crest stem cell markers in high and low proliferative DPSC sub-populations and assessed the impact of oxidative stress-induced senescence on their overall stem cell characteristics. In accordance with previous studies, all DPSC sub-populations demonstrated positive expression of MSC markers, CD73 (ecto-5-nucleotidase), CD90 (Thy-1) and CD105 (endoglin) and negative for haematopoietic stem cell marker, CD45 (Huang et al., 2009; Waddington et al., 2009; Kawashima, 2012; Harrington et al., 2014; Alraies et al., 2017). However, MSC marker, CD29 (integrin β_1), expression was only evident to varying degrees in low proliferative DPSCs (Pittenger et al., 1999). Furthermore, although CD90 expression was largely retained in high and low proliferative DPSCs through culture expansion and irrespective of H_2O_2 treatment, CD73 and CD105 expression showed declined detection at later PDs, as previously described (Alraies et al., 2017), whilst reductions in CD73 and CD105 expression were less apparent for low proliferative DPSCs, in a H_2O_2-dependent and -independent manner, respectively. Therefore, the loss of these MSC marker expression by DPSC sub-population, A1, with prolonged culture expansion in the absence or presence of H_2O_2 treatment, may result in altered differentiation capabilities, as previously described (Rallapalli et al., 2009; Sivasankar and Ranganathan, 2015; Moraes et al., 2016; Alraies et al., 2017).

Overall, all sub-populations analysed were confirmed as being DPSCs, based on positive MSC marker (CD73, CD90 and CD105) expression and absence of haematopoietic marker (CD45) expression. However, despite displaying expression of established MSC markers, DPSC sub-populations presented more diverse expression for other stem cell markers analysed, a likely consequence of DPSC heterogeneity, with each sub-population having contrasting phenotypic properties and/or biological activities (Gronthos et al., 2000, 2002; Huang et al., 2009; Ducret et al., 2016; Ledesma-Martínez et al., 2016). Indeed, this is a key consideration, in terms of whether the contrasting senescence properties between high and low proliferative DPSC sub-populations reflect their isolation from different mesodermal or neuro-ectodermal origins or stem cell niches within the dental pulp (Sloan and Waddington, 2009). Other studies have reported the presence of various embryonic stem cell marker gene expression profiles for DPSCs, including Oct4, Nanog, SSEA4, CD146 and CD166 (Aghajani et al., 2016, Akpinar et al., 2014, Shoi et al., 2014), in addition to neural crest markers, such as CD271 and Slug (LaBonne and Bronner-Fraser, 2000; Martens et al., 2012; Sonoda et al., 2015). All DPSC sub-populations showed strong positive gene expression for self-renewal and multipotent adult stem cell marker, BMI-1, at early PDs. Loss of BMI-1 expression has been proposed to be a marker of impaired odontogenic differentiation capacity in senescent DPSCs (Mehrazarin et al., 2011). Therefore BMI-1 may be a factor in maintaining the differentiation and regenerative abilities of dental mesenchymal cells.

The established MSC multi-lineage differentiation marker, CD146 (Xu et al., 2008; Russell et al., 2010) and multipotent stem cell marker (CD271), were both positively expressed in all low proliferative DPSCs only. CD146 presents an early expressed MSC marker within the dental pulp, being linked to MSC multipotency (Miura et al., 2003; Russell et al., 2010). CD146 is also regarded as a pericyte marker, indicating the presence of DPSCs from the perivascular niche within the cell rich zone (Shi and Gronthos 2003; Lizier et al., 2012). Therefore, despite CD146 expression in suggested to decline during DPSC expansion (Sivasankar and Ranganathan, 2015), CD146 positive expression in all low proliferative DPSC sub-populations examined, may indicate that these DPSCs are of perivascular origin. The absence of CD271 (nerve growth factor receptor p75, LNGFR), expression in high proliferative DPSCs has been reported previously (Alraies et al., 2017) and may

suggest that such populations are not neural crest- or sub-odontoblast layer-derived, as $CD271^{+}$ cells are regarded as being of neural crest origin, located within the cell-rich, sub-odontoblast region of dental pulp (Martens et al., 2012; Alvarez et al., 2015). Studies have also reported that $CD271^{low+}/CD90^{high+}$ DPSCs population present a highly enriched clonogenic population and exhibit log-term proliferation and multi-lineage differentiation potential *in vitro* (Mikami et al., 2011; Yasui et al., 2016), although not all studies have demonstrated complete inhibition of multipotent differentiation in CD271-expressing DPSCs (Alvarez et al., 2014; Pisciotta et al., 2015). Nonetheless, positive CD271 expression in low proliferative capacity DPSCs (A2, C3 and D4), may provide further support to these cells being neural crest-derived and more lineage restricted than high proliferative capacity DPSCs, such as A1. Stem cell differentiation regulator, CD166 (Sloan and Waddington, 2009; Kang et al., 2016), was negligibly expressed in high proliferative DPSCs, although low proliferative DPSC sub-populations, C3 and D4, expressed strong CD166 expression.

In terms of embryonic stem cell markers, Oct4, was absent in high proliferative DPSCs, but expressed by all low proliferative DPSCs analysed. In contrast, other embryonic stem cell markers assessed, SSEA4 and Slug, were positively expressed in high proliferative DPSCs and low proliferative DPSCs, C3 and D4, but both absent from low proliferative DPSC, A2. No or negligible expression of the pluripotent stem cell (Nanog) was found in all DPSCs examined. Oct4, SSEA4 and Nanog are embryonic stem cell markers that indicate pluripotency (Huang et al., 2009; Ishkitiev et al., 2010, Kawanabe et al., 2012; Ponnaiyan, 2014). Previous study have shown weak Oct4 and Slug expression in high and low proliferative DPSC sub-populations (Alraies et al., 2017). Oct4 and Nanog are known to play key role in maintaining the process of self-renewal and pluripotency in embryonic stem cells (Wang et al., 2006). Both Oct4 and Nanog are also reported to maintain the MSC characteristics of DPSCs (Lizier et al., 2012), with depletion of Oct4/Nanog expression decreasing DPSC proliferation and osteogenic differentiation properties, whilst Oct4/Nanog overexpression enhances proliferation and multipotent differentiation properties (Huang et al., 2014). Despite such findings, Oct4 function in MSCs has been subject of debate and although it is essential for maintaining pluripotency, it may also have other function (Ulloa-Montoya et al., 2007; Lengner et al., 2008).

SSEA-4 is an embryonic stem cell-associated antigen, suggested to regulate stem cell differentiation (Gang et al., 2007). Kawanabe et al. (2012), found that SSEA-4+ DPSCs represent 45% of the total dental pulp cells. SSEA-4+ DPSCs were further shown to be clonogenic and to possess with multi-lineage (osteogenic, chondrogenic and neurogenic) differentiation capacities *in vitro*. Embryonic/neural crest marker, Slug, is required for neural crest migration and specification and implicated in promoting commitment to a mesenchymal lineage (LaBonne and Bronner-Fraser, 2000; Batlle et al., 2013). No or negligible expression of the neural crest marker, CD117 (c-kit), was found in all DPSCs examine (Miettinen and Lasota, 2005). CD117+ cells have been reported in both the cell-rich zone and the dental pulpal core. However, while CD117+/CD44- cells in the cell-rich zone may indicate stemness, CD117+/CD44+ stromal cells in the pulpal core may indicate undifferentiated perivascular or perineural cells (Rusu et al., 2014). Furthermore, cells in this layer have a role participating in stromal network of the dental pulp and contact nerves (Rusu et al., 2014). Consequently, this may indicate a possibility of functional role of DPSCs in signalling and neurotransmission within dental pulp.

Overall, expression of these stem cell markers was largely unaffected by increasing H_2O_2 treatment or culture expansion. However, SSEA4 and Slug expression declined at later PDs (45-60PDs) in high proliferative DPSCs, whilst increasing H_2O_2 treatment reduced the expression of Oct4 and CD271 in low proliferative DPSCs (A2 and C3 and D4, respectively). Oct4 expression has been previously highlighted as being susceptible to extended *in vitro* cell expansion (Takahashi and Yamanaka, 2006; Boroujeni et al., 2012; Tsai et al., 2012), in addition to increased DPSC exposure to oxidative stress and senescence induction in culture (Forristal et al., 2013; Mas-Bargues et al., 2017). Intriguingly, despite BMI-1 expression also being proposed to be sensitive to oxidative stress and senescence, due to its role in repressing $p16^{INK4a}$ expression and maintaining cellular hyper-proliferation (Itahana et al., 2003; Park et al., 2004; Mas-Bargues et al., 2017), no obvious differences in BMI-1 expression were identified between the high and low proliferative DPSCs assessed herein, despite the apparent variations identified in their overall susceptibilities to oxidative stress-induced senescence.

Therefore, as with the previously reported scenario with DPSC susceptibility to telomere-dependent senescence (Alraies et al., 2017), this Chapter has confirmed that significant variations also exist in the susceptibilities of DPSCs to oxidative

stress-induced (telomere-independent) senescence. As DPSCs exhibit negligible hTERT expression, this suggests that other intrinsic mechanisms help protect high proliferative DPSCs from oxidative stress and cellular senescence. As oxidative stress-induced senescence is well-established to be accompanied by an accumulation of oxidative stress-related damage to cellular biomolecules, including DNA, proteins and lipids (Beckman and Ames, 1998), further investigations assessed oxidative damage responses in these high and low DPSC sub-populations, to further explain the senescence findings presented in this Chapter.

Chapter 3

Assessment of Dental Pulp Stem Cell Susceptibility to Oxidative Stress-Induced Biomarker Formation

Chapter 3

Assessment of Dental Pulp Stem Cell Susceptibility to Oxidative Stress-Induced Biomarker Formation

3.1 Introduction

The previous Chapter confirmed that distinct differences exist in the relative susceptibilities of dental pulp stem cell (DPSC) sub-populations to oxidative stress-induced (telomere-independent) senescence. While both high and low proliferative DPSC populations exhibited accelerated susceptibilities to oxidative stress-induced senescence in a hydrogen peroxide (H_2O_2) dose-dependent manner, low proliferative DPSC sub-populations demonstrated a markedly earlier onset of oxidative stress-induced senescence, compared to high proliferative DPSCs.

Oxidative stress is a well-established promoter of telomere-independent cellular senescence, also known as premature senescence or stress-induced premature senescence, SIPS (Campisi and d'Adda di Fagagna F, 2007), which refers to the balance between the production of intracellular reactive oxygen species (ROS), such as superoxide and hydroxyl radical species ($O_2^{\cdot -}$ and $^{\cdot}OH$, respectively) and non-radical species, such as hydrogen peroxide (H_2O_2) and cellular antioxidant defence mechanisms. Thus, elevated ROS levels at the expense of antioxidant levels accelerate cellular senescence via telomere-independent mechanisms (Ben-Porath and Weinberg, 2004; Victorelli and Passos, 2017). However, exposure to oxidative stress can also promote biomolecular damage, due to continuous production of ROS that readily interact with cellular macromolecules, causing accumulative damage to cellular components (Comporti, 1989; Chaudhari et al., 2014). Although DNA is a strong candidate of oxidative modification and damage (Cooke et al., 2003; Evans et al., 2004), proteins (Halliwell, 1999; Levine and Stadtman, 2001) and lipids (Halliwell, 1999; Spiteller, 2001), are also highly susceptible to oxidative stress-induced, damage. The resulting damage to these components was found to ultimately promote cell senescence, causing several pathologies that impair tissue function (Sikora et al., 2011).

Several pathological conditions can occur as a consequence of oxidative damage to DNA. For this reason, oxidative DNA damage has been widely studied in various cell lines. For instance, fibroblasts exhibit higher levels of 8-hydroxy-deoxy-guanosine (8-OHdG) in senescent cells than in young cells, indicating increased oxidative DNA damage (Chen et al., 1995). Notably, oxidative DNA damage results in different types of DNA lesions depending on the ROS involved, the duration of exposure and the cellular reparative ability (Halliwell and Chirico, 1993). ROS interactions with DNA are mainly attributed to ·OH species causing DNA base modification, deoxyribose sugar damage, formation of DNA protein cross-links, single and double strand breaks and the formation of apurinic lesions, many of which are mutagenic (Shibutani et al., 1991; Finkel and Holbrook, 2000). DNA bases are particularly vulnerable to oxidation by ROS, with guanine base having the lowest reduction/oxidation potential of the four bases (Neeley and Essigmann, 2006). Consequently, ·OH species induce DNA damage at the C-8 position of guanine base yielding 8-OHdG as an oxidation product, which has led to 8-OHdG becoming a well-established marker of *in vitro* and *in vivo* oxidative DNA damage (Toussaint et al., 2000; Tahara et al., 2001; David et al., 2007). Nonetheless, ROS can also attack other positions in the structures of guanine or other purine/pyrimidine DNA bases, yielding a wide range of oxidative DNA base damage products (Cooke et al., 2003; Evans et al., 2004). In contrast to ·OH species, less reactive species, such as $O_2^{\cdot -}$ and H_2O_2, cause indirect DNA damage by acting as a source for other ROS, such as ·OH species (Halliwell, 1999; Poulsen et al., 2000; Ames, 2001; Kasprzak, 2002). Indeed, DNA oxidation is a complex process and many studies have reported that ROS can promote site-specific damage (Enright et al., 1996). Accordingly, the cellular repair responses towards ROS-induced DNA lesions are also complex, as different DNA repair mechanisms may exist depending on the source and extent of oxidative damage induced (Giorgio et al., 2007; Hazane-Puch et al., 2010).

Previous research has recognized a strong association between protein oxidation and oxidative stress, senescence and aging, with numerous studies utilising oxidative protein damage as a marker of oxidative stress in research on many different pathological conditions (Flint Beal, 2002), such as diabetes (Almogbel and Rasheed, 2017), Alzheimer's disease (Greilberger et al., 2010) and arthritis (Pullaiah et al., 2018). Protein oxidation results from ROS accumulation (Berlett and

Stadtman, 1997). Upon oxidation, protein damage is presented as modifications to amino acid structure leading to fragmentation to lower molecular weight products. ROS modification of amino acids results in the formation of numerous oxidized by-products, including oxo acids, aldehydes, hydroperoxides and hydroxylated derivatives, due to a range of chemical reactions, including the hydroxylation of amino acid side chains, decarboxylation and deamination reactions. Studies have highlighted a number of amino acids that are more susceptible to modification by ROS, including proline, histidine, lysine, arginine and tyrosine, resulting in formation of chemically stable moieties (Dean et al, 1997; Dalle-Donne et al., 2003). Additionally, carbonyl derivatives can be produced indirectly on the nucleophilic side chains of cysteine, histidine and lysine by secondary reaction with aldehydes produced during lipid peroxidation, or with reactive carbonyl derivatives generated during glycation and glycoxidation reactions (Berlett and Stadtman, 1997; Halliwell, 1999; Uchida, 2000). Indeed, the increased presence of protein carbonyl groups as a consequence of oxidative stress exposure and amino acid side chain modifications, is the most widely used biomarker to implicate oxidative protein damage in the aetiology and/or progression of various diseases and conditions (Dalle-Donne et al., 2003), and many studies have documented age-associated accumulation of oxidized proteins in fibroblasts (Sitte et al., 2000; Grune et al, 2005; Torres and Perez, 2008; Baraibar et al., 2012; Rodríguez-Sureda et al., 2015).

The phospholipid constituents of cell membranes are also liable to oxidative damage due to their high unsaturated fatty acid contents, resulting in lipid peroxidation (Halliwell, 1999; Spiteller, 2001; Kohen and Nyska, 2002). Although lipid peroxidation is a normal physiological process necessary for renovation of biological membranes, excessive activation is linked to many pathologies. Lipid peroxidation of membranes affects several aspects of cellular machinery, such as the loss of membrane fluidity and integrity leading to impaired cellular function (Halliwell, 1999). The end products formed, such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and the prostaglandin-like products, isoprostanes, are now commonly used as markers of oxidative lipid peroxidation (Halliwell and Chirico, 1993; Spiteller, 2001; Kohen and Nyska, 2002).

3.2 Chapter Aims

The previous Chapter confirmed significant variations in oxidative stress-induced (telomere-independent) senescence susceptibilities between DPSC sub-populations. However, as oxidative stress-induced senescence is commonly associated with the increased induction of cellular oxidative biomolecular damage, this Chapter examined whether high and low proliferative DPSC sub-populations also differed in their respective susceptibilities to oxidative stress-induced biomarker formation via the analysis of established oxidative stress marker levels of DNA (8-OHdG), protein (carbonyl content) and lipid (peroxidation) damage. By confirming such differences between high and low proliferative DPSCs, this would provide further evidence to support high proliferative DPSCs being more resistant to SIPS, compared to low proliferative DPSCs, thereby enhancing our understanding of DPSC biology and its inter-relationship between oxidative stress and cellular ageing.

3.3 Materials and Methods

3.3.1 Isolation, Culture and Expansion of Dental Pulp Stem Cells Under Oxidative Stress Conditions

Human DPSCs were isolated, cultured and expanded in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, ThermoFisher Scientific, Paisley, UK), as previously described (Chapters 2.3.1, 2.3.2 and 2.3.3).

3.3.2 Oxidative DNA Damage Detection

Oxidative DNA damage (in the form of 8-OHdG levels), in high and low proliferative DPSC sub-populations in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), was detected by immunohistochemistry using an OxyDNA Assay Kit (Merck Millipore, Watford, UK). This is a fluorescence-based Kit, based on direct binding of a fluorescent probe to 8-OHdG in the DNA of fixed permeabilised cells. 8-OHdG detection was performed, as per manufacturer's instructions.

DPSCs at selected population doublings (PDs) throughout their proliferative life-spans, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, Chapter 2), were seeded into 8-well chamber slides (VWR International Ltd., Lutterworth, UK), at 5,000 cells/cm^2 (n=3 wells/experimental group) and maintained at 37ºC/5% CO_2 overnight. Following culture medium

removal, DPSCs were washed (x1) at room temperature in phosphate buffered saline (PBS, 300µl/well) and fixed in 4% paraformaldehyde solution in PBS (150µl/well, Santa Cruz Biotechnology, Dallas, USA), for 10min and re-washed (x1) in PBS (300µl/well). Subsequently, cells were permeabilised in 0.1% Triton X-100 (250µl/well, Sigma-Aldrich Company Ltd.) for 5min and washed (x1) in Wash Solution (in Kit). To visualise oxidized DNA, DPSCs were incubated in fluorescein isothiocyanate (FITC)-Conjugate (100µl/well, in Kit), for 1h at room temperature under darkness, prior to washing (3min x5) at room temperature under agitation in Wash Solution (300µl/well) and rinsing (x1) in double-distilled water (500µl/well). DPSCs were then incubated in Hoechst Solution (300µl/well, 1:2000 in 0.1% bovine serum albumin-PBS, Sigma-Aldrich Company Ltd.), for 5min at room temperature under darkness. DPSCs were subsequently washed (x2) in double-distilled water (300µl/well) and the chambers removed. Slides were mounted with Gerhard Menzel coverslips (Thermo Fisher Scientific) using Fluor Save Reagent (Merck Millipore), for 10min under darkness. Slides were viewed using Leica Dialux 20 Fluorescent Microscope (Leica Microsystems UK Ltd., Milton Keynes, UK). Images were captured at a magnification of x200 using HCImage acquisition and analysis software (Hamamatsu Corporation, Sewickley, USA) and processed using Adobe Photoshop software (Adobe Systems, San Jose, USA). Oxidative DNA damage was demonstrated as increased DNA green fluorescence intensities in the cytoplasmic (mitochondrial DNA) and nuclear DNA regions, due to bound FITC-Conjugate. Control wells were also included consisting of PBS, instead of FITC-Conjugate. Each experiment performed on n=3 separate occasions.

3.3.3 Oxidative Protein Damage Detection

Oxidative protein damage (in the form of protein carbonyl content), in high and low proliferative DPSC sub-populations in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), was detected by immunohistochemistry using an OxyICC™ Oxidized Protein Detection Kit (Merck Millipore). This is a fluorescence-based Kit, based on the chemical derivatisation of protein carbonyl groups using 2,4-dinitrophenylhydrazine (DNPH), which can then be detected by a specific biotinylated anti-DNPH primary antibody and

tetramethylrhodamine (TRITC)-conjugated streptavidin secondary antibody. Protein carbonyl group detection was performed, as per manufacturer's instructions.

DPSCs at selected PDs throughout their proliferative life-spans, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, Chapter 2), were seeded into 8-well chamber slides at 5,000 cells/cm^2 (n=3 wells/experimental group) and maintained at 37ºC/5% CO_2 overnight. Following culture medium removal, DPSCs were washed (x2) at room temperature in PBS (300µl/well) and fixed in 100% cold methanol (300µl/well, ThermoFisher Scientific), for 5min and re-washed (x3) in PBS (300µl/well). Subsequently, freshly diluted DNPH solution was added (300µl/well) and DPSCs incubated for 45min at room temperature, under darkness. DPSCs were washed (x4) in PBS (300µl/well), blocked with Blocking Buffer (300µl/well, in Kit) and incubated overnight at 4°C. Cells were washed (x3) in PBS (300µl/well) and incubated in streptavidin (300µl/well, pre-diluted 1:1000 in Block Buffer, in Kit), for 1h at 37ºC. DPSCs were washed (x3) in PBS (300µl/well) and incubated in Hoechst Solution (300µl/well, 1:2000 in 0.1% bovine serum albumin-PBS), for 5min at room temperature under darkness. The chambers were removed, and slides mounted with Gerhard Menzel coverslips using Fluor Save Reagent, for 10min under darkness. Slides were viewed using Leica Dialux 20 Fluorescent Microscope, with images captured at a magnification of x200 using HCImage acquisition and analysis software and processed using Adobe Photoshop software. Oxidative protein damage was demonstrated as the increase in the red fluorescence intensity of the rhodamine conjugate. Control wells were also included consisting of PBS instead of anti-DNPH primary antibody. Each experiment performed on n=3 separate occasions.

3.3.4 Oxidative Lipid Damage Detection

Oxidative lipid damage (in the form of lipid peroxidation), in high and low proliferative DPSC sub-populations in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), was detected by immunohistochemistry using an Image-iT® Lipid Peroxidation Kit (ThermoFisher Scientific). This is a fluorescence-based Kit, which allows the detection of lipid peroxidation in live cells through the oxidation of boron dipyrromethene (BODIPY®) 581/591 C11 reagent. BODIPY® 581/591 C11 reagent is an oxidation sensitive fatty acid which localises to the membranes of live cells and upon oxidation

by lipid hydroperoxides (Drummen et al., 2002), displays a shift in peak fluorescence emission of its phenylbutadiene segment from ~590nm to ~510nm. This fluorescence shift from red to green (Pap et al., 1999), providing a ratiometric indication of lipid peroxidation by fluorescence microscopy analysis. Lipid peroxidation detection was performed, as per manufacturer's instructions.

DPSCs at selected PDs throughout their proliferative life-spans, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, Chapter 2), were seeded into 8-well chamber slides at 5,000 cells/cm^2 (n=3 wells/experimental group) and maintained at 37ºC/5% CO_2 overnight. Culture medium was removed and replaced with fresh culture medium, containing Image-iT® Lipid Peroxidation Sensor (10µM, 300µl/well, Component A, in Kit) and incubated at 37°C for 30min. Following culture medium removal, DPSCs were washed (x3) at room temperature in PBS (300µl/well) and incubated in Hoechst Solution (300µl/well, 1:2000 in 0.1% bovine serum albumin-PBS), for 5min at room temperature under darkness. The chambers were removed, and slides mounted with Gerhard Menzel coverslips using Fluor Save Reagent, for 10min under darkness. Slides were viewed using Leica Dialux 20 Fluorescent Microscope. Slides were viewed at two separate wavelengths, one at excitation/emission 581/591nm (Texas Red filter) for the detection of reduced BODIPY® 581/591 C11 reagent and the other at excitation/emission of 488/510nm (FITC filter) for the oxidized BODIPY® 581/591 C11 reagent. Images were captured at a magnification of x200 using HCImage acquisition and analysis software and processed using Adobe Photoshop software. Oxidative lipid damage was demonstrated as the increase in the green fluorescence intensity due to FITC detection. Control wells were also included consisting of PBS instead of Image-iT® Lipid Peroxidation Sensor. Each experiment performed on n=3 separate occasions.

3.4 Results

3.4.1 Oxidative DNA Damage in Dental Pulp Stem Cells Under Oxidative Stress

A number of different DPSC sub-populations previously isolated and characterised from 3 individual patient donors (patients A, C and D), for oxidative stress-induced senescence susceptibilities (Chapter 2), were also assessed for their susceptibilities to oxidative DNA damage (8-OHdG detection) throughout their proliferative life-spans, following treatment with increasing H_2O_2 concentrations (0, 50µM, 100µM,

200μM). These included previously identified high proliferative (highly resistant to oxidative stress-induced senescence) sub-population, A1 and low proliferative (highly susceptible to oxidative stress-induced senescence) sub-populations, A2, C3 and D4. Overall, oxidative DNA detection showed marked variations in the susceptibilities to oxidative stress-induced damage between high and low proliferative DPSCs, with each demonstrating differences in the extent of positive nuclear DNA fluorescent staining detected, irrespective of whether DPSCs were derived from the same or different patients.

Highly proliferative DPSC population (A1, Patient A) at early PDs (2-10PDs), exhibited the least amounts of FITC detection for oxidative DNA damage overall, especially in untreated and 50μM H_2O_2-treated cultures (Figure 3.1, A-C and D-F, respectively). Although low intensity background staining was evident in the cellular cytoplasmic regions due to increased cell permeability and mitochondrial DNA detection, limited intense positive staining was evident in the nuclear DNA regions of these cultures. However, increased detection of nuclear DNA fluorescent staining intensities were identified for A1 at early PDs (2-10PDs), treated with 100μM and 200μM H_2O_2 (*arrowed*, Figure 3.1, G-I and J-L, respectively). It was also apparent that there was a strong co-localisation between the oxidative DNA damage (FITC) staining and the Hoechst nuclear stain, thereby further confirming that the oxidative DNA damage detected was particularly exclusive to the nuclei of A1 at early PDs (2-10PDs), treated with 100μM and 200μM H_2O_2.

In contrast, low proliferative DPSC sub-populations (A2, Patient A, C3, patient C and D4, patient D) at early PDs (2-10PDs), all exhibited enhanced susceptibilities to increased nuclear oxidative DNA damage, even in untreated controls, with increased detection of cytoplasmic and nuclear FITC staining, with nuclear staining further showing strong co-localisation with the corresponding Hoechst nuclear stain (*arrowed*, Figures 3.2, 3.3 and 3.4 for A2, C3 and D4, respectively). Highly proliferative DPSC population, A1, only exhibited similar cytoplasmic and nuclear FITC staining intensities and oxidative DNA damage detection to low proliferative DPSC sub-populations, at much later PDs (45-60PDs), both in untreated and H_2O_2-treated cultures (*arrowed*, Figure 3.5). FITC-Conjugate-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative DNA damage detection (image P, Figures 3.1-3.5, respectively).

Figure 3.1. Immunocytochemical detection of oxidative DNA damage in DPSC (A1, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A1 demonstrated low intensity, positive FITC staining in the cytoplasmic and nuclear regions of untreated and 50µM H_2O_2-treated cultures, although increased detection of nuclear DNA fluorescent staining was identified in cultures treated with 100µM and 200µM H_2O_2 (*arrowed*). Oxidative DNA damage (FITC) staining also showed a strong co-localisation with the Hoechst nuclear stain, confirming that oxidative DNA damage was particularly exclusive to the cell nuclei. FITC-Conjugate-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative DNA damage detection (image P). Scale bar 100µm, ×200 magnification.

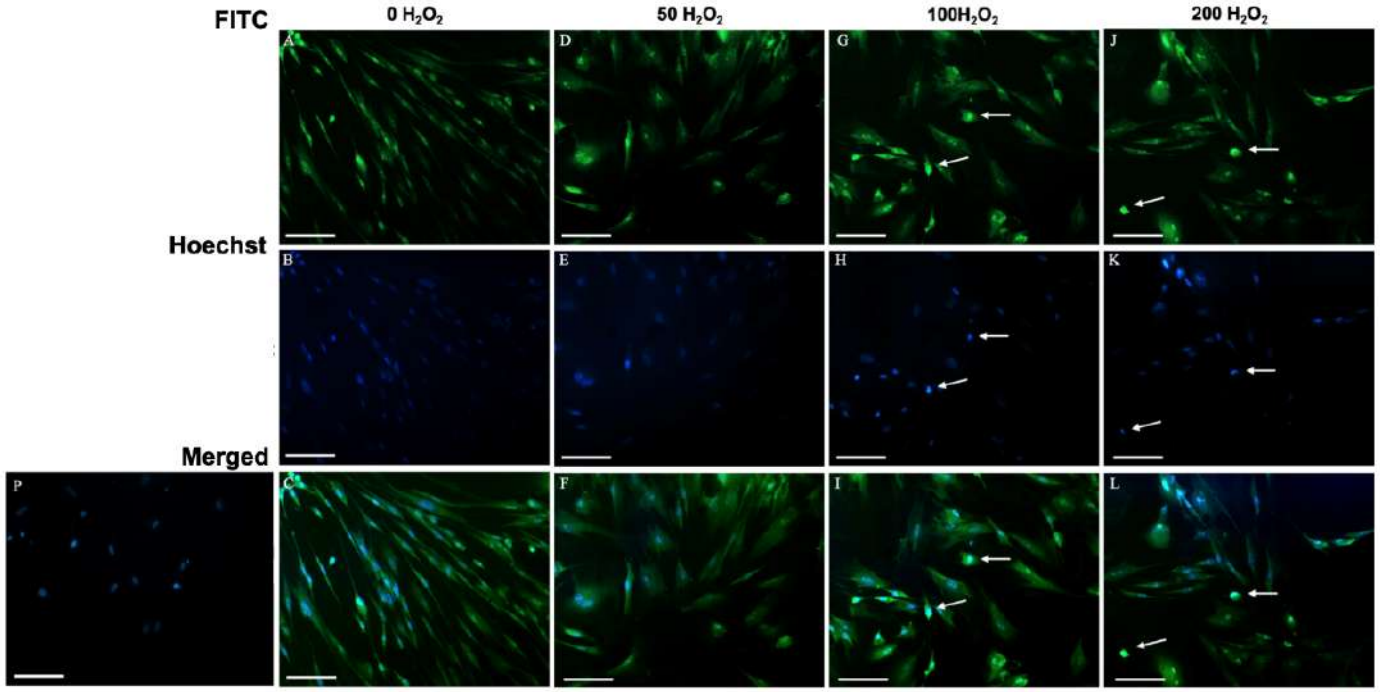


Figure 3.2. Immunocytochemical detection of oxidative DNA damage in DPSC (A2, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A2 demonstrated increased nuclear oxidative DNA damage, even in untreated controls, with increased detection of cytoplasmic and nuclear fluorescent staining (*arrowed*). Oxidative DNA damage (FITC) staining also showed a strong co-localisation with the Hoechst nuclear stain, confirming that oxidative DNA damage was particularly exclusive to the cell nuclei. FITC-Conjugate-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative DNA damage detection (image P). Scale bar 100µm, ×200 magnification.

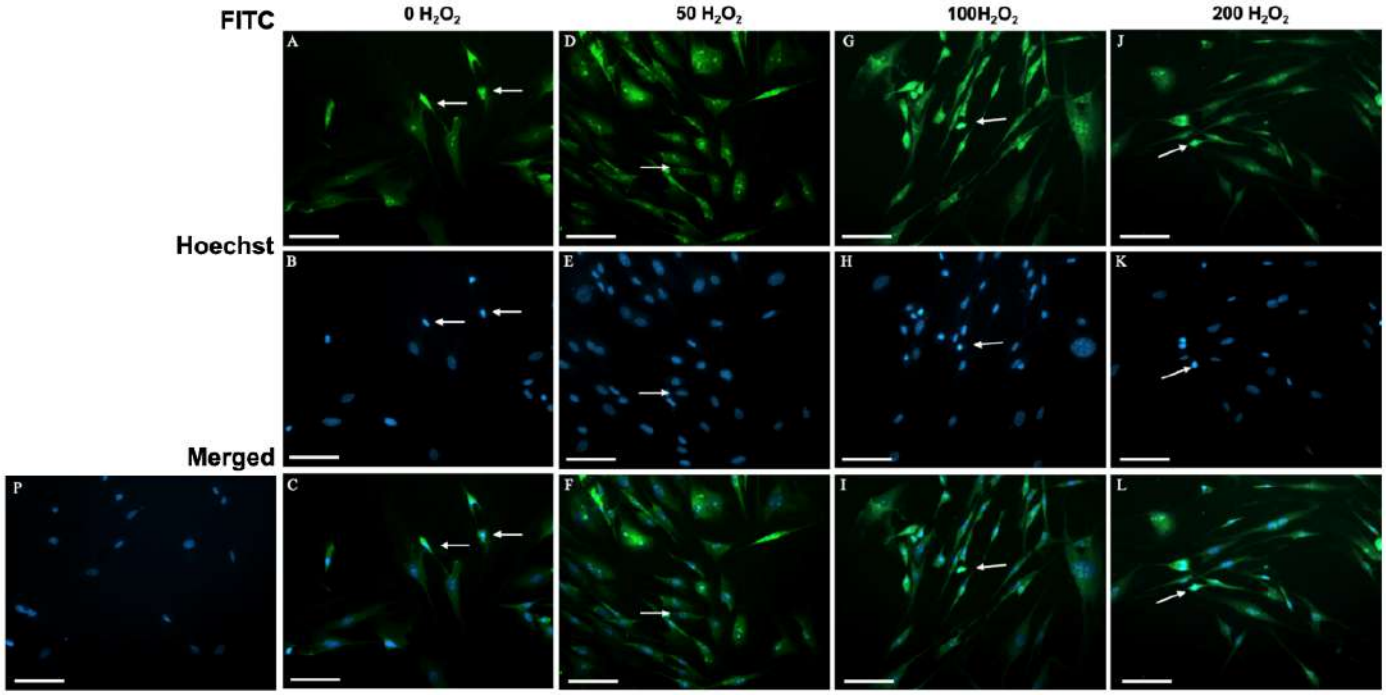


Figure 3.3. Immunocytochemical detection of oxidative DNA damage in DPSC (C3, Patient C), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. C3 demonstrated increased nuclear oxidative DNA damage, even in untreated controls, with increased detection of cytoplasmic and nuclear fluorescent staining (*arrowed*). Oxidative DNA damage (FITC) staining also showed a strong co-localisation with the Hoechst nuclear stain, confirming that oxidative DNA damage was particularly exclusive to the cell nuclei. FITC-Conjugate-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative DNA damage detection (image P). Scale bar 100µm, ×200 magnification.

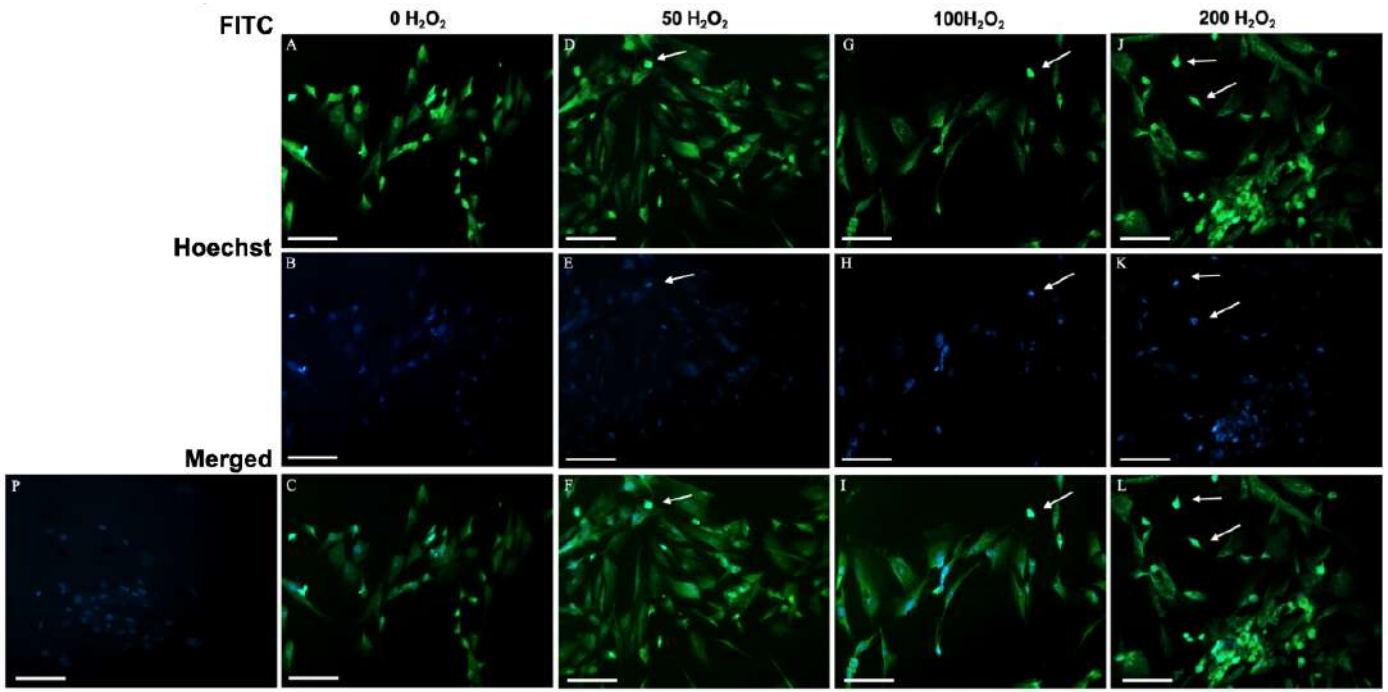


Figure 3.4. Immunocytochemical detection of oxidative DNA damage in DPSC (D4, Patient C), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. D4 demonstrated increased nuclear oxidative DNA damage, even in untreated controls, with increased detection of cytoplasmic and nuclear fluorescent staining (*arrowed*). Oxidative DNA damage (FITC) staining also showed a strong co-localisation with the Hoechst nuclear stain, confirming that oxidative DNA damage was particularly exclusive to the cell nuclei. FITC-Conjugate-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative DNA damage detection (image P). Scale bar 100µm, ×200 magnification.

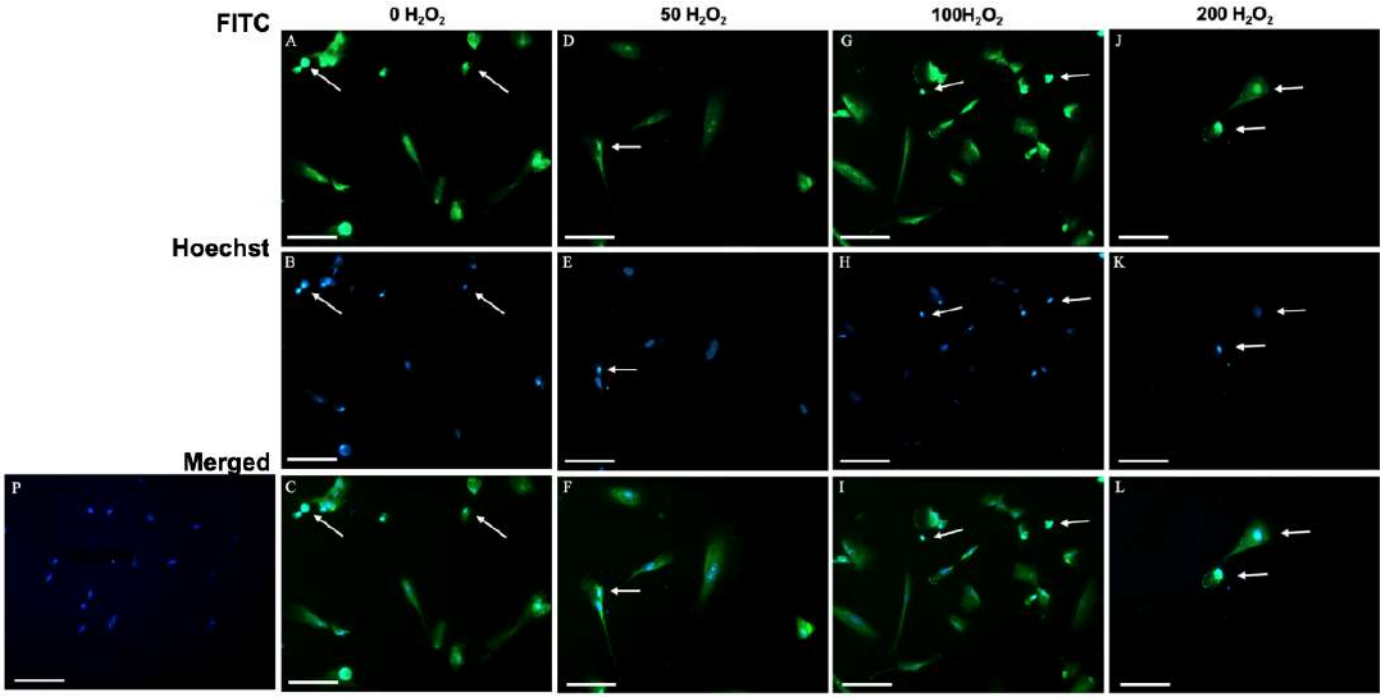
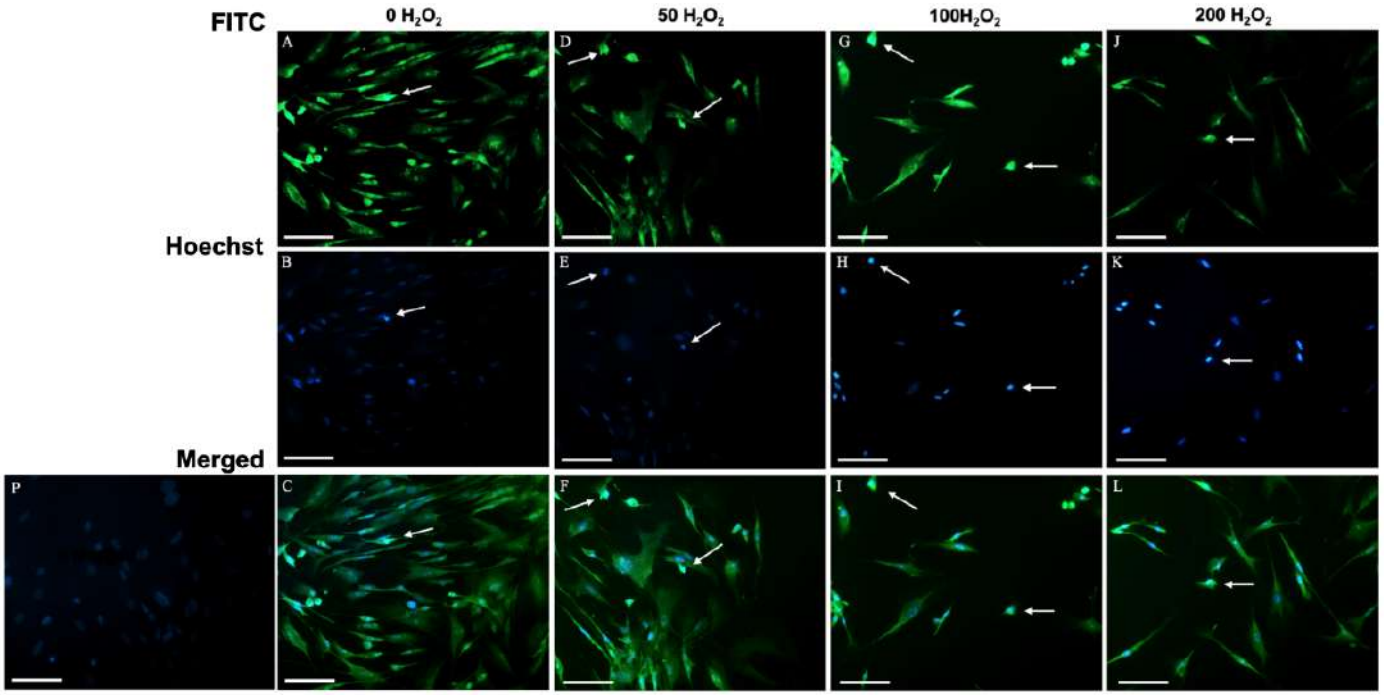


Figure 3.5. Immunocytochemical detection of oxidative DNA damage in DPSC (A1, Patient A), at late population doublings (45-60PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A1 demonstrated increased nuclear oxidative DNA damage, even in untreated controls, with increased detection of cytoplasmic and nuclear fluorescent staining (*arrowed*). Oxidative DNA damage (FITC) staining also showed a strong co-localisation with the Hoechst nuclear stain, confirming that oxidative DNA damage was particularly exclusive to the cell nuclei. FITC-Conjugate-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative DNA damage detection (image P). Scale bar 100µm, ×200 magnification.



3.4.2 Oxidative Protein Damage in Dental Pulp Stem Cells Under Oxidative Stress

High proliferative DPSC sub-population, A1 and low proliferative DPSC sub-populations, A2, C3 and D4 were also assessed for their susceptibilities to oxidative protein damage (protein carbonyl contents) throughout their proliferative lifespans, following treatment with increasing H_2O_2 concentrations (0, 50μM, 100μM, 200μM). Overall, oxidative protein detection showed marked variations in the susceptibilities to oxidative stress-induced damage between high and low proliferative DPSCs, with each demonstrating differences in the extent of positive protein carbonyl fluorescent staining detected, irrespective of whether DPSCs were derived from the same or different patients.

Highly proliferative DPSC population, A1, at early PDs (2-10PDs), exhibited the least amounts of rhodamine detection for oxidative protein damage overall, with comparable minimal intensity positive cellular staining detectable in both untreated and H_2O_2-treated cultures (Figure 3.6). However, the presence of viable DPSCs within these cultures were confirmed by Hoechst nuclear stain. In contrast, low proliferative DPSC sub-populations, A2, C3 and D4, at equivalent early PDs (2-10PDs), all exhibited increased rhodamine detection and enhanced susceptibilities to increased oxidative protein damage overall, even in untreated controls (Figures 3.7, 3.8 and 3.9 for A2, C3 and D4, respectively). Positive rhodamine detection in these low proliferative DPSC sub-populations was exhibited as intensely diffuse staining distributed throughout the cells, indicative of extensive oxidative protein damage. Highly proliferative DPSC population, A1, only exhibited similar extensive rhodamine staining intensities and oxidative protein damage detection to low proliferative DPSC sub-populations, at much later PDs (45-60PDs, Figure 3.10). This was particularly evident in H_2O_2-treated cultures, although lesser rhodamine staining was detectable in untreated controls, even at much later PDs (45-60PDs). Rhodamine-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative protein damage detection (image P, Figures 3.6-3.10, respectively).

Figure 3.6. Immunocytochemical detection of oxidative protein damage in DPSC (A1, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A1 demonstrated comparable minimal intensity positive cellular staining detectable in both untreated and H_2O_2-treated cultures overall. However, the presence of viable DPSCs within these cultures were confirmed by Hoechst nuclear stain. Rhodamine-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative protein damage detection (image P). Scale bar 100µm, ×200 magnification.

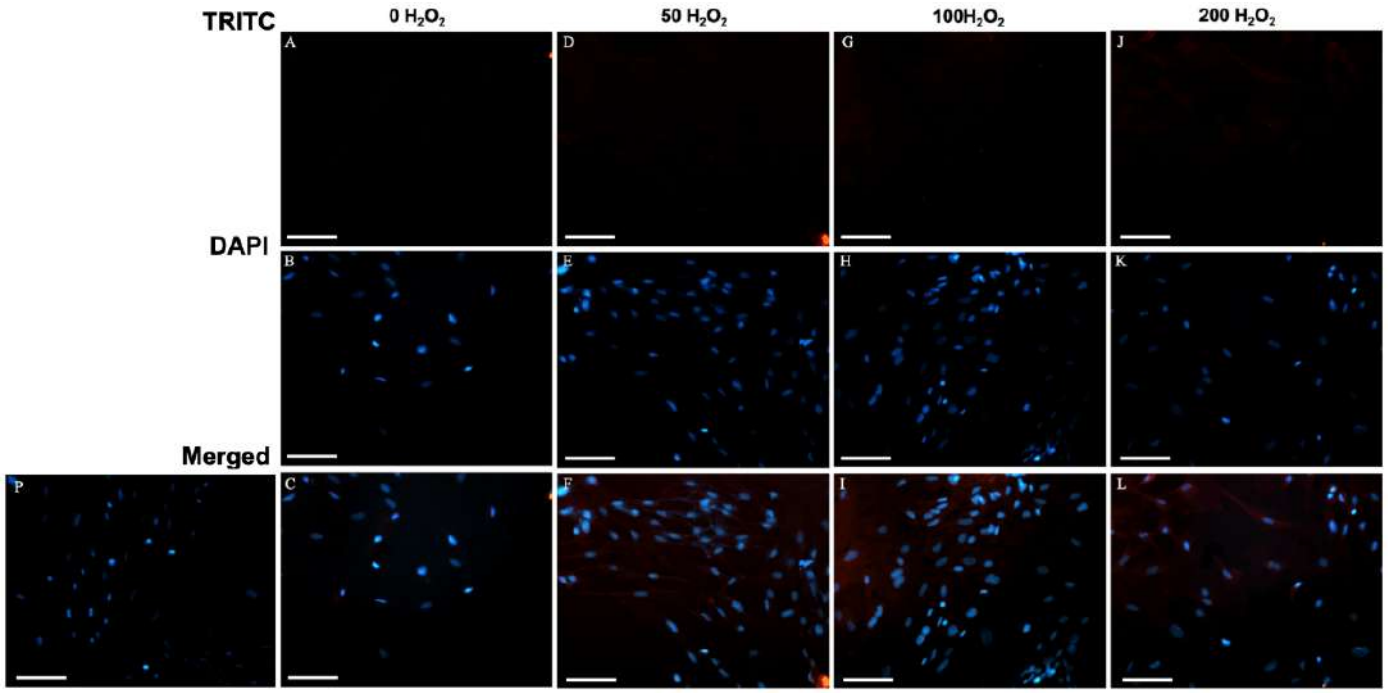


Figure 3.7. Immunocytochemical detection of oxidative protein damage in DPSC (A2, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A2 demonstrated increased rhodamine detection and enhanced susceptibilities to increased oxidative protein damage overall, even in untreated controls. Positive rhodamine detection was exhibited as intensely diffuse staining distributed throughout the cells, indicative of extensive oxidative protein damage. The presence of viable DPSCs within these cultures were confirmed by Hoechst nuclear stain. Rhodamine-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative protein damage detection (image P). Scale bar 100µm, ×200 magnification.

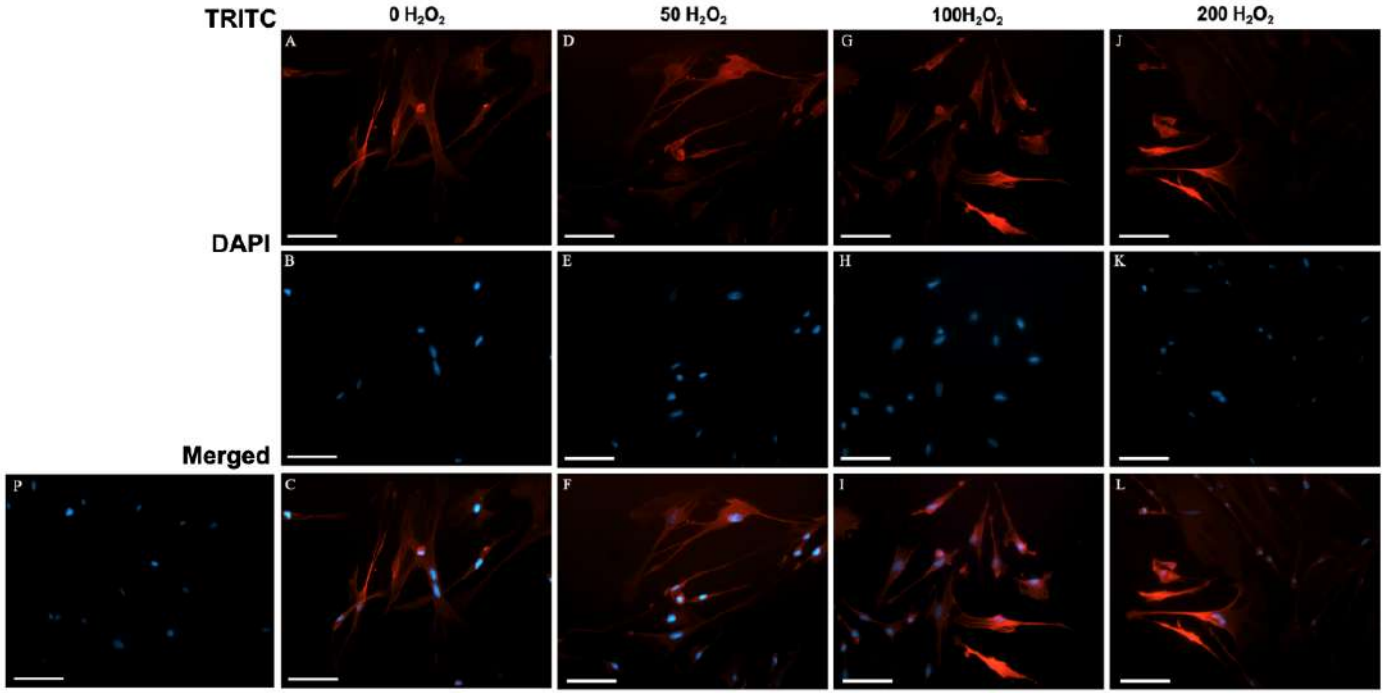


Figure 3.8. Immunocytochemical detection of oxidative protein damage in DPSC (C3, Patient C), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. C3 demonstrated increased rhodamine detection and enhanced susceptibilities to increased oxidative protein damage overall, even in untreated controls. Positive rhodamine detection was exhibited as intensely diffuse staining distributed throughout the cells, indicative of extensive oxidative protein damage. The presence of viable DPSCs within these cultures were confirmed by Hoechst nuclear stain. Rhodamine-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative protein damage detection (image P). Scale bar 100µm, ×200 magnification.

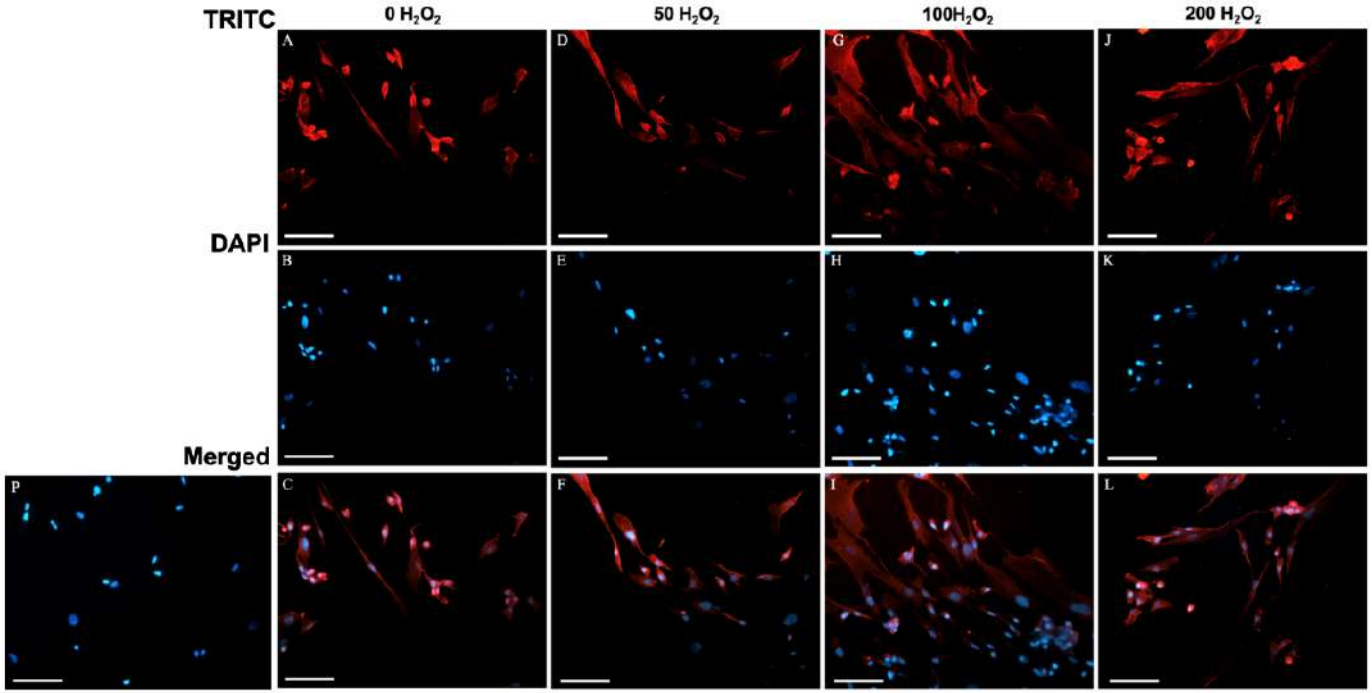


Figure 3.9. Immunocytochemical detection of oxidative protein damage in DPSC (D4, Patient D), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. D4 demonstrated increased rhodamine detection and enhanced susceptibilities to increased oxidative protein damage overall, even in untreated controls. Positive rhodamine detection was exhibited as intensely diffuse staining distributed throughout the cells, indicative of extensive oxidative protein damage. The presence of viable DPSCs within these cultures were confirmed by Hoechst nuclear stain. Rhodamine-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative protein damage detection (image P). Scale bar 100µm, ×200 magnification.

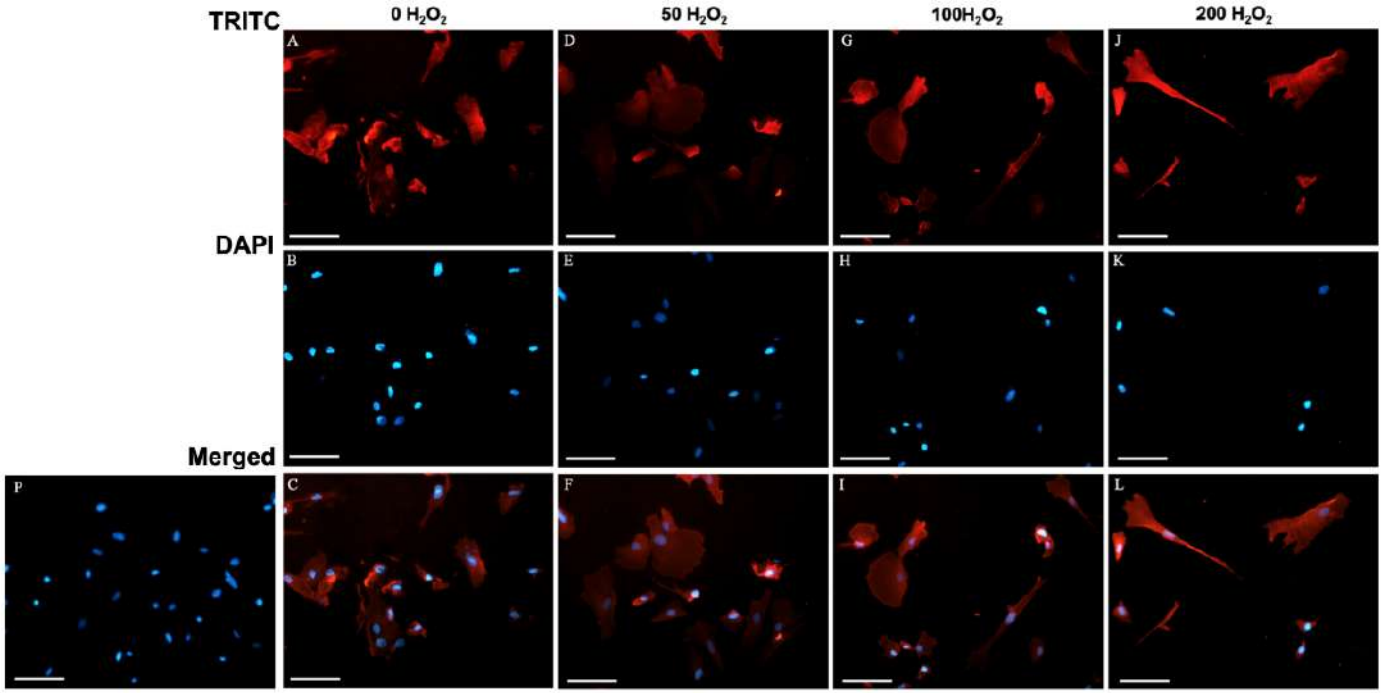
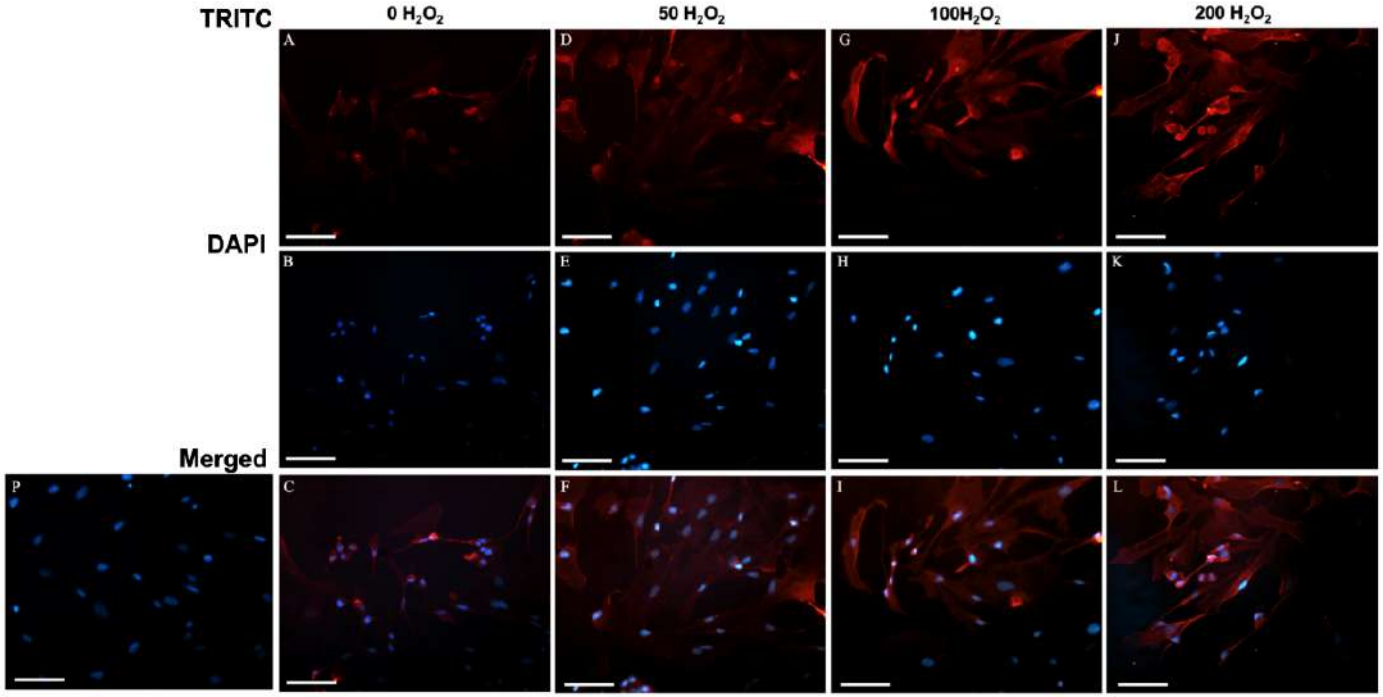


Figure 3.10. Immunocytochemical detection of oxidative protein damage in DPSC (A1, Patient A), at late population doublings (45-60PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A1 demonstrated increased rhodamine detection and enhanced susceptibilities to increased oxidative protein damage overall. This was particularly evident in H_2O_2-treated cultures, although lesser rhodamine staining was detectable in untreated controls. Positive rhodamine detection was exhibited as intensely diffuse staining distributed throughout the cells, indicative of extensive oxidative protein damage. The presence of viable DPSCs within these cultures were confirmed by Hoechst nuclear stain. Rhodamine-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative protein damage detection (image P). Scale bar 100µm, ×200 magnification.



3.4.3 Oxidative Lipid Damage in Dental Pulp Stem Cells Under Oxidative Stress

High proliferative DPSC sub-population, A1 and low proliferative DPSC sub-populations, A2, C3 and D4 were also assessed for their susceptibilities to oxidative lipid damage (lipid peroxidation) throughout their proliferative lifespans, following treatment with increasing H_2O_2 concentrations (0, 50µM, 100µM, 200µM). Overall, oxidative lipid detection showed marked variations in the susceptibilities to oxidative stress-induced damage between high and low proliferative DPSCs, with each demonstrating differences in the extent of positive lipid peroxidation fluorescent staining detected, irrespective of whether DPSCs were derived from the same or different patients.

Highly proliferative DPSC population, A1, at early PDs (2-10PDs), exhibited the lowest detectable fluorescence colour shift from red (Texas Red) to green (FITC), indicative of the increased oxidative lipid damage overall, especially in untreated and 50µM H_2O_2-treated cultures, where no or negligible FITC staining was determined (Figure 3.11). However, increased dose-dependent detection of positive FITC staining intensities were apparent for A1 at early PDs (2-10PDs), treated with 100µM and 200µM H_2O_2. In contrast, low proliferative DPSC sub-populations, A2, C3 and D4, at equivalent early PDs (2-10PDs), all exhibited the increased detection of shifts from Texas Red-FITC staining and enhanced susceptibilities to increased oxidative lipid damage overall, even in untreated controls (Figures 3.12, 3.13 and 3.14 for A2, C3 and D4, respectively). Positive FITC detection in these low proliferative DPSC sub-populations was exhibited as diffuse staining distributed throughout the cells, being particularly localised to the cell membranes indicative of extensive oxidative lipid damage in these regions. Highly proliferative DPSC population, A1, only exhibited similar significant shifts in Texas Red-FITC staining intensities and oxidative lipid damage detection to low proliferative DPSC sub-populations, at much later PDs (45-60PDs, Figure 3.15). A1 demonstrated increased oxidative lipid damage, even in untreated controls, with increased detection of FITC staining, particularly localised to the cell membranes. Image-iT® Lipid Peroxidation Sensor-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative lipid damage detection (image P, Figures 3.11-3.15, respectively).

Figure 3.11. Immunocytochemical detection of oxidative lipid damage in DPSC (A1, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A1 demonstrated the lowest detectable fluorescence colour shift from red (Texas Red) to green (FITC), indicative of the increased oxidative lipid damage overall, especially in untreated and 50µM H_2O_2-treated cultures, where no or negligible FITC staining was determined. However, increased dose-dependent detection of positive FITC staining intensities were apparent for A1 at early PDs (2-10PDs), treated with 100µM and 200µM H_2O_2. Image-iT® Lipid Peroxidation Sensor-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative lipid damage detection. Scale bar 100µm, ×200 magnification.

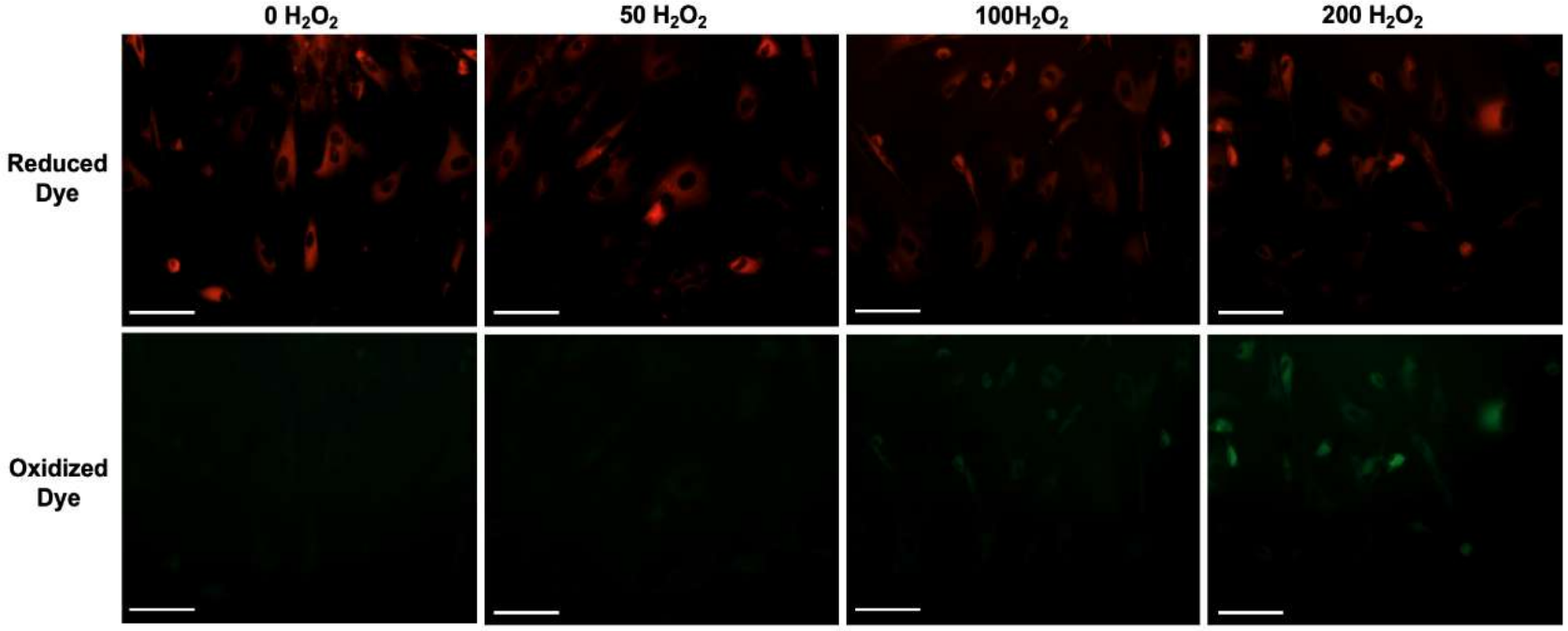


Figure 3.12. Immunocytochemical detection of oxidative lipid damage in DPSC (A2, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. A2 demonstrated increased detection of shifts from Texas Red-FITC staining and enhanced susceptibility to oxidative lipid damage, even in untreated controls. Positive FITC detection in these low proliferative DPSC sub-populations was exhibited as diffuse staining distributed throughout the cells, particularly localised to the cell membranes. Image-iT® Lipid Peroxidation Sensor-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative lipid damage detection. Scale bar 100µm, ×200 magnification.

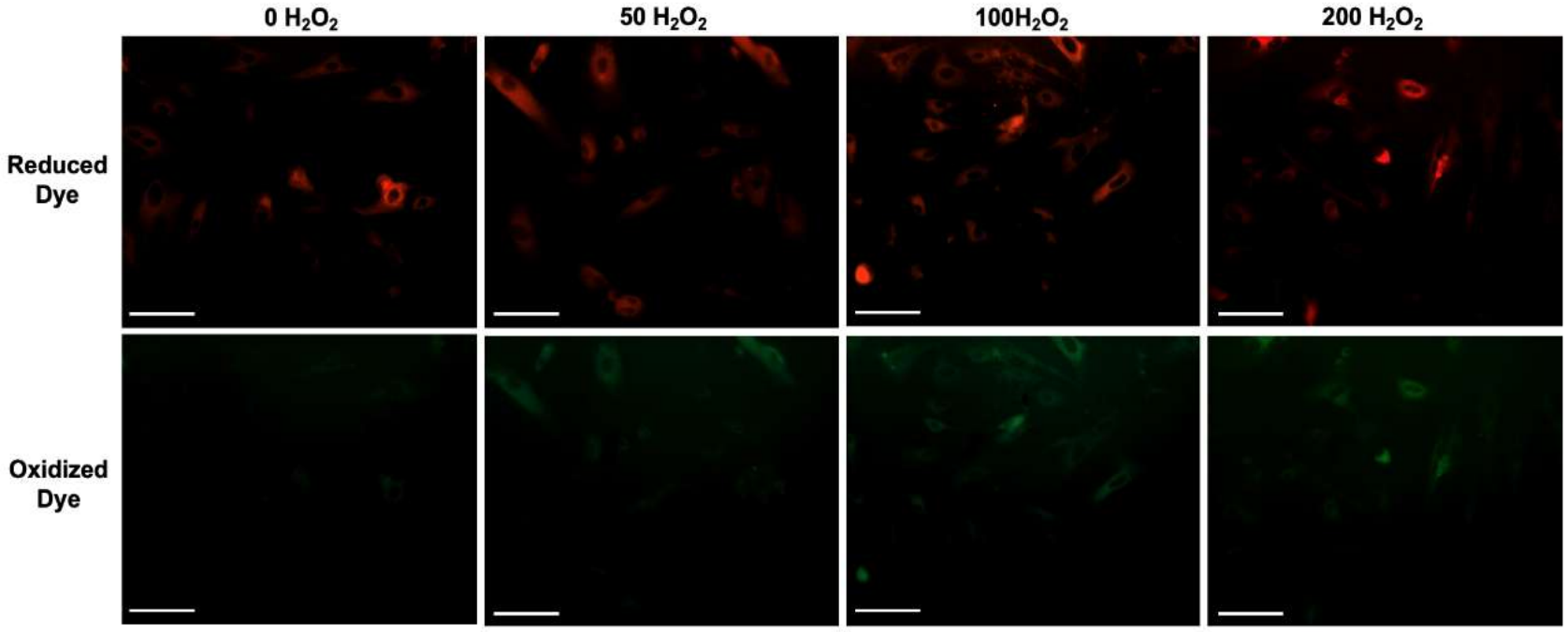


Figure 3.13. Immunocytochemical detection of oxidative lipid damage in DPSC (C3, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. C3 demonstrated increased detection of shifts from Texas Red-FITC staining and enhanced susceptibility to oxidative lipid damage, even in untreated controls. Positive FITC detection in these low proliferative DPSC sub-populations was exhibited as diffuse staining distributed throughout the cells, particularly localised to the cell membranes. Image-iT® Lipid Peroxidation Sensor-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative lipid damage detection. Scale bar 100µm, ×200 magnification.

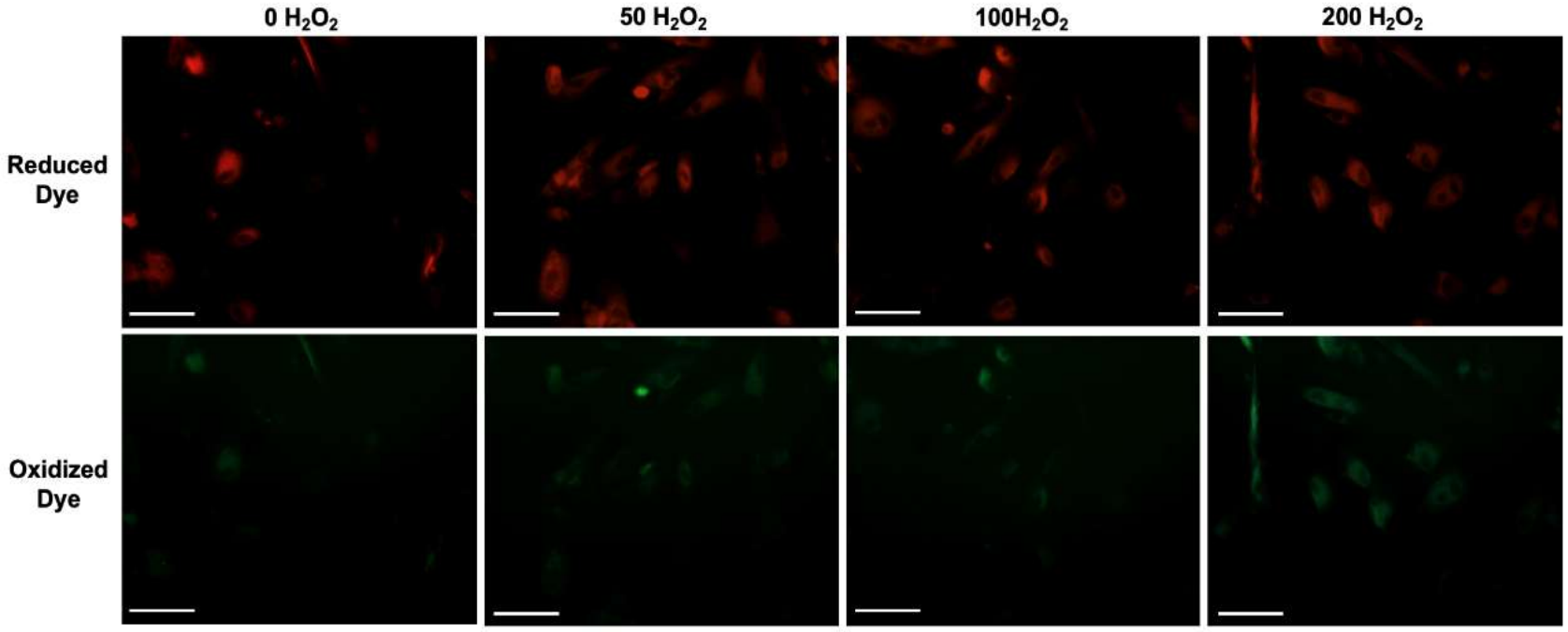


Figure 3.14. Immunocytochemical detection of oxidative lipid damage in DPSC (D4, Patient A), at early population doublings (2-10PDs) under increasing H_2O_2 concentrations (50µM, 100µM, 200µM), compared to untreated controls. D4 demonstrated increased detection of shifts from Texas Red-FITC staining and enhanced susceptibility to oxidative lipid damage, even in untreated controls. Positive FITC detection in these low proliferative DPSC sub-populations was exhibited as diffuse staining distributed throughout the cells, particularly localised to the cell membranes. Image-iT® Lipid Peroxidation Sensor-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative lipid damage detection. Scale bar 100µm, ×200 magnification.

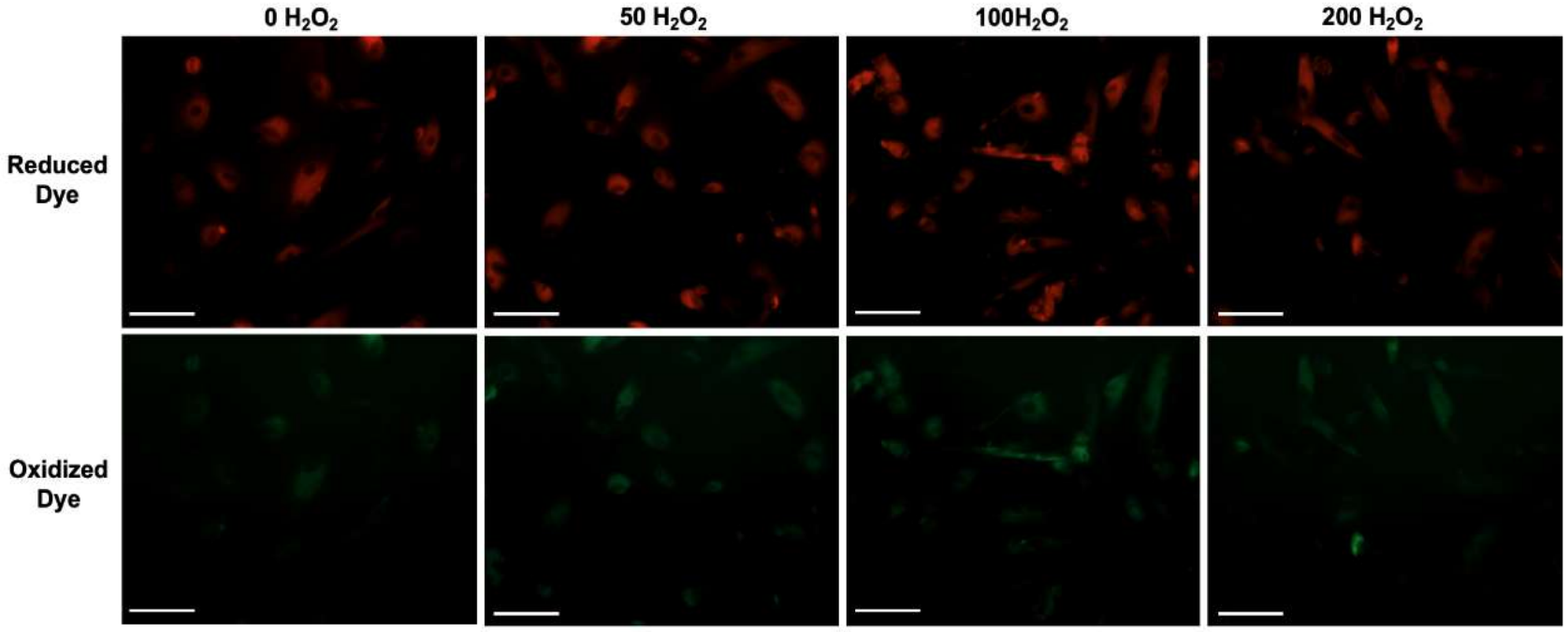
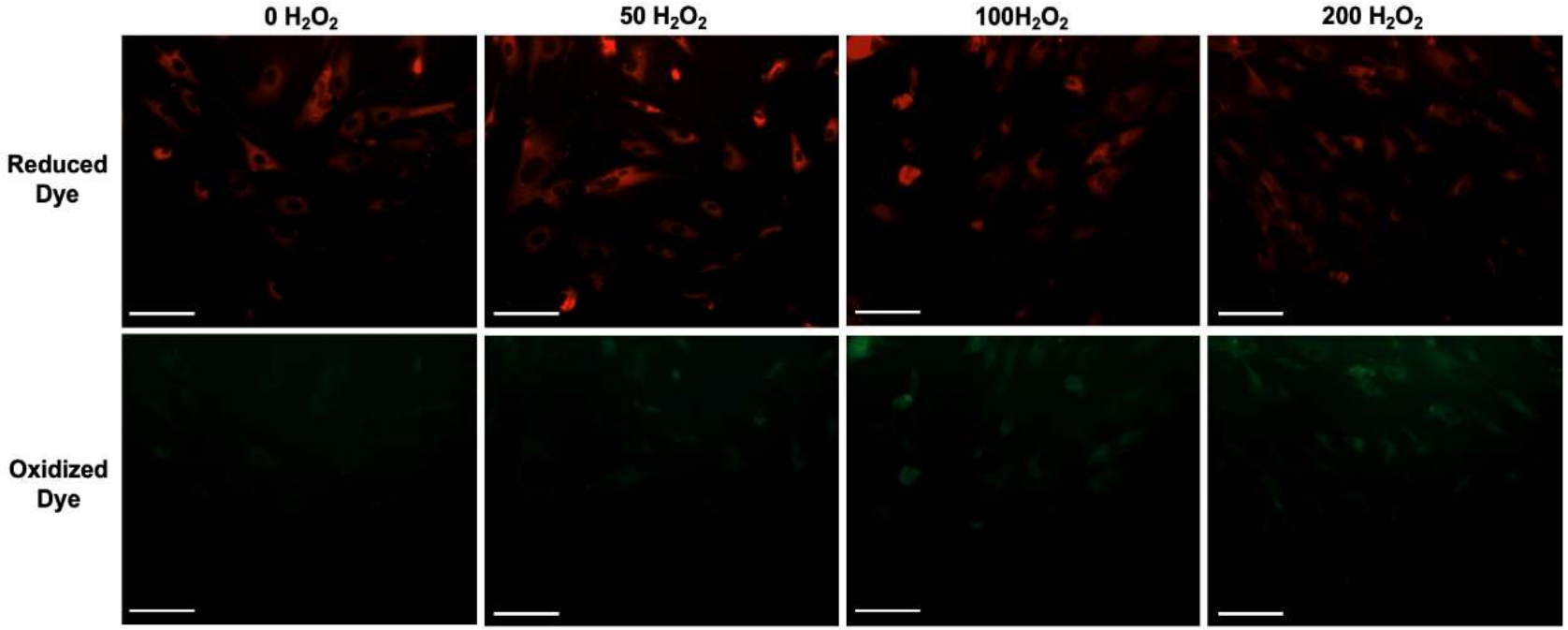


Figure 3.15. Immunocytochemical detection of oxidative lipid damage in DPSC (A1, Patient A), at late population doublings (45-60PDs) under increasing H_2O_2 concentrations (50μM, 100μM, 200μM), compared to untreated controls. A1 demonstrated increased oxidative lipid damage, even in untreated controls, with the increased detection fluorescence colour shift from red (Texas Red) to green (FITC), indicative of the increased oxidative lipid damage overall. Image-iT® Lipid Peroxidation Sensor-free/Hoechst-treated controls confirmed the absence of non-specific binding and oxidative lipid damage detection. Scale bar 100μm, ×200 magnification.



3.5 Discussion

It is a well-established concept that oxidative stress-induced (telomere-independent) senescence, is preceded or accompanied by increased oxidative stress-induced, biomolecular damage (Ben-Porath and Weinberg, 2004; Campisi and d'Adda di Fagagna F, 2007; Victorelli and Passos, 2017), with the associated elevations in ROS levels at the expense of endogenous antioxidant defences leading to accumulative damage to cellular components, including DNA, proteins and lipids (Halliwell, 1999; Levine and Stadtman, 2001; Spiteller, 2001; Cooke et al., 2003; Evans et al., 2004; Sikora et al., 2011; Chaudhari et al., 2014). In line with such previous findings, this Chapter confirmed that in addition to distinct differences in the respective susceptibilities of DPSC sub-populations to telomere-dependent senescence (Alraies et al., 2017) and telomere-independent senescence (Chapter 2), oxidative stress-induced senescence in DPSCs was associated with concomitant increased in oxidative stress-induced damage to DNA, proteins and lipids and that DPSC sub-populations also differ in their respective susceptibilities to oxidative damage. Therefore, these results provide additional biomolecular evidence to support the difference in DPSCs susceptibilities and response to oxidative stress-induced senescence overall.

Although it is established that mesenchymal stem cells (MSCs) are more resistant to H_2O_2-induced senescence, compared to non-somatic cell types, such as fibroblasts (Brandl et al., 2011; Choo et al., 2014), collectively, the most obvious differences identified herein were the relative susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress-induced damage overall, with high proliferative DPSC, A1, exhibiting significantly reduced oxidative DNA, protein and lipid damage overall at early PDs (2-10PDs), versus the elevated levels of damage identified in low proliferative DPSC, A2, C3 and D4, at equivalent PDs. Indeed, elevated oxidative damage was even evident in low proliferative DPSC sub-populations in the absence of culture supplementation with sub-lethal doses of exogenous H_2O_2. On the contrary, high proliferative DPSC, A1, only demonstrated similar oxidative biomolecular damage to these low proliferative DPSC sub-populations, at much later PDs in their proliferative lifespans. Such findings imply that high proliferative DPSC, A1, possesses higher resistance to H_2O_2-induced oxidative stress, which gradually diminishes during more extensive *in vitro* cell culture expansion.

Oxidative DNA damage and impaired DNA repair capabilities are established to accompany cellular senescence (Chen et al., 1995, 2003; Duan et al., 2005; Hazane et al., 2006; Hazane-Puch et al., 2010). Therefore, considering the increased susceptibilities of low proliferative DPSC sub-populations (A2, C3 and D4) to oxidative stress-induced senescence (Chapter 2), it is conceivable that these increases in oxidative DNA damage contribute to the early-onset of p53, $p21^{waf1}$ and $p16^{INK4a}$ induction and senescence in these DPSCs (Ben-Porath and Weinberg, 2004; Campisi and d'Adda di Fagagna, 2007). Indeed, in addition to differences in detectable levels of oxidative DNA damage between high and low proliferative DPSC sub-populations, it was apparent that there was a strong correlation between the localisation of the oxidative DNA damage and Hoechst nuclear stain, thereby further confirming that the oxidative DNA damage detected was particularly present within the nuclei of the DPSCs analysed. Such findings are comparable with the conclusions of previously reported studies, which have examined the effects of oxidative stress on the induction of nuclear DNA damage and senescence in various cell types, including MSCs (Yu et al., 2018) and fibroblasts (Roper et al., 2004; Kil et al., 2006; Martirosyan et al., 2006).

In addition to the nuclear oxidative DNA damage observed, both high and low proliferative DPSC sub-populations demonstrated cytoplasmic staining that was more visibly evident in all cells, compared to nuclear immunofluorescence, potentially indicating additional mitochondrial DNA damage. The susceptibility of mitochondrial DNA to oxidative damage has been described in several reports (Richter et al., 1988; Beckman and Ames, 1996; Yakes and Van Houten, 1997; Soultanakis et al., 2000), from which it has been proposed that mitochondrial DNA is more sensitive to oxidative damage than nuclear DNA, undergoing more extensive and persistent damage (Beckman and Ames, 1996; Yakes and Van Houten, 1997). Several reasons have been suggested for this, such as the higher sensitivity of mitochondrial DNA to ROS (Yakes and Van Houten, 1997), being in closer proximity to the electron transport chain and organized in a histone-free nucleoid, hence making it more exposed to oxidant attack than nuclear DNA (Bogenhagen, 2012; Blasiak et al., 2013). In addition, although mitochondrial-generated ROS normally cause mild mitochondrial DNA damage that is rapidly repaired, during chronic ROS exposure, the mitochondrial ability to repair DNA may be less efficient than the nucleus, due to malfunction of the electron transport system resulting in

more extensive mitochondrial DNA damage (Yakes and Van Houten, 1997; Blasiak et al., 2013).

Oxidative protein damage has also been well-documented as a marker of oxidative damage associated with cellular senescence and aging, due to direct protein modification and increased accumulation of oxidized cellular proteins, due to dysfunctions in the intracellular proteosome system under oxidative stress (Sitte et al., 2000; Chondrogianni and Gonos, 2004; Grune et al, 2005; Torres and Perez, 2008). Indeed, protein carbonyl groups are considered advantageous markers of oxidative stress compared to other oxidation products, due to their relatively simple formation and the relative stability of the oxidized protein. Previous research has reported that *in vitro* H_2O_2 treatment resulted in elevated levels of protein carbonyl residues in fibroblasts, compared to untreated fibroblasts (Dimon-Gadal et al., 2000; Grune et al, 2005; Jung et al., 2009). The current findings suggest an enhanced susceptibility of low proliferative DPSC sub-populations to oxidative protein damage, as protein damage was even observed early in untreated controls. In contrast, high proliferative DPSCs untreated with H_2O_2 showed resistance to oxidative protein damage, both early and late in their proliferative lifespans, with protein damage only observed in this sub-population upon prolonged dose-dependent exposure to higher H_2O_2 concentrations. These findings are in line with previous findings, in which H_2O_2-induced senescence in fibroblasts induced senescence, accompanied by significant increases in oxidized protein levels in a dose-dependent manner at both early and late stages in their proliferative lifespans (Jung et al., 2009). These results indicate the early existence of protein damage in the low proliferative DPSC sub-populations, reflecting enhanced susceptibility to oxidative stress compared to the high proliferative DPSCs.

Lipid peroxidation is another well-used cellular oxidative stress biomarker, due to the key role it plays in impairing cellular function (Halliwell, 1999) and the generation of harmful adducts, such as MDA and 4-HNE, that not only attack lipids, but DNA and proteins (Halliwell and Chirico, 1993; Spiteller, 2001; Kohen and Nyska, 2002). Although the results presented here demonstrated increased lipid peroxidation particularly localised to the cell membranes of low proliferative DPSC sub-populations, versus high proliferative DPSCs at equivalent PDs, the oxidative lipid damage detected appeared to be lower in intensity, compared to the corresponding DNA and protein damage. These findings concur with previous

studies, stating that oxidative damage biomarkers may occur concomitantly upon ROS accumulation. However, the extent of their damage and the time course over which such damage appears can vary greatly. For example, it is known that lipid peroxidation products are detoxified quickly within minutes, once formed (Levine et al., 1990; Siems et al., 1997; England et al., 2000). On the other hand, the degradation process for oxidized proteins is much slower, occurring over hours or days (Grune et al., 1995; 1996). Furthermore, oxidative stress biomarker level variations may occur depending on cell type, as H_2O_2-induced lipid peroxidation has been reported to occur more significantly in non-somatic cells, such as fibroblasts and endothelial cells, compared to embryonic stem cells (Barandalla et al., 2016).

The results described in the present Chapter provide strong evidence for the variation in susceptibility of DPSC sub-populations to biomolecular damage, as a consequence of ROS accumulation and oxidative stress. However, as enhanced oxidative stress-induced bimolecular damage and senescence are a consequence of excessive ROS production and exposure, at the expense of cellular antioxidant defence mechanisms, the significant variations in the contrasting susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress overall, may be related to differential antioxidant expression and activity profiles between these DPSCs, i.e. that high proliferative DPSC sub-populations possess superior antioxidant expression and activity profiles, compared to low proliferative DPSCs. Indeed, enzymic antioxidant status is critical in maintaining the intracellular redox balance and cell survival (i.e. cells with higher antioxidant capacities are more resistant to oxidative stress and senescence (Serra et al, 2000, 2003; Lorenz et al, 2001; Blander et al, 2003; Richter and von Zglinicki, 2007). As such, in order to address the hypothesis, the antioxidant expression/activities of well-established enzymic antioxidants with renowned roles in regulating cellular senescence were next investigated in these high and low proliferative DPSC sub-populations, as detailed in Chapter 4.

Chapter 4

Assessment of Enzymic Antioxidant Expression Profiles Between Dental Pulp Stem Cell Sub-Populations

Chapter 4

Assessment of Enzymic Antioxidant Expression Profiles Between Dental Pulp Stem Cell Sub-Populations

4.1 Introduction

The previous Chapters have clearly demonstrated that significant variations exist in the relative susceptibilities of dental pulp stem cell (DPSC) sub-populations to oxidative stress-induced biomolecular damage and telomere-independent senescence, with low proliferative DPSC sub-populations demonstrating significantly greater oxidative damage to cellular constituents, such as DNA, proteins and lipids and the earlier onset of oxidative stress-induced senescence, compared to high proliferative DPSCs. As differences in cellular susceptibilities to oxidative stress-induced biomolecular damage and senescence are often associated with contrasting enzymic antioxidant profiles between different cell types, variation in the relative susceptibilities of DPSC sub-populations to oxidative stress-induced damage and senescence may indicate differences in the respective antioxidant defence capabilities of DPSCs, in terms of the enzymic antioxidant expression and activity profiles between these sub-populations.

Oxidative stress is a well-established mediator of biomolecular damage and telomere-independent senescence in various cell types, including mesenchymal stem cells (MSCs, Ben-Porath and Weinberg, 2004; Liu et al., 2011; Shyh-Chang et al., 2013; Chaudhari et al., 2014; Li et al., 2017; Victorelli and Passos, 2017). Such events are associated with cellular overproduction and/or increased exposure to reactive oxygen species (ROS), such as superoxide radical species ($O_2^{\cdot -}$), hydroxyl radical species ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2), leading to an imbalance with the endogenous cellular antioxidant defence mechanisms. Thus, elevated ROS levels at the expense of antioxidant levels accelerate oxidative damage accumulation and telomere-independent senescence. Under normal physiological conditions, ROS are generated via a wide range of cellular mechanisms, mainly occurring in the mitochondria, in addition to NADPH oxidase complexes (Balaban et al., 2005; Chaudhari et al., 2014).

To counteract the excessive accumulation of ROS and to regulate cellular redox homeostasis, cells possess antioxidant defence mechanisms. These defences vary from simple, low molecular weight compounds, such as ascorbate and α-tocopherol, to enzymes, such as superoxide dismutases (SODs), catalase and glutathione-metabolising enzymes, including glutathione peroxidases (GPXs), transferases, reductases and synthetases. These enzymic and non-enzymic antioxidants protect cells from damage by directly scavenging ROS or by sequestering transition metal ions (Waddington et al., 2000). Enzymic antioxidant defence mechanisms are mainly orchestrated by Nf-E2 related factor 2 (Nrf2), which activates the various antioxidant responsive element (ARE)-dependent genes that encode enzymic antioxidants, ultimately mitigating oxidative stress (Kensler et al., 2007; Nguyen et al., 2009; Ma, 2013). SODs catalyse $O_2^{\cdot-}$ dismutation into H_2O_2. Three SOD isoenzymes which have been identified in cells. SOD1 occurs in the cytoplasm, the intermembrane space of mitochondria and in the nucleus, SOD2 is located to the mitochondria matrix and SOD3 is secreted into the extracellular matrix (Zelko et al., 2002; Fattman et al., 2003; Nozik-Grayck et al., 2005; Fukai and Ushio-Fukai, 2011). Most aerobic cells contain catalase, which catalyses the dismutation of H_2O_2 and mostly localised intracellularly in peroxisomes within the cytosol (Kirkman and Gaetani, 2007). GPXs provide another mechanism to remove excessive intracellular H_2O_2 levels, by catalysing the oxidation of reduced glutathione (GSH) by H_2O_2. GPXs are tetrameric proteins localised within the cytoplasm, with each subunit containing an atom of selenium present at the active site as selenocysteine (Deponte, 2013, Lu, 2013). Reduced GSH is produced by the formation of γ-glutamylcysteine from glutamate and cysteine, followed by the formation of GSH from γ-glutamylcysteine, catalysed by γ-glutamylcysteine synthetase and glutathione synthetase (GSS), respectively (Lu, 2013). Glutathione S-transferases (GSTs) are located within the cytoplasm, mitochondria and microsomal compartments of cells, where these catalyse the conjugation of GSH via sulfhydryl groups, to electrophilic centres on a wide variety of substrates, in order to detoxify compounds and make them more water-soluble (Wu and Dong, 2012; Board and Menon, 2013, Lu, 2013). Reduced GSH is regenerated by the activity of glutathione reductase (GSR), at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH, Deponte, 2013). The GST superfamily also

contains glutathione transferase ζ (GSTZ1) amongst other isoforms, which catalyses the reaction of GSH with xenobiotics and endobiotics (Board and Anders, 2011). Although GSTZ1 is located in the cytosol, it is also localised within cellular mitochondria. Some properties of cytosolic and mitochondrial GSTZ1 differ, these are not related to differences in amino acid sequence or post-translationally modified residues (Zhong et al., 2018).

Despite possessing such an array of tightly-regulated, cellular antioxidant defence mechanisms, imbalance between ROS overproduction and cellular antioxidant capacity are strongly correlated with increased oxidative stress-induced biomolecular damage and the induction of telomere-independent senescence in a variety of cell types, particularly fibroblasts (Serra et al., 2000, 2003; Lorenz et al., 2001; Blander et al., 2003; Brown and Stuart, 2007; Richter and von Zglinicki 2007; Hammad et al., 2018). However, more recent studies into the effects of oxidative stress on embryonic stem cell (ESC) behaviour have confirmed that stem cells have contrasting responses to oxidative stress than somatic cells, due to the activation of different antioxidant defence mechanisms (Barandalla et al., 2016). Furthermore, correlations between the susceptibilities of MSCs from various sources to cellular senescence, with their relative enzymic antioxidant expression and activity capabilities, have also been established (Valle-Prieto and Conget, 2010; Ko et al., 2012; Jeong and Cho, 2015; Yu et al., 2018; Chen et al., 2019). This is also the case, in terms of MSC susceptibilities to oxidative stress-induced biomolecular damage, with cellular resistance to oxidative damage being attributed to their relatively high enzymic antioxidant capacities (He et al., 2004; Ko et al., 2012; Yu et al., 2018; Chen et al., 2019). Thus, although the reasons behind such contrasting oxidative stress responses between different stem cell populations is still under debate, the majority of theories are increasingly focussed on the roles of antioxidants as being major influences in providing stem cell resistance to oxidative stress, via their possession of superior enzymic antioxidant induction capabilities (Liu et al., 2011; Shyh-Chang et al., 2013; Urao and Ushio-Fukai, 2013; Benameur et al., 2015; Sheshadri and Kumar, 2016).

4.2 Chapter Aims

The previous Chapters confirmed significant variations in oxidative stress-induced biomolecular damage and telomere-independent senescence between DPSC sub-

populations. However, given the strong correlations identified between cellular susceptibilities to oxidative stress-induced damage and telomere-independent senescence, with endogenous enzymic antioxidant expression and activity levels, this Chapter examined whether the differences in enzymic antioxidant capabilities further contributed to the contrasting susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress-induced biomolecular damage and telomere-independent senescence. Confirming differences in enzymic antioxidant capacities between high and low proliferative DPSCs would provide a greater understanding of DPSC biology and its inter-relationship with oxidative stress and cellular ageing.

4.3 Materials and Methods

4.3.1 Isolation, Culture and Expansion of Dental Pulp Stem Cells Under Oxidative Stress Conditions

Human DPSCs were isolated, cultured and expanded in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, ThermoFisher Scientific, Paisley, UK), as previously described (Chapters 2.3.1, 2.3.2 and 2.3.3).

4.3.2 Enzymic Antioxidant Gene Expression Analysis

4.3.2.1 Real Time Quantitative Polymerase Chain Reaction (RT-QPCR)

Expression of enzymic antioxidants genes (SOD1, SOD2, SOD3, CAT1, GPX1, GPX2, GPX3, GPX4, GPX5, GSR, GSS and GSTZ1), in high and low proliferative DPSC sub-populations in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), were investigated using Real Time Quantitative Polymerase Chain Reaction (RT-QPCR) techniques. DPSCs at selected population doublings (PDs) throughout their proliferative life-spans, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), were seeded, RNA extracted and complimentary DNA (cDNA) synthesised, as previously described (Chapters 2.3.6.1 and 2.3.6.2).

4.3.2.2 TaqMan QPCR

Following RNA extraction and the preparation of cDNA, cDNA was amplified using the Applied Biosystems™ ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific), via the use of Applied Biosystems™ TaqMan® primer probes (Table 2.1,

all purchased from ThermoFisher Scientific) according to manufacturer's protocols. Post-thermal cycling, QPCR was performed in MicroAmp™ Fast Optical 96-Well Reaction Plates (ThermoFisher Scientific). Each reaction had a final volume of 20µl/well, consisting of cDNA (4µl), TaqMan® Fast Universal PCR Master Mix (2X) no AmpErase® UNG (10µl), RNase-free water (4µl), TaqMan® Gene Expression Assay Primer/Probe Mix (FAM™ reporter/MGB-NFQ quencher, 1µl) and eukaryotic 18S Ribosomal RNA (rRNA) Endogenous Control (VIC® reporter/TAMRA™ quencher, 1µl). The TaqMan® target and reference gene expression assay mixes (Table 4.1), contained forward and reverse PCR primers and TaqMan® probes. Negative control reactions were also included for each plate, with RNase-free water replacing cDNA to account for any undesired reagent contamination and undesirable signal. Reaction Plates were then covered with MicroAmp™ Optical Adhesive Film (ThermoFisher Scientific), to reduce possibility of cross contamination between wells and centrifuged at 500g for 30s, before transferring the plates to the Applied Biosystems™ ViiA™ 7 Real-Time PCR System, for cDNA amplification. Reactions were run with an initial denaturing step of 95°C for 20s, followed by 40 cycles at a denaturing step temperature of 95ºC (1s) and 1 cycle at an annealing temperature of 60ºC (20s).

4.3.2.3 Quantification of Gene Expression

The comparative cycle threshold (ΔΔCT) method was used to calculate the relative quantification (RQ) of gene expression, with 18S rRNA used as a reference gene for normalisation. CT values, where amplification is within the curve's linear range, were used in this process. CTs of the standard reference gene (18S rRNA) were subtracted from target gene CT values, to obtain delta CT (dCT) values (known as the cycle threshold differences). Subsequently, mean dCT values for the experimental and control group samples were calculated. Expression of each target gene in experimental group samples relative to the mean control group expression values were subsequently determined, using the following equation:

$$2^{-(dCT(\text{Experimental Target}) - dCT(\text{Mean Control Group}))}$$

Table 4.1. Applied Biosystems™ TaqMan® gene expression assay mixes. Catalogue and assay IDs are provided *in lieu* of primer sequences, as Applied Biosystems™ do not supply this information.

Gene Target	TaqMan® Gene Expression Assay
SOD1	Hs00533490_m1
SOD2	Hs00167309_m1
SOD3	Hs04973910_s1
CAT	Hs00156308_m1
GPX1	Hs00829989_Gh
GPX2	Hs01591589_m1
GPX3	Hs01041668_m1
GPX4	Hs00989766_m1
GPX5	Hs00559733_m1
GSR	Hs00167317_m1
GSS	Hs00609286_m1
GSTZ1	Hs01041668_m1
18S rRNA	4310893E

Resulting RQ values for every sample group/experimental condition were averaged and standard error of the mean (SEM) values were calculated. Each experiment was performed on n=3 independent occasions.

4.3.3 Enzymic Antioxidant Protein Level Analysis

The gene expression levels of particular enzymic antioxidants (SOD1, SOD2, SOD3 and GSTZ1), in high and low proliferative DPSC sub-populations in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), were also verified at the protein level by Western blotting. DPSCs at selected population doublings (PDs) throughout their proliferative life-spans, were seeded at 5,000 cells/cm^2 in T-75 flasks and maintained at 37ºC/5% CO_2 in αMEM medium (ThermoFisher Scientific), in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM, ThermoFisher Scientific). Culture medium was changed every two days. Cells were grown to 80-90% confluence, the culture medium was removed, and the cells were washed briefly in ice-cold phosphate buffered saline (PBS, x1). DPSCs were subsequently harvested for total protein extraction.

4.3.3.1 Protein Extraction

Total protein was extracted using Pierce® Radio-Immunoprecipitation Assay (RIPA) extraction buffer (ThermoFisher Scientific), according to the manufacturer's instructions. Prior to use, one cOmplete™ Protease Inhibitor Cocktail tablet (Roche Ltd., Welwyn Garden City, UK), was dissolved in 25 ml ice-cold RIPA buffer (Table 4.2). DPSCs were lysed by the addition of RIPA/inhibitor buffer (400µl/flask), followed by scraping the contents of each flask into sterile Eppendorf® safe-lock tubes (1.5ml, Eppendorf Ltd., Stevenage, UK), to minimise contamination. To maximise protein yields, samples were sonicated with Digital SLPe™ Cell Disruptor (Branson Ultrasonics Corp., Slough, UK), then briefly vortexed before centrifuging at 10,000xg for 15min at 4°C. Resulting supernatants were subsequently transferred into fresh pre-chilled 1.5ml Eppendorf tubes and samples were stored at -80°C until required. In order to determine extract protein concentrations, supernatants were assessed using the Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (ThermoFisher Scientific), according to manufacturer's instructions. Bovine serum albumin (BSA) standard curves were prepared ranging between 125-2000µg/ml,

with PBS serving as the diluent. Standards and samples were vortexed briefly and centrifuged at 8000xg for 30s, at 4°C. Both standards and extracted supernatants were then added to a 96-well plate (10µl/well, in duplicate) and Pierce® Working Reagent (200µl/well, in Kit) added. Plates were then shaken for 30s and incubated at 37°C for 30min. Protein quantification was performed through measuring endpoint absorbance at 562nm, using a FLUOstar® Omega Plate Reader (BMG Labtech, Aylesbury, UK). Absorbance values were calculated and the BSA standard curves used to determine the protein contents in each extract, with mean extract protein concentrations expressed as µg/ml.

4.3.3.2 Western Blotting

DPSC protein extracts were separated and proteins of interest identified, using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Aliquots of each protein extract (10µg) were loaded into sterile Eppendorf® PCR tubes (0.2ml, Eppendorf Ltd.) and 4x reducing buffer (Bio-Rad Laboratories, Hemel Hempstead, UK, Table 4.2) added to each sample (3 parts sample:1 part reducing buffer). Tubes were briefly vortexed, centrifuged for 30s, incubated at 95°C for 5min and left to cool on ice. Protein samples and pre-stained Precision Plus Protein™ Kaleidoscope™ ladders (Bio-Rad Laboratories), were separated on pre-formed 4-15% TGX™ gels using the Mini-Protean® Tetra Cell System (Bio-Rad Laboratories), at 100V for 80min. The Tetra Cell tank was filled with 1X running buffer (Table 4.2).

During the electrophoresis period, membranes were prepared by consecutively soaking cut to size pieces of Hybond™-P polyvinylidene difluoride (PVDF) blotting membrane (GE Healthcare, Amersham, UK), in methanol, double-distilled water and transfer buffer (Table 4.2) for 5min each. Electroblot sponges were also prepared by soaking in transfer buffer. Post-separation, gels were removed from casts and assembled into a transfer 'sandwich' comprising the transfer cassette, electroblot sponges, gels, PVDF membranes and the extra thick filter paper. The completed transfer cassettes were placed with an ice block into the Mini Trans-Blot® Electrophoretic Transfer Cell System (Bio-Rad Laboratories) and filled with ice-cold 1X transfer buffer (Table 4.2). Protein samples and pre-stained Kaleidoscope™ ladders were electroblotting onto PVDF membranes at 100V for 1h. The protein transfer efficiency was determined for each blot by staining the PVDF membranes

with Ponceau S solution (Sigma, Poole, UK), for 5min at room temperature on a plate rocker, followed by removal of stain after effective transfer confirmation by washing with double-distilled water (x3, 10min each).

Post-transfer, membranes were incubated with blocking buffer (Table 4.2), on a plate rocker for 1h at room temperature, to prevent non-specific protein binding. Membranes were subsequently rinsed on a plate rocker with 1% Tris-buffered saline (TBS)-Tween (x1, Table 4.2), for 1min at room temperature. Membranes were incubated with the appropriate primary antibody, diluted in blocking buffer. Primary antibodies utilised and incubation conditions are listed in Table 4.3 (all raised in rabbit, Abcam, Cambridge, UK). Normalised protein loading on each blot was confirmed using a β-actin Loading Control (1:20,000, Abcam). After primary antibody incubation, membranes were washed with 1% TBS-Tween (3x5min) at room temperature on a plate rocker and incubated in horseradish peroxidase (HRP)-conjugated, swine anti-rabbit secondary antibody (1:5000, Dako, Ely, UK), in blocking buffer on a plate rocker for 1 h at room temperature. Membranes were then washed with 1% TBS-Tween (3x5min) at room temperature on a plate rocker, followed by two additional washes with TBS alone (5min each, Table 4.2), to reduce background. Proteins were visualised utilising the enhanced chemiluminescence (ECL) method. Membranes were incubated in ECL™ Prime Detection Reagent (VWR International, Lutterworth, UK) for 3min in the dark at room temperature, to ensure full coverage and reactivity, prior to placing in a Hypercassette™ (GE Healthcare). Autoradiographic films (Hyperfilm™-ECL, ThermoFisher Scientific) was placed on the membranes and exposed for the required amount of time, prior to being developed using a Curix-60 Auto-Developer (AGFA Healthcare, Greenville, USA). Immunoblot images were captured, and densitometry performed using ImageJ® Software (NIH Software, Version 1.49). Each experiment was performed on n=3 independent occasions.

Table 4.2. List of buffers used during protein extraction, SDS-PAGE separation and Western blot transfer and probing.

Buffer	Contents
Pierce® RIPA/cOmplete™ inhibitor lysis and extraction buffer (25ml).	25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 cOmplete™ protease inhibitor cocktail tablet.
Reducing buffer (1ml).	900µl 4x Laemmli sample buffer, 100µl β-mercaptoethanol.
Running buffer (10X).	0.25M Tris-HCl (30.3g/l), 1.92M glycine (144g/l), 1% SDS (10g), pH 8.3.
Transfer buffer (10X).	0.25M Tris-HCl (30.3g/l), 1.92M glycine (144g/l), pH 8.3.
Transfer buffer (1X).	100ml Transfer buffer (10X), 200ml methanol, 700ml double-distilled water.
Tris-buffered saline (TBS, 10X).	0.25M Tris-HCl (30.3g/l), 0.15M NaCl (80g/l), pH 7.4.
1% TBS-Tween	1l TBS (1X), 1ml Tween® 20.
Blocking buffer (50ml)	5% powdered skimmed milk (2.5g/50ml), 50ml 1% TBS-Tween.

Table 4.3. Summary of the primary antibodies and conditions used during Western blot analysis (all supplied by Abcam, Cambridge, UK).

Primary Antibody	Type and Host	Dilution	Expected Molecular Weight	Incubation Conditions
SOD1	Polyclonal, rabbit	1:1000	17kDa	4°C, overnight
SOD2	Polyclonal, rabbit	1:1000	26kDa	4°C, overnight
SOD3	Polyclonal, rabbit	1:1000	31kDa	4°C, overnight
GSTZ1	Polyclonal, rabbit	1:500	24kDa	4°C, overnight
β-actin	Polyclonal, rabbit	1:20,000	42kDa	1h, room temperature

4.3.4 Enzymic Antioxidant Activity Analysis

The total activities of particular enzymic antioxidants (SODs, catalase and GPX), in high and low proliferative DPSC sub-populations in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM), were also verified in cell extracts using commercial activity assays, according to manufacturer's instructions. Upon collection of cell extracts for total SOD, catalase and GPX activity analyses, the protein concentrations of each extract were quantified using the Pierce® BCA Protein Assay Kit described above, according to manufacturer's instructions. Each sample was loaded in duplicates and all experiments were carried out in triplicates.

4.3.4.1 Superoxide Dismutase Activity

Total SOD activities were determined using SOD Activity Colorimetric Assay Kits (Abcam). The Kit is based upon the reduction of the tetrazolium salt, WST-1, to formazan dye in the presence of $O_2^{\cdot-}$, produced by xanthine oxidase activity. WST-1 rates of reduction are linearly related to xanthine oxidase inhibition by SODs, with the extent of inhibition being quantifiable spectrophotometrically at 450nm.

High and low proliferative DPSCs at selected population doublings (PDs) throughout their proliferative life-spans, were seeded at 5,000 cells/cm^2 in T-75 flasks and maintained at 37ºC/5% CO_2 in αMEM medium, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM). Culture medium was changed every two days. Cells were harvested at densities of 2x10^6 cells/flask and lysed in ice-cold 0.1M Tris-HCl buffer, pH 7.4, containing 0.5% Triton X-100, 5mM β-mercaptoethanol and 0.1mg/ml phenylmethylsulfonyl fluoride (PMSF). Upon lysis, extracts were centrifuged at 14,000xg for 5min at 4°C; and the resultant supernatants collected into 1.5ml Eppendorf tubes on ice.

Samples, standards (SOD Human Standard, in Kit) and internal assay controls (in Kit) were aliquoted into wells of 96-well plates (20µl/well in duplicate), followed by the addition of Enzyme Working Solution (20µl, in Kit) to each well. The plates were mixed and incubated at 37°C for 20min, prior to absorbance values being read using a FLUOstar® Omega Plate Reader, at 450nm. Sample readings were obtained for each extract using standard curves and relative total SOD activities calculated using the following equation, with A= absorbance. Each experiment was performed on n=3 independent occasions.

$$\text{Total SOD Activity} = \frac{(\text{Ablank1} - \text{Ablank3}) - (\text{Asample} - \text{Ablank2})}{(\text{Ablank1} - \text{Ablank3})} \times 100$$

4.3.4.2 Catalase Activity

Total catalase activities were determined using Catalase Specific Activity Assay Kits (Abcam). The Kit measures catalase activity based on spectrophotometric changes in absorbance which occur due to catalase rapidly removing H_2O_2 preventing it reacting with luminescent substrate. The extent to which catalase prevents H_2O_2 reacting with the luminescent substrate can subsequently be determined spectrophotometrically at 450nm.

High and low proliferative DPSCs at selected population doublings (PDs) throughout their proliferative life-spans, were seeded at 5,000 cells/cm^2 in T-75 flasks and maintained at 37ºC/5% CO_2 in αMEM medium, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM). Culture medium was changed every two days. DPSCs ($2x10^6$ cells in total for each experimental condition) were harvested by scraping adherent cells from the culture flasks. Flasks were rinsed with PBS (x2) and the cellular contents solubilised in Extraction Buffer (200µl, in Kit), on ice for 20 min. Extracts were centrifuged at 16,000xg for 20min at 4°C; and the cell pellets discarded. The remaining supernatants were collected into 1.5ml Eppendorf tubes on ice.

Samples and standards (native catalase from human erythrocytes, Abcam) were aliquoted into wells of 96-well plates (100µl/well in triplicate) and incubated at room temperature for 3h, on a plate rocker at 300rpm. Wells were subsequently washed with Wash Buffer (300µl x2, in Kit), followed by the addition of Anti-Catalase Primary Detector Antibody (100µl, in Kit) to each well. The plates were mixed and incubated at room temperature for 1h, on a plate rocker at 300rpm. Wells were washed with Wash Buffer (300µl x2), to remove unbound detector antibody. HRP-Conjugated Secondary Antibody (100µl, in Kit) was added to each well and plates incubated at room temperature for 1h. Wells were washed with Wash Buffer (300µl x3), prior to the addition of HRP Development Solution (100µl, in Kit) to each well. Absorbance values were read over a 15min period, using a FLUOstar® Omega Plate Reader at 600nm. Sample readings were obtained for each extract using standard curves and relative total catalase activities calculated. Each experiment was performed on n=3 independent occasions.

4.3.4.3 Glutathione Peroxidase Activity

Total GPX activities were determined using Glutathione Peroxidase Assay Kit (Cambridge Bioscience, Cambridge, UK). The Kit indirectly measures GPX activity, based on spectrophotometric changes in absorbance which occur due to the oxidation of NADPH to $NADP^+$. GPX reduces hydroperoxide to oxidized glutathione (GSSG), which is then recycled to its reduced state by GR in the presence of NADPH. The oxidation of NADPH can subsequently be determined spectrophotometrically, due to decreases in absorbance at 340nm.

High and low proliferative DPSCs at selected population doublings (PDs) throughout their proliferative life-spans, were seeded at 5,000 cells/cm^2 in T-75 flasks and maintained at 37ºC/5% CO_2 in αMEM medium, in the absence and presence of sub-lethal doses of exogenous H_2O_2 (0, 50µM, 100µM or 200µM). Culture medium was changed every two days. Cells were harvested at densities of $2x10^6$ cells/flask and washed with ice-cold 50mM Tris-HCl buffer, pH 7.5, containing 5mM EDTA and 1mM dithiothreitol (DTT), prior to resuspension cold Assay Buffer (200µl, in Kit) and homogenisation on ice. Homogenised extracts were centrifuged for at 10,000g for 15min at 4°C to remove insoluble material and the resultant supernatants collected into 1.5ml Eppendorf tubes on ice.

Samples, standards (glutathione peroxidase from human erythrocytes, Sigma) and internal assay controls (in Kit) were aliquoted into wells of 96-well plates (20µl/well in triplicate), with Assay Buffer (120µl, 50mM Tris-HCl, pH 7.6, containing 5mM EDTA, in Kit) and Co-Substrate Mixture (50µl, consisting of NADPH, glutathione and GR, in Kit). Reactions were initiated by the addition of Cumene Hydroperoxide (20µl, in Kit) to each well and the plates agitated. Absorbance values were read over a 6min period, using a FLUOstar® Omega Plate Reader at 340nm. Sample readings were obtained for each extract using standard curves and total GPX activities calculated using the following equations, with A= absorbance. Each experiment was performed on n=3 independent occasions.

$$\Delta A_{340}/\text{min} = (\Delta A_{340} \text{ @ Time 2} - \Delta A_{340} \text{ @ Time 1}) / \text{Time 2 (min)} - \text{Time 1 (min)}$$

$$\text{GPX activity} = (|\Delta A_{340}/\text{min}| / 0.00373\mu M^{-1}) \times (0.19\text{ml} / 0.02\text{ml}) = \text{nmol/min/ml}$$

4.4 Statistical Analysis

Statistical analyses were performed via use of GraphPad Prism Software (GraphPad Software Inc., La Jolla, USA). Graphical data for each experimental condition/sample group are exhibited as average±standard error of the mean (SEM). For QPCR data, statistical significance was determined using Analysis of Variance (ANOVA), with post-hoc Tukey's multiple comparisons tests. Significance was considered at $p<0.05$.

4.5 Results

4.5.1 Enzymic Antioxidant Gene Expression

A number of different DPSC sub-populations previously isolated and characterised from 3 individual patient donors (patients A, C and D), for oxidative stress-induced biomolecular damage and senescence susceptibilities (Chapters 2 and 3), were also assessed for their respective enzymic antioxidant expression and activity capabilities throughout their proliferative life-spans, following treatment with increasing H_2O_2 concentrations (0, 50μM, 100μM, 200μM). These included previously identified high proliferative (highly resistant to oxidative stress-induced senescence) sub-population, A1 and low proliferative (highly susceptible to oxidative stress-induced senescence) sub-populations, A2, C3 and D4. Overall, marked differences in the expression and activities of particular enzymic antioxidants were identified between high and low proliferative DPSCs, irrespective of whether DPSCs were derived from the same or different patients.

QPCR analysis demonstrated distinct differences in the antioxidant gene expression profiles between high and low proliferative DPSC sub-populations. For the SOD genes analysed (SOD1, SOD2 and SOD3), although SOD1 and SOD3 expression were undetectable in all high proliferative DPSC sub-populations at early PDs (10-25PDs) and late PDs (40-60PDs) analysed (*data not shown*), significantly higher SOD1 and SOD3 expression were detectable in low proliferative DPSCs at early PDs (2-10PDs, both $p<0.001$), albeit at low levels with no significant increases in SOD expression with increasing H_2O_2 treatment ($p>0.05$, Figure 4.1). Despite no significant differences in SOD2 expression between high and low proliferative DPSCs at early PDs (10-25PDs and 2-10PDs, respectively) in the absence of H_2O_2 treatment ($p>0.05$, Figure 4.2), high proliferative DPSCs at early PDs (10-25PDs) demonstrated significantly higher (10-15 fold) inductions in SOD2 expression when

treated with increasing H_2O_2 concentrations ($p<0.001$-0.05), compared to H_2O_2-treated low proliferative DPSC sub-populations at early PDs (2-10PDs, $p<0.001$-0.05). However, high proliferative DPSCs at late PDs (45-60PDs) lost the ability to induce significant SOD2 expression upon treatment with H_2O_2, with no significant differences in SOD2 expression identified ($p>0.05$), compared to low proliferative DPSCs at early PDs (2-10PDs). Therefore, this resulted in H_2O_2-treated high proliferative DPSCs at late PDs (45-60PDs) having significantly lower SOD2 expression levels, compared to their early PDs (10-25PDs) counterparts ($p<0.001$-0.05).

Comparison of catalase gene expression demonstrated that expression was maintained at relatively low levels in all high and low DPSCs analysed (Figure 4.3). Although negligible basal catalase expression was determined in high proliferative DPSC sub-populations at early PDs (10-25PDs) in the absence of H_2O_2 treatment, low proliferative DPSCs at early PDs (2-10PDs) exhibited higher expression levels overall ($p<0.05$). However, whereas high proliferative DPSCs at early PDs (10-25PDs) demonstrated minor, non-significant inductions in catalase expression equivalent to levels in low proliferative DPSCs, when treated with increasing H_2O_2 concentrations ($p>0.05$), further inductions in catalase expression were not evident with H_2O_2-treated low proliferative DPSC sub-populations at early PDs ($p>0.05$, 2-10PDs). Similar to low proliferative DPSCs at early PDs (2-10PDs), high proliferative DPSCs at late PDs (45-60PDs) without H_2O_2 treatment, also demonstrated higher basal levels of catalase expression than at early PDs (10-25PDs, $p<0.05$), although high proliferative DPSCs at late PDs (45-60PDs) also failed to promote further significant inductions in catalase expression with H_2O_2 treatment ($p>0.05$), except at 200μM H_2O_2 treatment, compared to their early PDs (10-25PDs) counterparts ($p<0.01$).

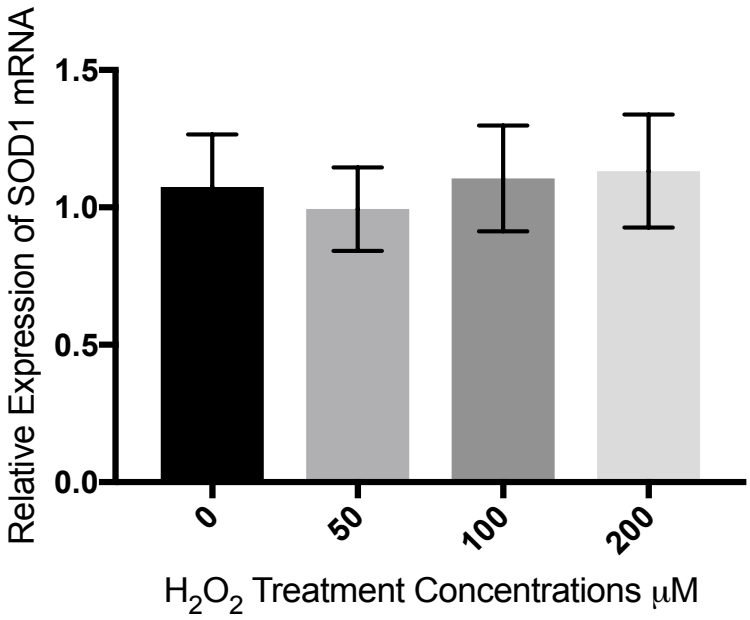
In terms of the expressions of antioxidant genes involved in glutathione metabolism, GPX1, GPX2, GPX3, GPX4, GPX5, GSR and GSS expression were undetectable in all high proliferative DPSC sub-populations at early PDs (10-25PDs) and late PDs (40-60PDs) analysed (*data not shown*). Similarly, GPX2 and GPX5 expression were undetectable in all low proliferative DPSC sub-populations at early PDs (2-10PDs) analysed (*data not shown*). However, despite exhibiting GPX1, GPX3, GPX4, GSR and GSS expression in low proliferative DPSCs at early PDs (2-10PDs, all $p<0.001$ versus high proliferative DPSCs at early and late PDs),

expression levels were only detectable at relatively low levels overall (Figures 4.4 and 4.5). Furthermore, GPX1 and GPX3 both demonstrated no significant inductions in expression by low proliferative DPSCs at early PDs (2-10PDs) with increasing H_2O_2 treatment ($p>0.05$, Figures 4.4). However, significant increases in GPX4 expression were shown by low proliferative DPSCs at early PDs (2-10PDs) with 200μM H_2O_2 treatment, compared to their untreated, 50μM H_2O_2 and 100μM H_2O_2 treated counterparts ($p<0.001$-0.01, Figures 4.4). Furthermore, GSR and GSS expression were significantly increased in low proliferative DPSCs at early PDs (2-10PDs), following treatment with 100μM and 200μM H_2O_2 and 100μM H_2O_2 treatments, respectively (all $p<0.05$, Figure 4.5).

Despite no significant differences in the expression of another glutathione metabolism enzyme, GSTZ1, between high and low proliferative DPSCs at early PDs (10-25PDs and 2-10PDs, respectively) in the absence of H_2O_2 treatment ($p>0.05$, Figure 4.6), high proliferative DPSCs at early PDs (10-25PDs) demonstrated significant inductions (100-125 fold) in GSTZ1 expression when treated with increasing H_2O_2 concentrations ($p<0.001$), compared to H_2O_2-treated low proliferative DPSC sub-populations at early PDs (2-10PDs). However, high proliferative DPSCs at late PDs (45-60PDs) lost the ability to induce significant GSTZ1 expression upon treatment with H_2O_2, with no significant differences in GSTZ1 expression identified ($p>0.05$), compared to low proliferative DPSCs at early PDs (2-10PDs). Therefore, H_2O_2-treated high proliferative DPSCs at late PDs (45-60PDs) possessed significantly lower GSTZ1 expression levels, compared to their early PDs (10-25PDs) counterparts (all $p<0.001$).

Figure 4.1. QPCR analysis of (A) SOD1 and (B) SOD3 gene expression by low proliferative DPSC sub-populations at early PDs (2-10PDs), following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, $p>0.05$. SOD1 and SOD3 expression were undetectable in all high proliferative DPSCs analyzed.

A)



B)

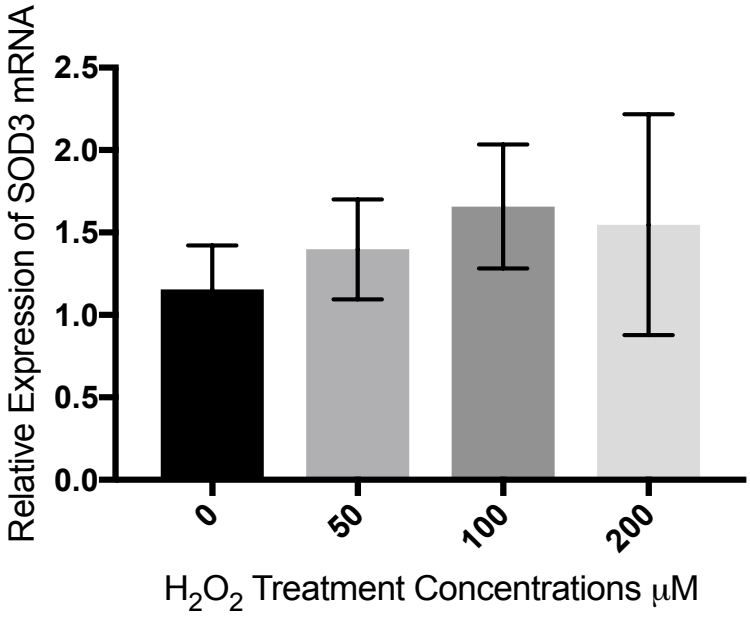


Figure 4.2. QPCR analysis of SOD2 gene expression by high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, *p<0.05, ***p<0.001.

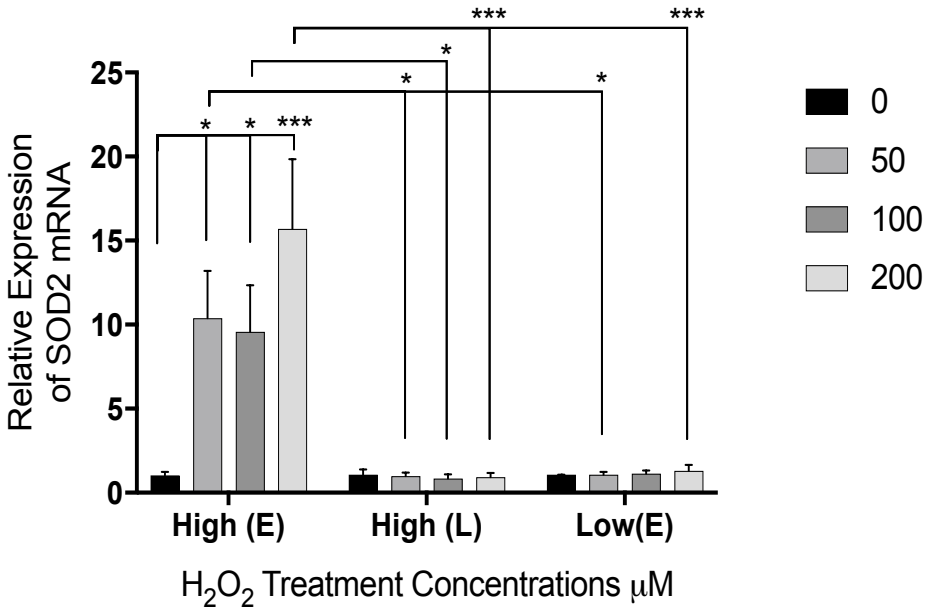


Figure 4.3. QPCR analysis of catalase gene expression by high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, *p<0.05, **p<0.01.

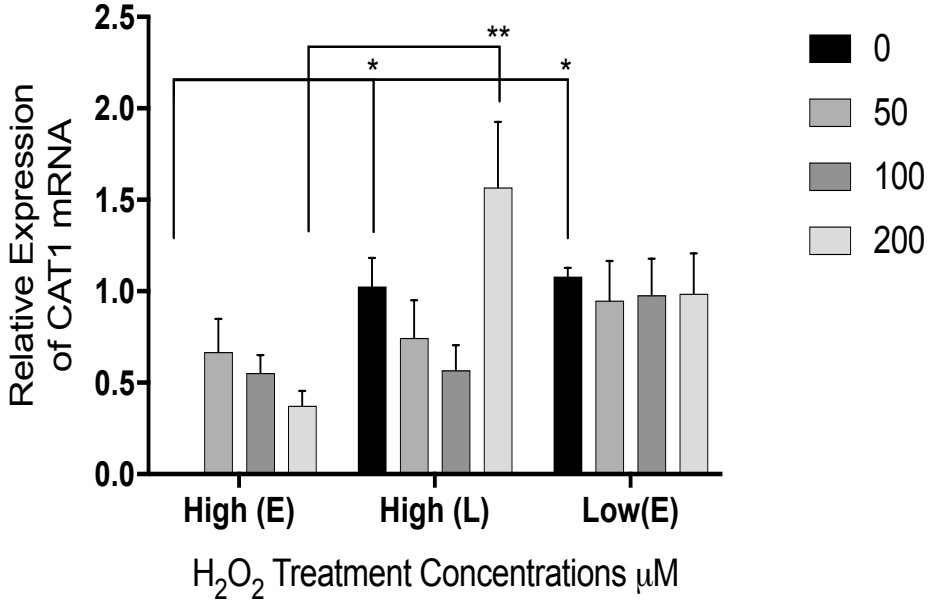
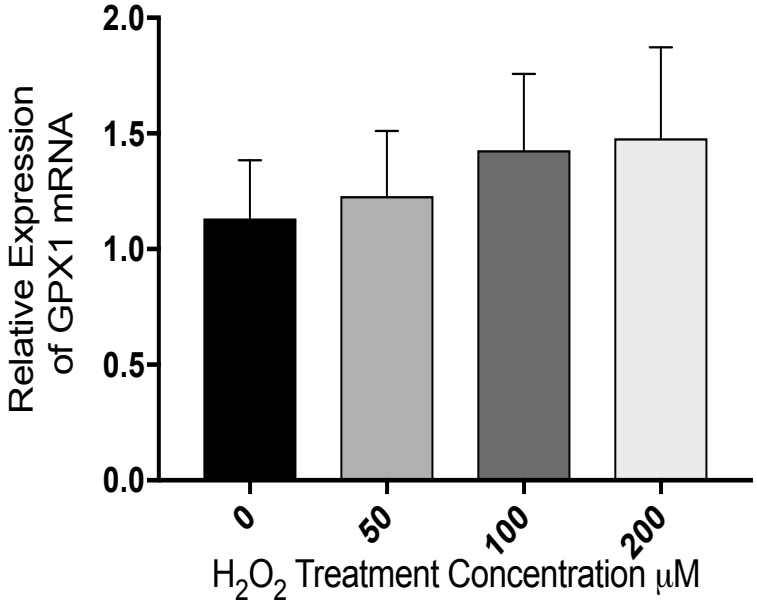
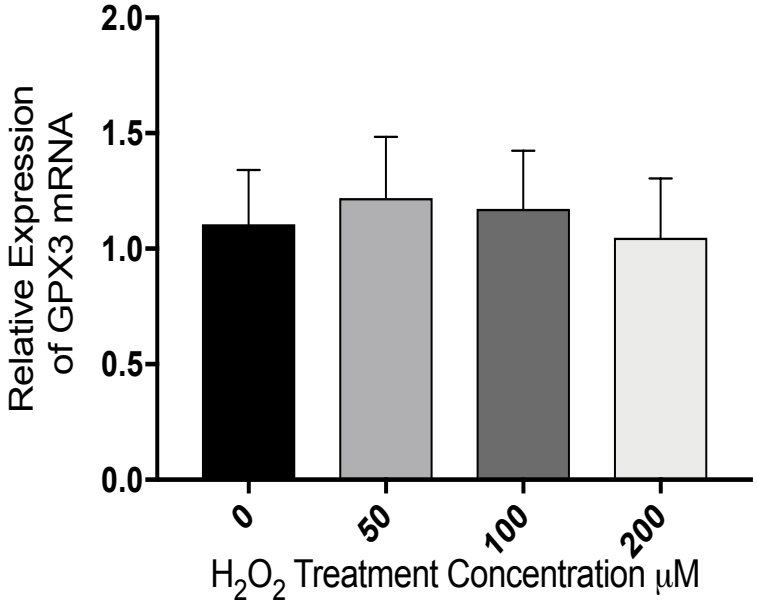


Figure 4.4. QPCR analysis of (A) GPX1, (B) GPX3 and (C) GPX4 gene expression by low proliferative DPSC sub-populations at early PDs (2-10PDs), following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, **p<0.01, ***p<0.001. GPX1, GPX3 and GPX4 expression were undetectable in all high proliferative DPSCs analyzed.

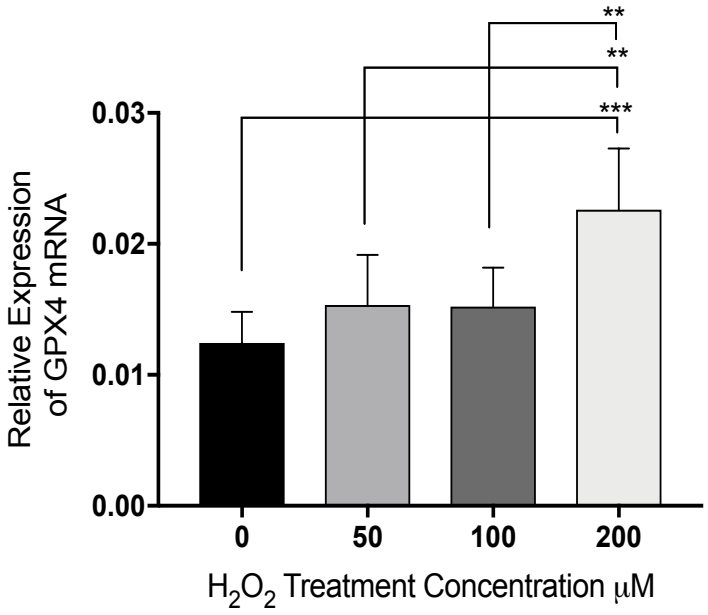
A)



B)



C)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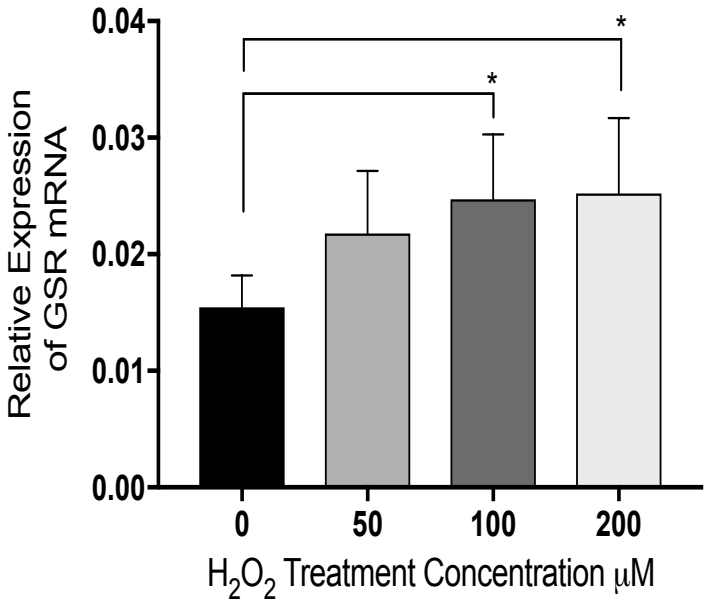
**
***
0.03
0.02
0.01
0.00
Relative Expression of GPX4 mRNA
0
50
100
200
H2O2 Treatment Concentration μM

Figure 4.5. QPCR analysis of (A) GSR and (B) GSS gene expression by low proliferative DPSC sub-populations at early PDs (2-10PDs), following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, *p<0.05. GSR and GSS expression were undetectable in all high proliferative DPSCs analyzed.

A)



B)

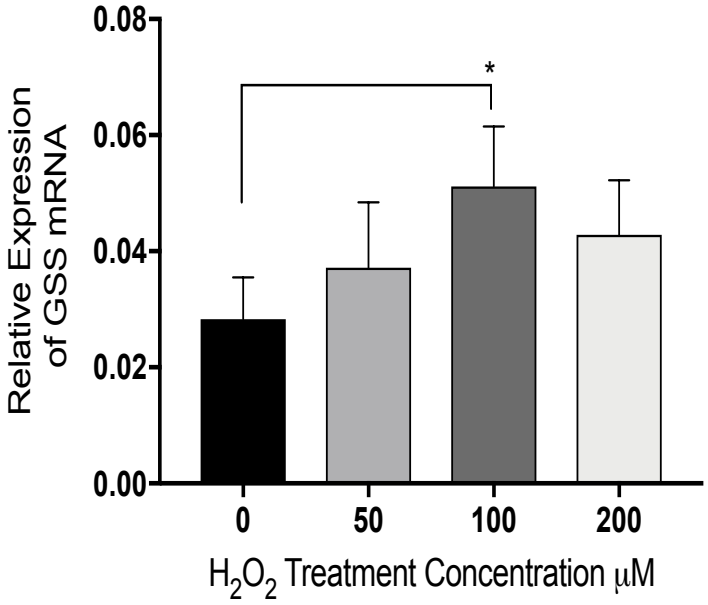
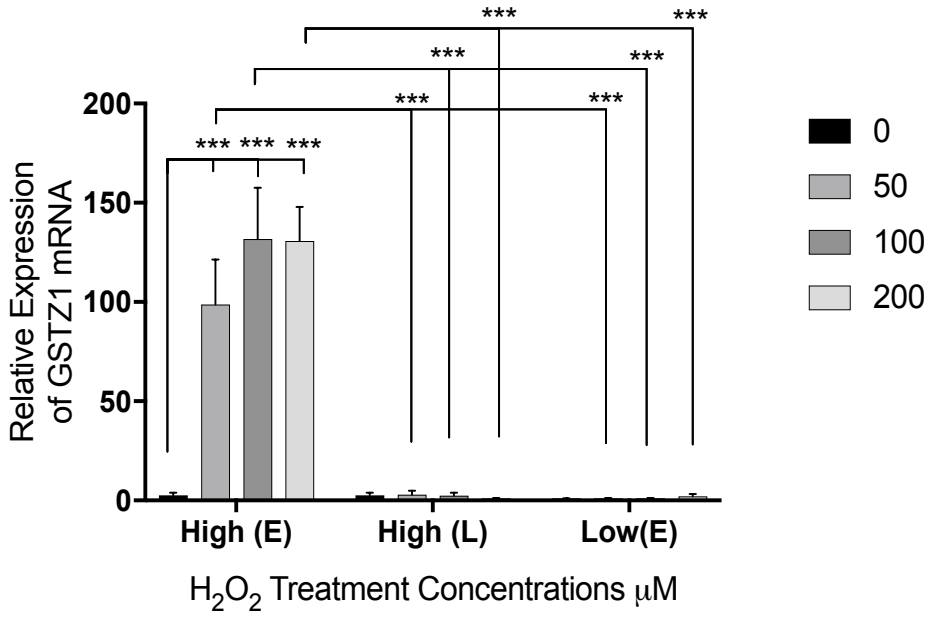


Figure 4.6. QPCR analysis of GSTZ1 gene expression by high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, ***p<0.001.



4.5.2 Enzymic Antioxidant Protein Levels

In line with the significant inductions in SOD2 and GSTZ1 expression levels identified in H_2O_2-treated high proliferative DPSCs at early PDs (10-25PDs) by QPCR, compared to low proliferative DPSCs at early PDs (2-10PDs) and high proliferative DPSCs at late PDs (45-60PDs), Western blot analysis was performed to validate SOD isoform and GSTZ1 findings at a protein level.

Western blot analysis of SOD1 levels demonstrated low levels of detectable SOD1 protein in high proliferative DPSCs at early PDs (10-25PDs), with no significant inductions in SOD1 levels at early PDs with increasing H_2O_2 treatment, although significant reductions in SOD1 protein levels were evident following 50μM H_2O_2 treatments ($p<0.01$). High proliferative DPSCs at late PDs (45-60PDs) exhibited similar SOD1 levels to their early PD counterparts on the whole, although significant increases in detectable SOD1 were identified with 50μM H_2O_2 treatments ($p<0.001$). In contrast, untreated low proliferative DPSC sub-populations at early PDs (2-10PDs) demonstrated significantly higher SOD1 protein levels ($p<0.01$-0.05), compared to high proliferative DPSCs at early (10-25PDs) and late PDs (45-60PDs). Furthermore, low proliferative DPSC sub-populations at early PDs (2-10PDs) showed further significant increases in SOD1 levels with 100μM and 200μM H_2O_2 treatments ($p<0.05$ and $p<0.01$, respectively). Consequently, H_2O_2-treated low proliferative DPSCs at early PDs (2-10PDs) possessed significantly higher SOD1 protein levels (all $p<0.001$), versus high proliferative DPSCs at early (10-25PDs) and late PDs (45-60PDs).

Analysis of SOD2 showed much higher levels of detectable SOD2 protein in high proliferative DPSCs at early PDs (10-25PDs), compared to low proliferative DPSCs at early PDs (2-10PDs, $p<0.001$, Figure 4.8). Although SOD2 levels significantly declined in high proliferative DPSCs at early PDs with 50μM H_2O_2 treatment ($p<0.001$), significant inductions in SOD2 levels were identified with 100μM and 200μM H_2O_2 treatments (both $p<0.001$). In contrast, SOD2 levels were virtually undetectable in low proliferative DPSCs at early PDs (2-10PDs), both in the absence and presence of H_2O_2 treatment (all $p<0.001$ versus untreated and H_2O_2-treated high proliferative DPSCs at early PDs). SOD2 levels in untreated high proliferative DPSCs at late PDs (45-60PDs) showed significant reductions compared to their early PD counterparts ($p<0.001$). Furthermore, despite significant increases in SOD2 levels with 50μM H_2O_2 treatments ($p<0.001$), significant decreases in

SOD2 levels were shown with 100μM and 200μM H_2O_2 treatments, compared to untreated controls ($p<0.01$ and $p<0.001$, respectively). However, despite such reductions, SOD2 levels in untreated and H_2O_2-treated high proliferative DPSCs at late PDs (45-60PDs) were still significantly higher than those quantified for untreated and H_2O_2-treated low proliferative DPSCs at early PDs (2-10PDs, all $p<0.001$).

SOD3 protein levels were undetectable in all high and low proliferative DPSCs analysed, irrespective of early or late PDs and H_2O_2 treatments (Figure 4.9). Consequently, no significant differences in densitometry were shown (all $p>0.05$).

GSTZ1 demonstrated significantly higher levels of detectable protein in high proliferative DPSCs at early PDs (10-25PDs), compared to low proliferative DPSCs at early PDs (2-10PDs), with further significant increases in GSTZ1 levels evident following each H_2O_2 treatment (all $p<0.001$, Figure 4.10). In contrast, GSTZ1 levels were undetectable in low proliferative DPSCs at early PDs (2-10PDs), both in the absence and presence of H_2O_2 treatment (all $p<0.001$ versus untreated and H_2O_2-treated high proliferative DPSCs at early PDs). This was also the case in high proliferative DPSCs at late PDs (45-60PDs), with no GSTZ1 protein detectable in control and H_2O_2-treated cultures (all $p<0.001$ versus untreated and H_2O_2 treated high proliferative DPSCs at early PDs).

Figure 4.7. Western blot and densitometric analysis of SOD1 protein levels in high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). For all Western blots, images from one representative experiment of three are shown. Densitometry data was normalised versus β-actin loading controls, with values subsequently normalised versus untreated high proliferative DPSCs at early PDs (10-25PDs). N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001. A.U. = Arbitrary units.

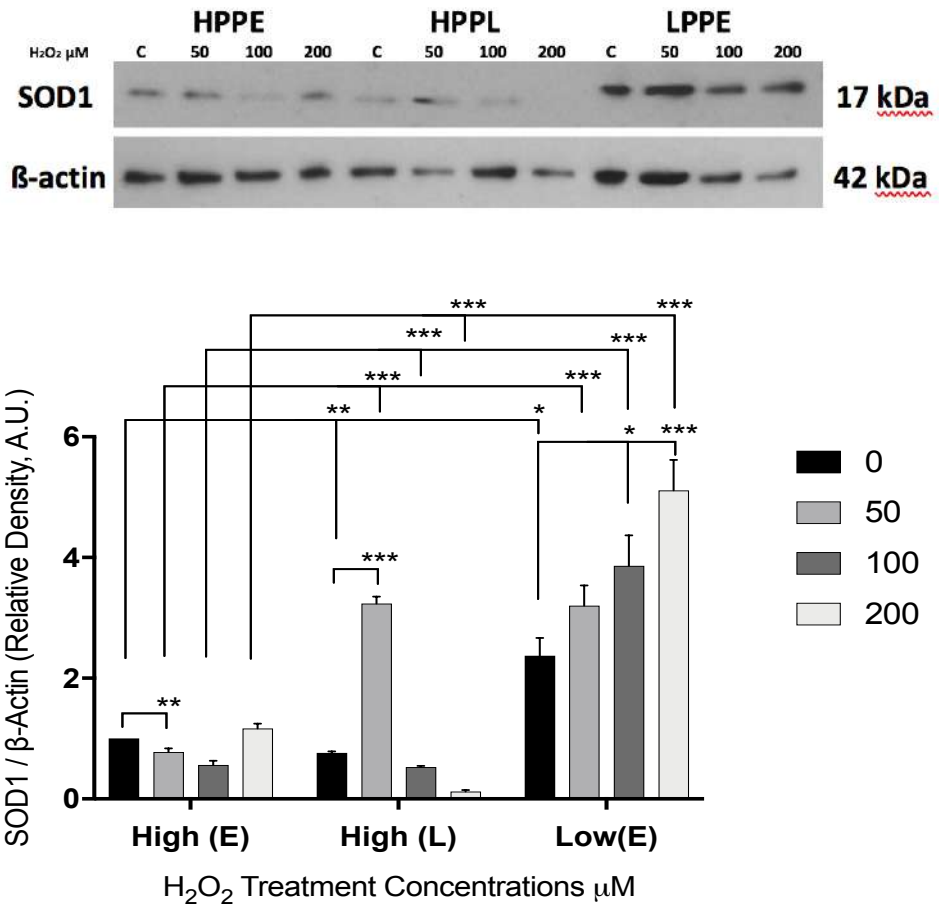


Figure 4.8. Western blot and densitometric analysis of SOD2 protein levels in high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). For all Western blots, images from one representative experiment of three are shown. Densitometry data was normalised versus β-actin loading controls, with values subsequently normalised versus untreated high proliferative DPSCs at early PDs (10-25PDs). N=3, average±SEM, **p<0.01, ***p<0.001. A.U. = Arbitrary units.

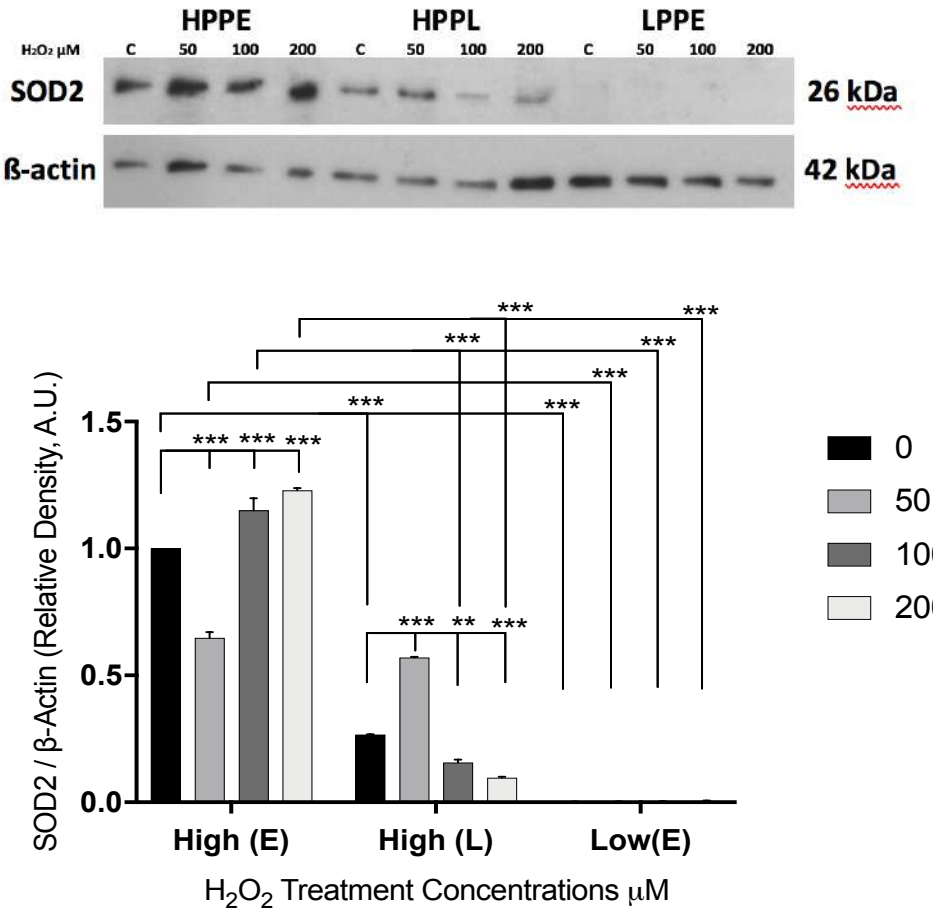


Figure 4.9. Western blot and densitometric analysis of SOD3 protein levels in high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). For all Western blots, images from one representative experiment of three are shown. Densitometry data was normalised versus β-actin loading controls, with values subsequently normalised versus untreated high proliferative DPSCs at early PDs (10-25PDs). N=3, average±SEM. A.U. = Arbitrary units.

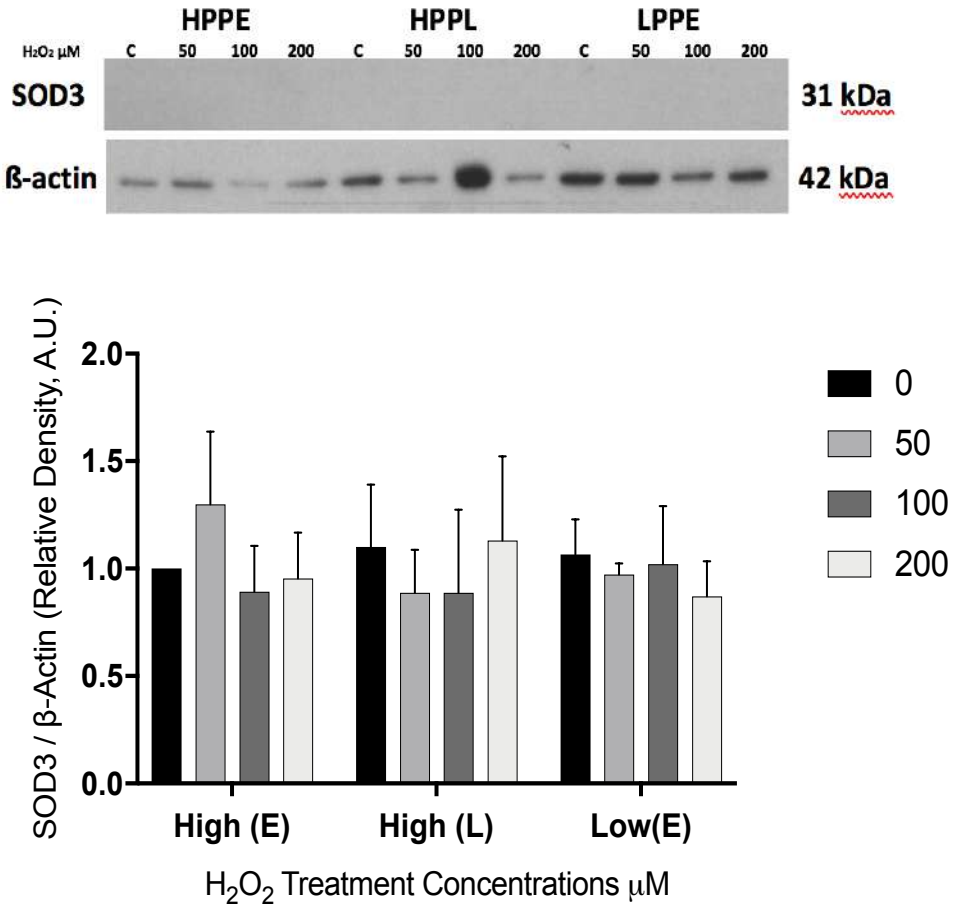
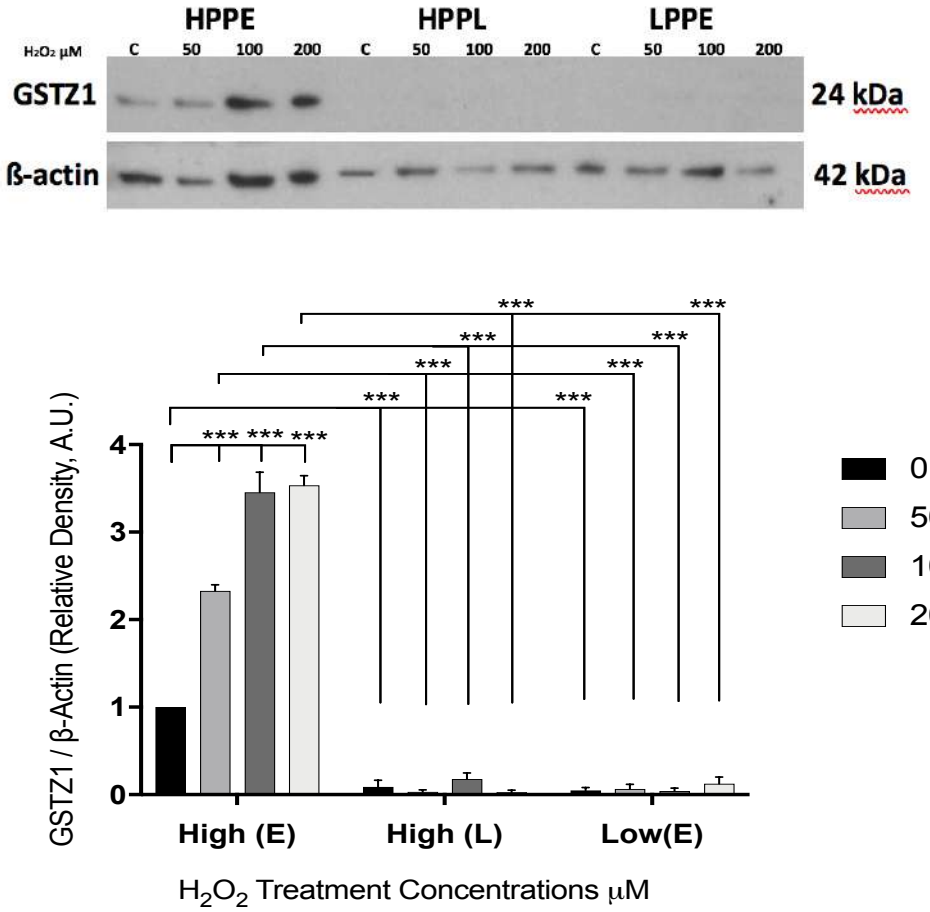


Figure 4.10. Western blot and densitometric analysis of GSTZ1 protein levels in high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). For all Western blots, images from one representative experiment of three are shown. Densitometry data was normalised versus β-actin loading controls, with values subsequently normalised versus untreated high proliferative DPSCs at early PDs (10-25PDs). N=3, average±SEM, ***p<0.001. A.U. = Arbitrary units.



4.5.3 Enzymic Antioxidant Activity Levels

In line with the relative expression and protein level difference identified for particular SOD, catalase and glutathione-related enzymes in high proliferative DPSCs at early PDs (10-25PDs), compared to low proliferative DPSCs at early PDs (2-10PDs) and high proliferative DPSCs at late PDs (45-60PDs), the total SOD, catalase and GPX activities within these DPSC sub-populations were quantified using commercial assays.

Total SOD activities were significantly increased in low proliferative DPSCs at early PDs (2-10PDs), compared to high proliferative DPSCs at early PDs (10-25PDs) and late PDs (45-60PDs), in the absence of H_2O_2 treatment (both $p<0.001$, Figure 4.11A). In contrast, high proliferative DPSCs at early PDs (10-25PDs) demonstrated significantly increased total SOD activities when treated with increasing H_2O_2 concentrations (all $p<0.001$), compared to H_2O_2-treated low proliferative DPSCs at early PDs (2-10PDs), which failed to induce further increases in SOD activities. However, high proliferative DPSCs at late PDs (45-60PDs) did not exhibit any increases in total SOD activities upon treatment with H_2O_2, with no significant differences in total SOD activities identified (all $p>0.05$), compared to low proliferative DPSCs at early PDs (2-10PDs). Therefore, H_2O_2-treated high proliferative DPSCs at late PDs (45-60PDs) demonstrated significantly lower total SOD activities, compared to their early PDs (10-25PDs) counterparts (all $p<0.001$).

Although total catalase activities were similarly at low levels in high and low proliferative DPSCs at early PDs (10-25PDs and 2-10PDs, respectively) in the absence of H_2O_2 treatment ($p>0.05$), significantly increased catalase activities were identified in high proliferative DPSCs at late PDs (45-60PDs) in the absence of H_2O_2 treatment, compared to high and low proliferative DPSCs at early PDs (both $p<0.001$, Figure 4.11B). However, high proliferative DPSCs at early PDs (10-25PDs) only demonstrated significantly increased total catalase activities when treated with 50μM H_2O_2 concentrations ($p<0.01$), compared to untreated high proliferative DPSCs at early PDs (2-10PDs), although levels were only equivalent to (i.e. at 100μM H_2O_2 concentrations, $p>0.05$) or significantly lesser than (i.e. at 50μM and at 200μM H_2O_2 concentrations, $p<0.05$ and $p<0.001$, respectively), catalase activities determined for untreated and H_2O_2-treated high proliferative DPSCs at late PDs (45-60PDs). In contrast, low proliferative DPSCs at early PDs (2-10PDs) exhibited low total catalase activities in the absence of increasing H_2O_2

treatment and an inability to induce further elevations in catalase activities upon increasing H_2O_2 treatment (all $p>0.05$). Consequently, total catalase activities for low proliferative DPSCs at early PDs (2-10PDs) treated with increasing H_2O_2 concentrations were significantly lower than high proliferative DPSCs at late PDs (40-65PDs) in the absence and presence of H_2O_2 treatment ($p<0.001$-0.05).

Total GPX activities were only detectable in low proliferative DPSCs at early PDs (2-10PDs, all $p<0.001$ versus high proliferative DPSCs at early and late PDs, Figures 4.11C), although non-significant inductions in total GPX activities were apparent following increasing H_2O_2 treatment (all $p>0.05$).

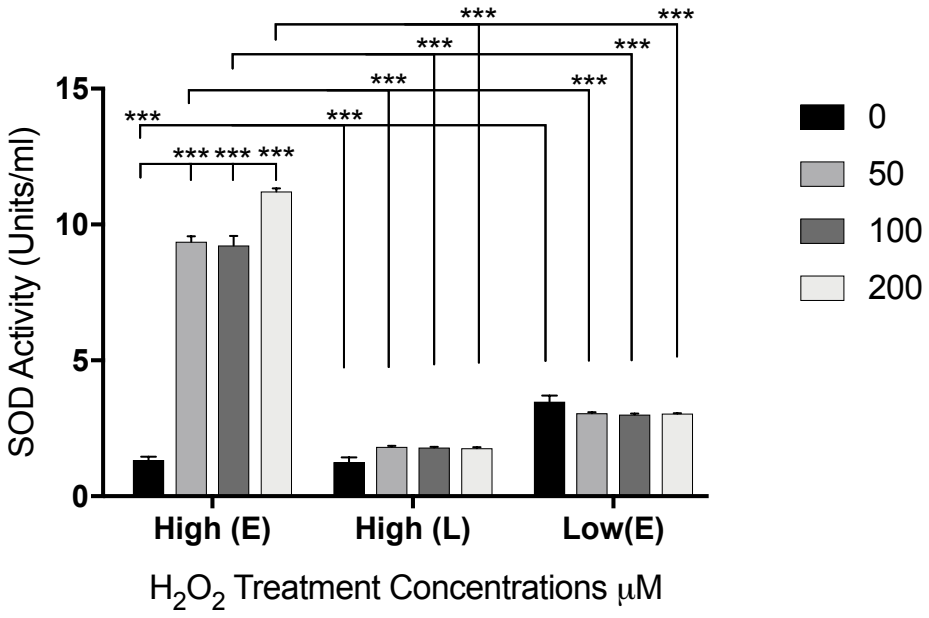
4.6 Discussion

The findings of previous Chapters 2 and 3, that significant variations exist in the relative susceptibilities of high and low proliferative DPSC sub-populations to oxidative stress-induced biomolecular damage and senescence, led to the hypothesis that differential enzymic antioxidant expression and activity profiles between high and low proliferative DPSCs contributed to such differences in the abilities to counteract oxidative stress exposure. From the findings presented in this Chapter and in support of this hypothesis, high proliferative DPSCs were demonstrated to possess distinct, and in some cases, superior abilities to induce enzymic antioxidant expression and activities upon H_2O_2 treatment, compared to low proliferative DPSCs.

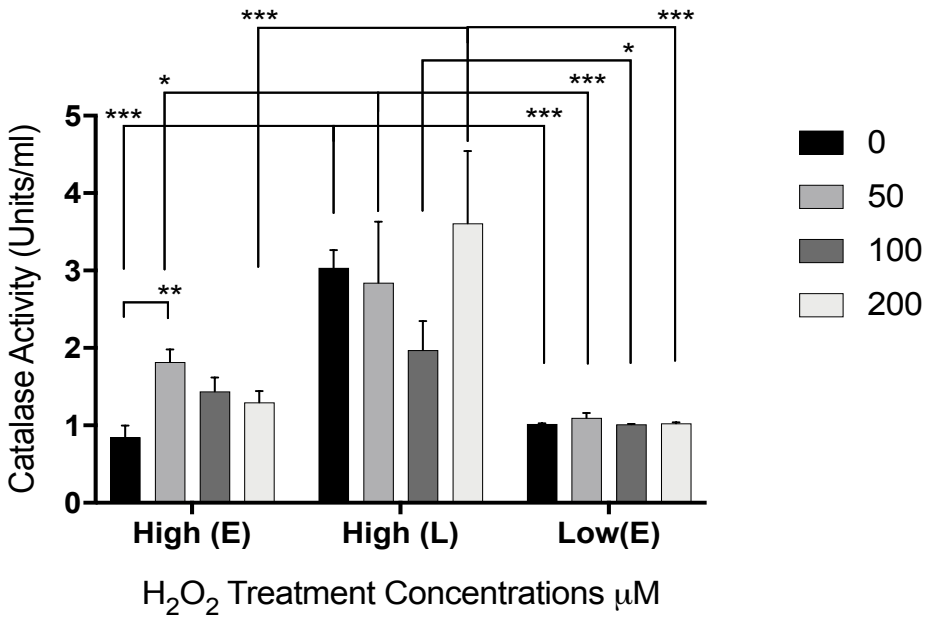
The ability of cells to induce upregulated antioxidant expression to counteract increasing ROS exposure, is a fundamental concept of oxidative stress and its role in disease, senescence and ageing (Tan et al., 2018). Indeed, enzymic antioxidant status has been shown to be critical in maintaining the intracellular redox balance and normal cellular functions, supported by studies demonstrating correlations between enzymic antioxidant expression/activity levels and cellular susceptibilities to oxidative stress-induced biomolecular damage and senescence in various cell types, including fibroblasts (Serra et al., 2000, 2003; Lorenz et al., 2001; Blander et al., 2003; Brown and Stuart, 2007; Richter and von Zglinicki 2007; Hammad et al., 2018) and MSCs (Valle-Prieto and Conget, 2010; Ko et al., 2012; Jeong and Cho, 2015; Yu et al., 2018; Chen et al., 2019). Thus, there is ever-increasing support for enzymic antioxidants as being major contributors to stem cell resistance to oxidative stress, via their possession of superior enzymic antioxidant

Figure 4.11. Activity assay analysis of (A) total SOD, (B) catalase and (C) GPX activities in high (10-25PDs and 45-60PDs) and low (2-10PDs) proliferative DPSC sub-populations at early (E) and late (L) PDs, following extended culture to senescence under increasing H_2O_2 concentrations (50μM, 100μM, 200μM). N=3, average±SEM, *p<0.05, **p<0.01, ***p<0.001. GPX activities were undetectable in all high proliferative DPSCs analyzed.

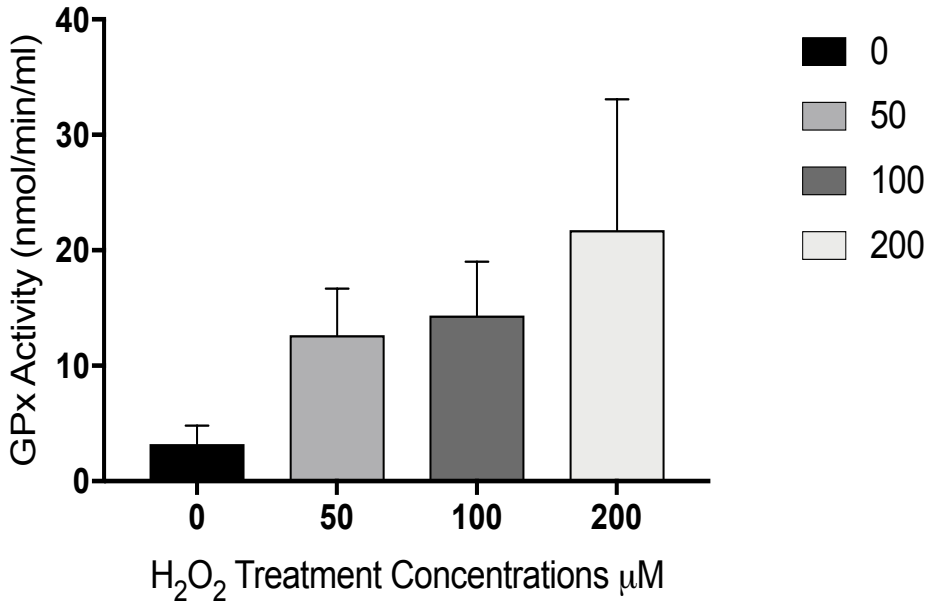
A)



B)



C)


40
30
20
10
0
GPx Activity (nmol/min/ml)
0
50
100
200
H2O2 Treatment Concentrations μM
0
50
100
200

induction capabilities (Liu et al., 2011; Shyh-Chang et al., 2013; Urao and Ushio-Fukai, 2013).

SOD profiles demonstrated distinct differences between high and low proliferative DPSCs, with SOD1 and SOD3 only being detectable in low proliferative DPSCs at low levels, with no significant inductions in expression with increasing H_2O_2 treatment. In contrast, despite similar basal SOD2 expression profiles in high and low proliferative DPSCs at early PDs (10-25PDs and 2-10PDs, respectively) in the absence of H_2O_2 treatment, only high proliferative DPSCs at early PDs (10-25PDs) demonstrated the ability to significantly induce SOD2 expression (10-15 fold) with increasing H_2O_2 treatment. However, this SOD2 induction capability was absent in low proliferative DPSCs at early PDs (2-10PDs). Such findings imply that SODs predominantly localised within cytoplasmic (SOD1) and extracellular (SOD3) regions do not contribute significantly to the antioxidant status of high proliferative DPSCs, although in line with the findings of previous Chapters 2 and 3, the induction of comparable SOD1 and SOD3 expression levels in untreated and H_2O_2-treated, low proliferative DPSCs may imply that these sub-populations are already experiencing increasing levels of oxidative stress and are reliant on these SODs to address these conditions (Zelko et al., 2002; Fukai and Ushio-Fukai, 2011; Tan et al., 2018). In contrast, the relatively high levels of SOD2 induction identified in high proliferative DPSCs, strongly suggests that SOD2 is a prominent mediator of antioxidant activity within these sub-populations. Indeed, similar contrasts between SOD1 and SOD2 expression profiles have previously been identified in high and low proliferative bone marrow-derived, MSCs (Bertolo et al., 2017). The relative total SOD activities determined between high and low proliferative DPSCs also appear to reflect these differences in expression. In light of the absence or limited detection of SOD1 and SOD3 expression and protein levels in high proliferative DPSCs at early PDs (10-25PDs), it is probable that the vast majority of the total SOD activity induced in high proliferative DPSCs by H_2O_2 treatment is accounted for by the significant upregulation in SOD2 expression. In contrast, gene expression and protein analyses suggest that SOD1 is the principle contributor to the lesser elevated total SOD activities identified in low proliferative DPSCs at early PDs (2-10PDs).

Mitochondria are the main cellular source of $O_2^{\cdot -}$ production as a by-product of oxidative phosphorylation, due to electron transport chain leakage. As a result,

mitochondrial-specific SOD2 is acknowledged as the primary antioxidant enzyme that protects cells from excessive ROS within the mitochondria and mitochondrial DNA against oxidative damage (Kienhöfer et al., 2009; Pervaiz et al., 2009; Sun et al., 2015; Sheshadri and Kumar, 2016). Unlike SOD1 and SOD3, the importance of SOD2 in mammals is highlighted by the fact that disruption/knockout leads to postnatal lethality, whilst SOD2 gene mutations result in premature ageing (Fukai and Ushio-Fukai, 2011; Sheshadri and Kumar, 2016). As mitochondria are also regarded as a principle source of endogenous ROS production during cellular senescence due to further alterations in oxidative phosphorylation, mitochondrial membrane potential and function (Passos and von Zglinicki, 2006; Passos et al., 2007; Geißle et al., 2012; Correia-Melo et al., 2016; Bertolo et al., 2017), these findings also imply that mitochondrial-derived ROS are significant mediators of cellular senescence in low proliferative DPSCs, whilst high proliferative DPSCs are able to resist oxidative stress-induced, senescence due to adaptations in SOD2 expression. As SOD1 and SOD2 are ubiquitously expressed by aerobic cells, the lack of SOD1 detection in high proliferative DPSCs is intriguing, although the absence of SOD3 expression in high proliferative DPSCs in particular, can be explained by its more specific expression profiles in cells and tissues where the required antioxidant activity exceeds that contributed by SOD1 and SOD2 (Zelko et al., 2002; Fattman et al., 2003; Nozik-Grayck et al., 2005; Fukai and Ushio-Fukai, 2011). However, although high proliferative DPSCs lacked SOD1 expression at the gene level, SOD1 was detectable in low quantities at a protein level which decreased further with H_2O_2 treatment. Such discrepancies between SOD1 gene and protein levels may be explained by very low or undetectable QPCR signals or unstable SOD1 mRNA levels that are subject to increased turnover, which were still accompanied by more stable protein signals detectable by Western blotting.

While SODs play a major role in the detoxification of $O_2^{\cdot -}$ and H_2O_2 formation, catalase and GPXs take major part in the enzymatic catabolism of these ROS, although excessive H_2O_2 accumulation is controlled via the coordinated actions of SOD2 and GSH within mitochondria (Kienhöfer et al., 2009; Pervaiz et al., 2009). Catalase profiles also demonstrated differences between high and low proliferative DPSCs, although only relatively low levels of catalase expression were detectable in high and low proliferative DPSCs overall. Despite being present at low/negligible levels in high proliferative DPSCs at early PDs (10-25PDs) in the

absence of H_2O_2 treatment, low proliferative DPSCs at early PDs (2-10PDs) exhibited higher expression and activity levels overall. However, neither high nor low proliferative DPSCs at early PDs were capable of consistently inducing significant inductions in catalase expression and activities with increasing H_2O_2 treatment, with similar levels identified between the sub-populations overall.

Similar catalase expression/activity profiles have been reported in high and low proliferative bone marrow-derived, MSCs (Bertolo et al., 2017). However, despite being a well-established and potent H_2O_2 detoxifying antioxidant, evidence suggests that catalase is particularly susceptible to down-regulated expression and/or enzymic inactivation following chronic ROS exposure, as reduced catalase activities have been reported in a number of tissues associated with excessive oxidative stress and disease (Góth and Eaton, 2000; Shi et al., 2007; Sadi et al., 2008; Waddington et al., 2011). Such findings suggest that low proliferative DPSCs at early PDs (2-10PDs) are exposed to chronic levels of oxidative stress, leading to an impaired ability to maintain catalase activities and degrade H_2O_2, resulting in enhanced oxidative stress-induced bimolecular damage and senescence. This would result in excessive H_2O_2 accumulation, especially as low proliferative DPSCs exhibited SOD activities capable of further increasing H_2O_2 formation via $O_2^{\cdot-}$ dismutation (Zelko et al., 2002; Fukai and Ushio-Fukai, 2011). Similar events may also be responsible for the dose-dependent reductions in total catalase activities in high proliferative DPSCs at early PDs (10-25PDs), with increasing H_2O_2 treatment, albeit to a much lesser extent than observed in low proliferative DPSCs at early PDs (2-10PDs). Therefore, although catalase appears to have a relatively minor role in mediating antioxidant responses in high proliferative DPSCs, as with SOD findings above, the induction of low levels of catalase expression in untreated low proliferative DPSCs may imply that these sub-populations are already experiencing increasing levels of oxidative stress and induce catalase expression as a consequence (Tan et al., 2018). However, despite increased catalase expression attempting to reduce intracellular H_2O_2 within the cytosol, the excessive oxidative stress that low proliferative DPSCs are exposed to inactivates catalase enzymic activities, preventing H_2O_2 removal.

The profiles of antioxidant enzymes associated with glutathione metabolism (GPX1, GPX2, GPX3, GPX4, GPX5, GSR and GSS), demonstrated that GPX, GSR and GSS expression or total GPX activities were undetectable in high proliferative DPSC sub-populations at early PDs (10-25PDs) and late PDs (40-60PDs).

Therefore, these findings imply that these glutathione-related antioxidant enzymes play negligible roles in the antioxidant status of high proliferative DPSCs. Similarly, although low proliferative DPSCs at early PDs (2-10PDs) exhibited GPX1, GPX3, GPX4, GSR and GSS expression and GPX4, GSR and GSS induction with increasing H_2O_2 treatment, the relatively low levels of gene expression and the total GPX activities detected overall further suggest that these GPXs, GSR and GSS are not major contributors to the antioxidant defence mechanisms of low proliferative DPSCs, to high proliferative DPSCs. However, high proliferative DPSCs at early PDs (10-25PDs) exhibited significant inductions in another glutathione metabolism enzyme, GSTZ1, at both gene and protein levels, compared to low proliferative DPSCs at early PDs (2-10PDs), with increasing H_2O_2 treatment in an attempt to adapt to the elevated oxidative stress.

While catalase convert H_2O_2 into water and oxygen without the requirement for cofactors, GPXs catalyse the decomposition of organic hydroperoxides (LOOH) and H_2O_2 utilising multiple cofactors, including GSH and GSR (Liu et al., 2004; Weydert and Cullen, 2010). Hence, GR does not act on ROS directly, but rather enable the GPX to function. In addition to GPXs playing crucial roles in oxidative stress defence mechanisms, GST has a primary function in the metabolism of ROS, via the conjugation of GSH to electrophilic centres on a wide variety of substrates, in order to detoxify xenobiotics (Wu and Dong, 2012; Board and Menon, 2013, Lu, 2013). The principle role of GSTZs is in catalysing GSH-dependent detoxification of xenobiotics and endobiotics (Board and Anders, 2011). Although GSTZ1 is located in the cytosol, it is also localised within the mitochondria. Indeed, relevant to the current study, increased mitochondrial GSTZ1 expression and activity has been shown to correlate with chronological ageing in human tissues, in contrast to cytosolic GSTZ1 (Zhong et al., 2018).

There is growing evidence that cellular oxidative stress tolerance is mainly due to the activity of the intrinsically expressed antioxidant enzymes (Liu et al., 2011; Shyh-Chang et al., 2013; Urao and Ushio-Fukai, 2013; Benameur et al., 2015; Sheshadri and Kumar, 2016). Although reports have shown the contrasting abilities of MSCs to resist oxidative stress, all agree that such variations are associated with the expression and activity levels of antioxidant enzymes, including SOD1, SOD2, catalase, GPX1 and GSH; with SOD2 in particular being implicated as having a prominent protective role (Valle-Prieto and Conget, 2010; Ko et al., 2012; Sheshadri

and Kumar, 2016). Indeed, in line with the induction of significantly elevated SOD2 and GSTZ1 expression in high proliferative DPSCs in particular, mouse embryonic fibroblasts from $SOD2^{-/-}$ mice demonstrate early-onset senescence, whilst MSCs from young mice lacking SOD2 exhibit increased senescent (Yang et al., 2013; Sart et al., 2015). Furthermore, highly proliferative bone marrow-derived MSCs possess lower mitochondrial membrane potential and reduced oxidative stress-induced senescence, due to the induction of elevated SOD2 expression (Bertolo et al., 2017). In contrast, endothelial progenitor cells are resistant to oxidative stress, due to high intrinsic SOD2 expression (He et al., 2004), whereas umbilical cord-derived MSCs have been shown to possess dramatically higher mitochondrial content, elevated $O_2^{\cdot -}$ and correspondingly increased SOD2 levels, compared to bone marrow-derived MSCs, leading to superior proliferation rates (Pietilä et al., 2012; Gan et al., 2015). Furthermore, human DPSCs have previously been shown to significantly increase SOD2 expression, in response to culture under increased oxygen tension (Mas-Bargues et al., 2017).

High SOD2 expression/activity levels have also been implicated as having essential roles in regulating ESC self-renewal and pluripotency, in addition to supporting superior MSC differentiation capabilities, via the regulation of ROS homeostasis (Chen et al., 2008; Pietilä et al., 2012; Gan et al., 2015; Sheshadri et al., 2015; Sheshadri and Kumar, 2016; Bertolo et al., 2017). Therefore, in addition to alleviating oxidative stress and promoting greater cell expansion in high proliferative DPSCs, their high induction of SOD2 expression and activities could also contribute to the maintenance of stem cell characteristics and multipotent differentiation capabilities in these DPSC sub-populations (Alraies et al., 2017). Thus, the relative levels of SOD2 expression appears to offer a useful characterisation of DPSCs and other MSC populations for *in vitro* assessment and translational development for clinical use. In contrast to SOD2, although GSTZ1 is an established antioxidant, few studies have correlated its expression with cellular susceptibilities to oxidative stress-induced biomolecular damage or senescence. However, increased GSTZ1 expression, especially mitochondrial GSTZ1, has been strongly associated with decreased human ageing and prolonged longevity, partly due to restrictions in cellular telomere shortening (Starr et al., 2008; Di Cianni et al., 2013; Zhong et al., 2018). Furthermore, $GSTZ1^{-/-}$ have also been shown to possess alterations in

mitochondrial ultrastructure, size and activity, confirming the important protective effects that GSTZ1 play in mitochondria (Lim et al., 2004; Board and Anders, 2011).

Although the expression and activities of certain enzymic antioxidants (SOD2 and GSTZ1) were demonstrated to be significantly induced beyond basal levels by increasing H_2O_2 treatment in high proliferative DPSCs at early PDs (10-25PDs), it was apparent that high proliferative DPSCs at late PDs (45-60PDs) did not share these abilities to induce antioxidant expression. However, high proliferative DPSCs at late PDs (45-60PDs) without H_2O_2 treatment demonstrated higher levels of catalase expression and activity than at early PDs (10-25PDs), although high proliferative DPSCs at late PDs (45-60PDs) failed to promote further inductions in catalase expression or activities with H_2O_2 treatments, compared to their early PDs (10-25PDs) counterparts. The absence of SOD2 and GSTZ1 antioxidant upregulation with increasing H_2O_2 exposure may suggest that the adaptive mechanisms of protection against oxidative stress are defective following extended expansion in culture. Previous studies have reported either a delayed antioxidant induction in fibroblasts until premature senescence is fully reached (Chen et al., 2004), or the severely impaired induction of antioxidant defence genes in senescent fibroblast populations (Kim et al., 2003), following oxidative stress exposure. Similar findings have also been reported in MSCs from various sources, with more aged cells exhibiting significantly lower SOD, catalase and GPX expression, leading to reduced antioxidant status and increased oxidative stress-induced bimolecular damage and senescence overall (Stolzing and Scutt, 2006; Geißle et al., 2012; Ko et al., 2012; Jeong and Cho, 2015; Yu et al., 2018; Chen et al., 2019). Thus, it appears that high proliferative DPSCs at late PDs (45-60PDs) possess certain impairments in their antioxidant defence capabilities due to persistent oxidative stress, culminating in their increased susceptibility to oxidative stress-induced bimolecular damage and senescence, in line with the findings presented in Chapters 2 and 3.

The results described in the present Chapter support the hypothesis that inherent differences exist in the enzymic antioxidant defence mechanisms and overall antioxidant status of high and low proliferative DPSCs, in line with similar correlations identified with other fibroblast and MSC populations, explaining the contrasting susceptibilities to oxidative stress-induced biomolecular damage and senescence between these DPSC sub-populations, as previously established

(Chapters 2 and 3). Specifically, the differential expression and activity profiles for SOD2 and GSTZ1 appear to be the main enzymic antioxidant mediators of the superior antioxidant profiles in high proliferative DPSCs at early PDs (10-25PDs). As SOD2 and GSTZ1 are predominantly localised within the mitochondria of cells, this implies that their antioxidant and anti-senescence properties principally protect high proliferative DPSCs from oxidative damage and senescence within mitochondria, proposed to be the major source of endogenous ROS production in senescent cell populations. In contrast, low proliferative DPSCs at early PDs (2-10PDs), fail to induce SOD2 or GSTZ1 upregulation upon oxidative stress exposure, being reliant on cytosolic and extracellular antioxidant defense mechanisms, such as SOD1, SOD3, catalase, GPXs, GSR and GSS. However, as these antioxidants are expressed at low/negligible levels in low proliferative DPSCs overall irrespective of H_2O_2 exposure, this suggests that low proliferative DPSCs are already exposed to excessive levels of oxidative stress which overcome normal antioxidant defences, resulting in defective antioxidant responses and enhanced oxidative stress-induced bimolecular damage and senescence. However, as identified with other fibroblast and MSC populations, the superior abilities of high proliferative DPSCs at early PDs (10-25PDs) to mount effective SOD2 and GSTZ1 defenses against oxidative stress exposure also become impaired at late PDs (45-60PDs) with progression through their proliferative lifespans towards senescence and despite significant inductions in catalase expression/activities, this leads to increased susceptibilities to oxidative stress-induced biomolecular damage and senescence, as previously described (Chapters 2 and 3). Therefore, overall, the results within this Chapter further highlight the complexity of cellular antioxidant systems, with MSCs derived from different stem cell niche and/or tissue sources exhibiting contrasting antioxidant responses and abilities to alleviate ROS accumulation and oxidative stress overall.

Chapter 5

General Discussion

Chapter 5

General Discussion

5.1 Overview

This PhD aimed to investigate the potential explanations for the contrasting differences in telomere length and proliferative potential between high and low proliferative dental pulp stem cells (DPSCs), focusing on whether differential oxidative stress responses and enzymic antioxidant profiles exist between DPSC sub-populations. The basis for undertaking this study was that despite significant heterogeneity in the *ex vivo* expansion capabilities of individual DPSCs being recognized for a number of years (Gronthos et al., 2000, 2002; Huang et al., 2009a), only recently had studies begun to address the impact of such variations in proliferative capabilities prior to replicative (telomere-dependent) senescence, on the multipotency of different DPSC sub-populations (Alraies et al., 2017). Although high proliferative DPSCs achieved >80 population doublings (PDs), low proliferating DPSCs only completed <40PDs before senescence, correlating with DPSCs with high proliferative capacities possessing longer telomeres (>18kb) and the absence of CD271 expression, compared to less proliferative populations (5-13kb). Low proliferative DPSC senescence was also associated with early loss of stem cell marker characteristics and impaired osteogenic and chondrogenic differentiation, in favour of adipogenesis. In contrast, high proliferative DPSCs retained multipotent differentiation capabilities, only demonstrating impaired differentiation following prolonged *in vitro* expansion (>60PDs, Alraies et al., 2017). However, since most studies report no or negligible human telomerase catalytic subunit (hTERT) expression in DPSCs (Egbuniwe et al., 2011; Mehrazarin et al., 2011a; Murakami et al., 2013; Alraies et al., 2017), such conclusions imply that other intrinsic mechanisms may be responsible for the maintenance of telomere lengths, proliferative capabilities and multipotency in highly proliferative DPSC sub-populations. Therefore, as oxidative stress is a prominent and well-established mediator of telomere-independent senescence (also known as premature senescence or stress-induced premature senescence, SIPS), in various cell types, including

somatic cells and MSCs (Liu et al., 2011; Shyh-Chang et al., 2013; Li et al., 2017); this PhD examined whether differential susceptibilities and responses to oxidative stress further contributed to these contrasting differences in proliferative and multipotency between high and low proliferative DPSCs.

In order to provide evidence for the enhanced ability of high proliferative DPSCs to resist oxidative stress, there was a need to confirm the differences between high and low proliferative DPSC sub-populations in their respective susceptibilities to oxidative stress induced via 50-200μM hydrogen peroxide (H_2O_2) treatments. While all DPSC sub-populations exhibited accelerated susceptibilities to oxidative stress-induced senescence in a H_2O_2 dose-dependent manner (especially at 100μM and 200μM concentrations), high proliferative DPSCs exhibited the highest resistance to H_2O_2-induced senescence, being capable of achieving 50-76PDs versus untreated controls (>80PDs). In contrast, low proliferative DPSCs exhibited accelerated earlier inductions of oxidative stress-induced senescence with H_2O_2 treatments (4-32PDs) and even in untreated controls (11-34PDs).

Such differences in relative susceptibilities to oxidative stress (H_2O_2-induced) senescence were accompanied by contrasting levels of detectable senescence-related markers (Ben-Porath and Weinberg, 2004; Campisi and d'Adda di Fagagna, 2007; Victorelli and Passos, 2017), with high proliferative DPSCs possessing reduced SA-β-galactosidase (SA-β-Gal) positivity and tumour suppressor marker (p53 and $p16^{INK4a}$) expression, at PDs where low proliferative DPSCs demonstrated overall increases in the detection of these and other senescence-associated markers, such as $p21^{waf1}$, particularly with increasing H_2O_2 concentrations. As both p53 and $p16^{INK4a}$ are both regarded as the principal mediators of senescence in MSCs (Shibata et al., 2007; Muthna et al., 2010; Mehrazarin et al., 2011; Feng et al., 2014) and as $p21^{waf1}$ also has a role in maintaining stem cell renewal due to its positive effects on cell cycle progression (Kippin et al., 2005; Ju et al., 2007), this may explain the presence of early $p21^{waf1}$ expression in all DPSC sub-populations analysed. Nonetheless, the contrasting p53 and $p16^{INK4a}$ expression between high and low proliferative DPSCs further confirms the earlier onset of oxidative stress-induced senescence with low proliferative DPSC sub-populations, versus high proliferative DPSCs at equivalent PDs.

The principle reason previously identified to be responsible for the contrasting proliferative responses and susceptibilities to telomere-dependent

senescence in DPSC sub-populations, were the respective average telomere lengths between these populations (>18kb versus 5-13kb for high and low proliferative DPSCs respectively, Alraies et al., 2017) with such superior telomere dynamic characteristics in high proliferative DPSCs sub-populations permitting extended PDS in culture, whilst protecting these DPSC from the onset of replicative senescence. However, in line with oxidative stress-induced senescence can occur irrespective to telomere length and without telomere shortening (Fumagalli et al., 2012; Bielak-Zmijewska et al., 2014, 2018), all DPSCs analysed generally retained their average telomere length profiles during extended culture, both in the absence and presence of oxidative stress.

In agreement with the majority of previous studies (Egbuniwe et al., 2011; Mehrazarin et al., 2011a; Murakami et al., 2013; Alraies et al., 2017), no or negligible hTERT expression was identified in the high and low proliferative DPSC sub-populations assessed herein. While hTERT expression is often an indication of the cell pluri-/multi-potency, down-regulation result in progressive telomere shortening and cellular senescence (Zimmermann et al., 2003, Flores and Blasco, 2010). Such findings imply that hTERT expression and activity appears not to be responsible for the maintenance of telomere lengths in high proliferative DPSCs or the variations in proliferative capabilities.

The expression of various mesenchymal (CD29, CD73, CD90 and CD105), hematopoietic (CD45), embryonic (Oct4, Nanog, SSEA4, CD146 and CD166) and neural crest stem cell markers (CD271 and Slug), were further assessed in high and low proliferative DPSC sub-populations (Huang et al., 2009; Waddington et al., 2009; Kawashima, 2012; Harrington et al., 2014; Alraies et al., 2017), to determine the impact of oxidative stress-induced senescence on the overall stem cell characteristics of high and low proliferative DPSCs. However, although all sub-populations were confirmed as being DPSCs, based on positive MSC marker (CD73, CD90, CD105) and absence of haematopoietic marker (CD45) expression, the expression of these and other stem cell markers were largely unaffected by increasing H_2O_2 treatment or culture expansion overall. However, certain markers, such as CD73, CD105, Oct4, SSEA4, CD271 and Slug, did decline with prolonged culture expansion in the absence or presence of H_2O_2 treatment, in line with previous findings (Takahashi and Yamanaka, 2006; Boroujeni et al., 2012; Tsai et al., 2012; Forristal et al., 2013; Alraies et al., 2017; Mas-Bargues et al., 2017). However,

intriguingly, despite BMI-1 expression also being proposed to be sensitive to oxidative stress and senescence, due to its role in repressing $p16^{INK4a}$ expression and maintaining cellular hyper-proliferation (Itahana et al., 2003; Park et al., 2004; Mas-Bargues et al., 2017), no obvious differences in BMI-1 expression were identified between the high and low proliferative DPSCs assessed herein, despite the apparent variations identified in their overall susceptibilities to oxidative stress-induced senescence. Nonetheless, the loss of these MSC markers by high or low proliferative DPSC sub-populations with prolonged culture expansion and/or H_2O_2 treatment, may impact on their retention of stem cell characteristics and altered differentiation capabilities (Rallapalli et al., 2009; Sivasankar and Ranganathan, 2015; Moraes et al., 2016; Alraies et al., 2017). Additionally, the display of such significant variations in stem cell marker expression between DPSC sub-populations further highlights the diverse heterogeneity which exists, with each sub-population having contrasting phenotypic properties and/or biological activities based on their origins or stem cell niches within the dental pulp (Gronthos et al., 2000, 2002; Huang et al., 2009; Sloan and Waddington, 2009; Ducret et al., 2016; Ledesma-Martínez et al., 2016).

Having established that significant variations in the susceptibilities of DPSCs to oxidative stress-induced (telomere-independent) senescence, the study sought to investigate whether contrasting susceptibilities to oxidative stress-related biomolecular damage also existed between high and low DPSC sub-populations. Oxidative stress-induced senescence is well-established to be accompanied by an accumulation of oxidative DNA, protein and lipid modification and damage (Beckman and Ames, 1998; Chaudhari et al., 2014), resulting in the formation of oxidative markers, such as 8-hydroxy-deoxy-guanosine (8-OHdG), protein carbonyl content, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE; Chen et al., 1995; Halliwell, 1999; Levine and Stadtman, 2001; Spiteller, 2001; Tahara et al., 2001; Cooke et al., 2003; Dalle-Donne et al., 2003; Evans et al., 2004; David et al., 2007).

In line with the contrasting susceptibilities of high and low proliferative DPSCs to oxidative stress-induced senescence, similar differences were also shown in the relative susceptibilities of such DPSC sub-populations to oxidative stress-induced DNA, protein and lipid damage. Indeed, low proliferative DPSC susceptibility to H_2O_2-induced senescence was accompanied by increased oxidative stress-induced bimolecular damage at early PDs (2-10PDs), with elevated DNA (8-

OHdG), protein (carbonyl content) and lipid (peroxidation) detection, irrespective of H_2O_2 treatments. In contrast, high proliferative DPSCs exhibited limited oxidative biomolecular damage at early PDs (2-10PDs), only equivalent to low proliferative DPSCs at much later PDs (45-60PDs).

Oxidative DNA damage and impaired DNA repair capabilities are established to accompany cellular senescence (Chen et al., 1995, 2003; Duan et al., 2005; Hazane et al., 2006; Hazane-Puch et al., 2010). Therefore, considering the increased susceptibilities of low proliferative DPSC sub-populations to oxidative stress-induced senescence, it is conceivable that these increases in oxidative DNA damage contribute to the early-onset of p53, $p21^{waf1}$ and $p16^{INK4a}$ induction and senescence in these DPSCs (Ben-Porath and Weinberg, 2004; Campisi and d'Adda di Fagagna, 2007; Victorelli and Passos, 2017). Furthermore, the early existence of elevated oxidative protein and lipid detection in low proliferative DPSC sub-populations reflects their enhanced susceptibility to oxidative stress-induced modification/damage and the accumulation of their oxidized products (Halliwell, 1999; Sitte et al., 2000; Spiteller, 2001; Kohen and Nyska, 2002; Chondrogianni and Gonos, 2004; Grune et al, 2005; Torres and Perez, 2008), compared to the high proliferative DPSCs.

As enhanced oxidative stress-induced bimolecular damage and senescence are a consequence of excessive reactive oxygen species (ROS) production and exposure, at the expense of cellular antioxidant defence mechanisms, the evidence supporting distinct variations in the susceptibilities of high and low proliferative DPSCs to oxidative stress-induced senescence and biomolecular damage, led to the hypothesis that such differences were related to differential enzymic antioxidant expression and activity profiles between these DPSC sub-populations. Indeed, enzymic antioxidant status is critical in maintaining the intracellular redox balance and cell survival, with strong correlations existing between superior cellular enzymic antioxidant capacities and resistance to oxidative stress and senescence, with such findings established in fibroblasts (Serra et al, 2000, 2003; Lorenz et al, 2001; Blander et al, 2003; Richter and von Zglinicki, 2007; Hammad et al., 2018) and mesenchymal stem cells (MSCs) from various sources (He et al., 2004; Valle-Prieto and Conget, 2010; Ko et al., 2012; Jeong and Cho, 2015; Yu et al., 2018; Chen et al., 2019).

In line with their resistance to oxidative stress-induced senescence and biomolecular damage, significant increases in superoxide dismutase (SOD2) and glutathione S-transferase ζ (GSTZ1) expression, in addition to significantly increased total SOD activities, were identified in high proliferative DPSCs at early PDs (10-25PDs). Such findings suggest that the differential expression and activity profiles for SOD2 and GSTZ1 are the main enzymic antioxidant mediators of the superior antioxidant profiles in high proliferative DPSCs at early PDs (10-25PDs). Mitochondrial-specific SOD2 is acknowledged as the primary antioxidant enzyme that protects cells from excessive ROS within the mitochondria and premature senescence, including MSCs (He et al., 2004; Kienhöfer et al., 2009; Pervaiz et al., 2009; Valle-Prieto and Conget, 2010; Fukai and Ushio-Fukai, 2011; Pietilä et al., 2012; Yang et al., 2013; Gan et al., 2015; Sart et al., 2015; Sun et al., 2015; Sheshadri and Kumar, 2016). High SOD2 expression/activity levels have also been implicated as having essential roles in regulating ESC self-renewal and pluripotency, in addition to supporting superior MSC differentiation capabilities, via the regulation of ROS homeostasis (Chen et al., 2008; Pietilä et al., 2012; Gan et al., 2015; Sheshadri et al., 2015; Sheshadri and Kumar, 2016; Bertolo et al., 2017). Therefore, in addition to alleviating oxidative stress and promoting greater cell expansion in high proliferative DPSCs, their high induction of SOD2 expression and activities could also contribute to the maintenance of stem cell characteristics and multipotent differentiation capabilities in these DPSC sub-populations (Alraies et al., 2017). Furthermore, increased GSTZ1 expression, especially mitochondrial GSTZ1, has been strongly associated with decreased human ageing and prolonged longevity, partly due to restrictions in cellular telomere shortening (Starr et al., 2008; Di Cianni et al., 2013; Zhong et al., 2018). Furthermore, GSTZ1$^{-/-}$ mice have also been shown to possess alterations in mitochondrial ultrastructure, size and activity, confirming the important protective effects that GSTZ1 play in mitochondria (Lim et al., 2004; Board and Anders, 2011). Therefore, as mitochondria are regarded as a principle source of endogenous ROS production during cellular senescence (Passos and von Zglinicki, 2006; Passos et al., 2007; Geißle et al., 2012; Correia-Melo et al., 2016; Bertolo et al., 2017), these findings also imply that SOD2 and GSTZ1 expression/activity profiles in high proliferative DPSCs, mediate antioxidant and anti-senescence protection in these sub-populations against mitochondrial ROS production and subsequent cellular damage/senescence.

In contrast to high proliferative DPSCs, the findings obtained suggest that low proliferative DPSCs at early PDs (2-10PDs), fail to induce SOD2 or GSTZ1 upregulation upon oxidative stress exposure, being reliant on cytosolic and extracellular antioxidant defense mechanisms, such as cytosolic SOD1, extracellular SOD3, catalase, glutathione peroxidases (GPXs), glutathione reductase (GSR) and glutathione synthetase (GSS). Indeed, similar contrasts between SOD1/SOD2 and catalase expression/activity profiles have previously been identified in high and low proliferative bone marrow-derived, MSCs (Bertolo et al., 2017). However, as these antioxidants are expressed at low/negligible levels in low proliferative DPSCs overall irrespective of H_2O_2 exposure, this suggests that low proliferative DPSCs are already exposed to excessive levels of oxidative stress which overcome normal antioxidant defences, resulting in defective antioxidant responses and enhanced oxidative stress-induced bimolecular damage and senescence. Indeed, despite being a well-established and potent H_2O_2 detoxifying antioxidant, evidence suggests that catalase is particularly susceptible to down-regulated expression and/or enzymic inactivation following chronic ROS exposure, as reduced catalase activities have been reported in a number of tissues associated with excessive oxidative stress and disease (Góth and Eaton, 2000; Shi et al., 2007; Sadi et al., 2008; Waddington et al., 2011). Such findings suggest that low proliferative DPSCs at early PDs (2-10PDs) are exposed to chronic levels of oxidative stress, leading to an impaired ability to maintain catalase activities and degrade H_2O_2, resulting in enhanced oxidative stress-induced bimolecular damage and senescence.

However, despite the superior abilities of high proliferative DPSCs at early PDs (10-25PDs) to elevate SOD2 and GSTZ1 expression, as with other fibroblast and MSC populations, such antioxidant responses become impaired at late PDs (45-60PDs) with progression through their proliferative lifespans towards senescence. The absence of SOD2 and GSTZ1 antioxidant upregulation with increasing H_2O_2 exposure may suggest that the adaptive mechanisms of protection against oxidative stress are defective following extended expansion in culture. Previous studies have reported either a delayed antioxidant induction in fibroblasts until premature senescence is fully reached (Chen et al., 2004), or the severely impaired induction of antioxidant defence genes in senescent fibroblast populations (Kim et al., 2003), following oxidative stress exposure. Similar findings have also been reported in MSCs from various sources, with more aged cells exhibiting significantly lower

SOD, catalase and GPX expression, leading to reduced antioxidant status and increased oxidative stress-induced bimolecular damage and senescence overall (Stolzing and Scutt, 2006; Geißle et al., 2012; Ko et al., 2012; Jeong and Cho, 2015; Yu et al., 2018; Chen et al., 2019). As high SOD2 expression/activity levels have also been implicated as having essential roles in regulating ESC self-renewal/pluripotency and supporting MSC differentiation capabilities (Chen et al., 2008; Pietilä et al., 2012; Gan et al., 2015; Sheshadri et al., 2015; Sheshadri and Kumar, 2016; Bertolo et al., 2017), the loss of SOD2 expression and activities could account for the loss of multipotency in high proliferative DPSCs following extended culture expansion (Alraies et al., 2017). Thus, it appears that high proliferative DPSCs at late PDs (45-60PDs) possess certain impairments in their antioxidant defence capabilities due to persistent oxidative stress, culminating in their increased susceptibility to oxidative stress-induced bimolecular damage/senescence and potential loss of stemness and multipotency properties.

Overall, the findings presented in this Thesis provide further support for the hypothesis that inherent differences exist in the enzymic antioxidant defence mechanisms and overall antioxidant status of high and low proliferative DPSCs, in line with similar correlations identified with other fibroblast and MSC populations. Such conclusions help explain the contrasting susceptibilities to oxidative stress-induced biomolecular damage and senescence between these DPSC sub-populations. The data also highlights the complexity of cellular antioxidant systems, with MSCs derived from different stem cell niche and/or tissue sources exhibiting contrasting antioxidant responses and abilities to alleviate ROS accumulation and oxidative stress overall. It has been proposed that high proliferative/multipotent DPSCs are minority sub-populations within dental pulp tissues (Sloan and Waddington, 2009; Alraies et al., 2107). Therefore, in order to exploit these desirable DPSCs for regenerative purposes, it is imperative that strategies are developed which permit the optimisation of population selection through the selectively screening and isolation of superior quality DPSCs from dental pulp tissues for *in vitro* expansion, assessment, bioprocessing and prudent cell banking. Such initiatives will aid the translational development of more effective DPSC-based therapies for clinical evaluation and application (Hilkens et al., 2016; Ferrúa et al., 2017; Rodas-Junco et al., 2017; Deans et al., 2018). Indeed, there are an ever-increasing number of cellular characteristics, such as telomere lengths and the relative expression of cell surface

markers (BMI-1, SSEA-4, CD271, STRO-1, c-Kit), being purported to characterise the presence of high proliferative/multipotent DPSC sub-populations (Mehrazarin et al., 2011; Kawanabe et al., 2012; Pisciotta et al., 2015; Yasui et al., 2016; Alraies et al., 2017). Consequently, the identification of significantly elevated SOD2 and GSTZ1 expression levels in high proliferative, multipotent DPSC sub-populations will allow the optimization of population selection by selectively screening and isolating better quality DPSCs from intact dental pulp tissues for *in vitro* expansion and assessment, aiding the translational development of more effective DPSC-based therapies for clinical evaluation and application.

5.2 Future Work

This PhD study can serve as a base for future studies that can further our knowledge on the mechanisms underlying the contrasting regenerative capabilities of high and low proliferative DPSCs. This Thesis showed that SOD2 and GSTZ1 expression and activities ae significantly elevated in high proliferative, multipotent DPSC sub-populations. Therefore, it would be strongly advised to examine this further through the stable knockdown of SOD2 and GSTZ1 gene expression in high proliferative DPSCs, using short hairpin RNA or CRISPR-CAS9 technology (Dowdy, 2017) and subsequently investigate the impact of such gene expression knockdowns on their susceptibilities to oxidative stress-induced biomolecular damage and senescence, stemness and multipotency characteristics.

As SOD2 and GSTZ1 are particularly localised within the mitochondria of cells (Lim et al., 2004; Board and Anders, 2011; Fukai and Ushio-Fukai, 2011; Pietilä et al., 2012; Di Cianni et al., 2013; Yang et al., 2013; Gan et al., 2015; Sart et al., 2015; Sun et al., 2015; Sheshadri and Kumar, 2016; Zhong et al., 2018), their protection against mitochondrial oxidative stress may contribute to the maintenance of proliferative and differentiation capabilities in high proliferative/multipotent DPSCs. Therefore, an alternative approach would be to examine whether low proliferative/unipotent DPSCs can be manipulated and 'rescued' from the negative effects of oxidative stress and senescence, via their over-expression of SOD2 and GSTZ1 through their transfection of non-viral plasmid or viral vectors carrying the SOD2 or GSTZ1 genes (Zhang and Godbey, 2006). Alternatively, exogenous small molecules may be applied to culture medium to induce similar effects, by specifically preventing mitochondrial oxidative stress or cellular senescence, leading

to improved proliferative and differentiation responses. Potential small molecules to examine for efficacy, include mitochondrial-targeting antioxidants (such as MitoQ and SkQ), suggested to provide higher protection against oxidative damage within mitochondria due to their ability to cross the mitochondrial membrane, hence eliminating ROS within their source (Oyewole and Birch-Machin, 2015; Feniouk and Skulachev, 2017). Alternatively, established cellular senescence inhibitors (such as the ROCK inhibitor, Y27632), may be evaluated to increase the proliferative and differentiation capabilities of low proliferative DPSCs (Uzawa et al., 2016; Park et al., 2018). Indeed, a recent study revealed that ROCK inhibitor, Y27632, caused ROS reduction and induced functional recovery of mitochondria in senescent fibroblasts, ultimately resulting in amelioration of senescence (Kang et al., 2017). Furthermore, a new class of synthetic and natural, bioactive compounds increasingly being evaluated as anti-senescence compounds are the senolytics which act by eliminating senescent cells or reversing the senescence-associated secretory phenotype (SASP, Mária and Ingrid, 2017; Gurău et al., 2018). Following 2D confirmation of such effects, further work could subsequently investigate the antioxidant or anti-senescence protective roles of such small molecules in 3D biomaterial environments and whether such methods enhance DPSC behaviour and therapy development in such settings (Zhou et al., 2016).

Another key consideration relating to the findings, is whether the contrasting proliferative, differentiation and enzymic antioxidant properties between high and low proliferative DPSC sub-populations reflect their isolation from different mesodermal or neuro-ectodermal origins (Pisciotta et al., 2015; Alraies et al., 2017) or stem cell niches within the dental pulp (Sloan and Waddington, 2009). Although the nature and origins of these DPSCs within dental pulpal tissues have yet to be elucidated, a better insight into their cell surface marker profiles by Flow Cytometry and/or genotypic profiles by Microarray technology, may shed new light on the developmental origins and stem cell niche sources of these contrasting DPSC sub-populations within dental pulp tissues (Gronthos et al., 2000, 2002; Sloan and Waddington, 2009; Alraies et al., 2017).

In summary, this Thesis has established and provided a greater insight into the oxidative defence mechanisms underlying the contrasting proliferative and differentiation capabilities of high and low proliferative DPSC sub-populations. Thus, the Thesis has enhanced our understanding of DPSC biology and its inter-

relationship with cellular ageing, in addition to ultimately providing additional cellular characteristic to utilise in the successfully screening, identification and isolation of superior quality high proliferative/multipotent DPSCs from dental pulp tissues. Furthermore, the Thesis has suggested certain small molecule approaches to overcome the reduced proliferative and unipotent capabilities of low proliferative DPSCs, potentially enabling us to increase their expansion and differentiation capacities beyond current levels. Such initiatives, therefore, may permit us to exploit DPSC sub-populations even more, for improved *in vitro* and *in vivo* assessment and more successful stem cell therapy development for eventual clinical use in regenerative medicine.

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