

Mitochondrial Reactive Oxygen Species Signalling and Vascular Smooth Muscle Cell Senescence

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> By Morufat Abisola Sanusi, BSc.

> > Department of Cardiovascular Sciences University of Leicester 2016

Abstract

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Morufat Abisola Sanusi, Cardiovascular Sciences Department, University of Leicester

Ageing is a risk factor for the development of cardiovascular disease. In particular, senescent vascular smooth muscle cells (VSMCs) have been observed within atherosclerotic plaques. Oxidants are widely implicated in vascular ageing and cardiovascular disease with evidence of oxidative stress in cells undergoing senescence.

Our previous data showed that Angiotensin II caused stress induced premature senescence (SIPS) in primary human VSMC via oxidant generation. Prevention of senescence with a mitochondria targeted antioxidant, Mito-TEMPO, suggested the mechanism was dependent on mitochondrial superoxide. The current study aimed to investigate if modulation of mitochondrial reactive oxygen species signalling is a general mechanism for senescence induction in human VSMC. The electron transport chain inhibitors Antimycin A and rotenone and the mitochondrial redox cycler, MitoParaquat all stimulated SIPS in VSMC. Interestingly, Antimycin A and rotenone also lead to a reduction in overall H_2O_2 levels suggesting a possible protective mechanism and highlighting the complexity of the signalling mechanism involving mitochondrial oxidants. qPCR Analysis suggested that changes in antioxidant gene expression do not account for the reduction in peroxide levels.

Although there was no evidence that Angiotensin II induced senescence in human coronary artery SMC, there was evidence for enhanced mitochondrial hydrogen peroxide production.

Senescent cells acquire a senescence associated secretory phenotype (SASP). To determine the composition of VSMC SASP, the tryptically digested secretome of conditioned media was analysed by LC-MS/MS. Bioinformatic analysis identified the NRF2-mediated oxidative stress response pathway and several endogenous antioxidants as amongst the affected responses in the aged VSMC secretome. These new data suggest that senescent VSMC produce a SASP that has multiple effects on neighbouring cell types including the induction of cell senescence and death; but also elements that might serve to preserve cell integrity and function and may limit the expression of a pro-inflammatory phenotype.

For Mummy & Daddy...x

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List of Abbreviations

ACE	Angiotensin Converting Enzyme
Angli	Angiotensin II
ARB	Angiotensin II Receptor Blockers
ARE	Antioxidant Response Element
AT(1 or 2)R	Angiotensin II Receptor Type (1 or 2)
B2M	β-2 microglobulin
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
CAT	Catalase
CDK	Cyclin Dependent Kinase
cpYFP	circularly permuted Yellow Fluorescent Protein
ĊVD	Cardiovascular Disease
Cvs	Cvsteine
DDR	DNA Damage Response
DHE	Dihydroethidium
DMEM	Dulbecco's Modified Fagle's Medium
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
FCs	Endothelial Cells
FCM	Extracellular Matrix
FGF	Enidermal Growth Factor
FTC	Electron Transport Chain
ERS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
GAPDH	Glyceraldebyde-3-phosphate debydrogenase
GDY	Glutathione perovidase
GSH	Glutathione
	Human Coronary Artery Smooth Muscle Cell
HDE	Human Dermal Fibroblast
HE	Hydroethidine
ныс	High Pressure Liquid Chromatography
	hTEPT_transfected human Arterial Smooth Muscle Cell
	Human Umbilical Voin Endotholial Colls
	Indolo 2 Acotic Acid
	Inculia like growth factor
	Insulin-like growth factor binding protoin
IGFBF	
	Liquid Chromotography Tandom Mass Chastromatry
LC-IVIS/IVIS	Liquid Chromatography randem Mass Spectrometry
	Materix Metalleproteineses
	Microporticlo
MpSOD	Manganasa Sunarovida Dismutasa
MEC	Masanchumal Stromal Calls
	mite chandrial DNA
	mitochondrial Electron Transport Chain
	mitochondrial Electron Transport Chain
	N agetul Leurteine
	N-alely-L-Cystellie
INF-KB	NUCLEAR FACTOR-KB

NO	Nitric Oxide
NOX	NAD(P)H Oxidases
NRF2	NF-E2-related factor
PAI	Plasminogen Activator Inhibitor
PD	Population Doubling
pRb	Retinoblastoma protein
PRX / PRDX	Peroxiredoxin/gene
RAS	Renin Angiotensin System
ROS	Reactive Oxygen Species
SA-β-galactosidase	Senescence Associated-β-galactosidase
SASP	Senescence-Associated Secretory Phenotype
SAHF	Senescence-Associated Heterochromatin Foci
SFM	Serum Free Media
SIPS	Stress-Induced Premature senescence
SOD	Superoxide Dismutase
ТВР	TATA-binding protein
tBHP	Tert-butylhydroperoxide
TGF-β	Transforming Growth Factor-β
TIMP	Tissue inhibitors of metalloproteinases
TNF-α	Tumour Necrosis Factor-α
TPP ⁺	Triphenylphosphonium cation
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular Smooth Muscle Cells
H_2O_2	Hydrogen Peroxide
•ОН	Hydroxyl radical
ONOO-	Peroxynitrite
0 ₂ •-	Superoxide anion
E 1	Ethidium

U ₂	Superoxide anion
E+	Ethidium
2-OH-E+	2-hydroxy-Ethidium
Mito-E+	Mito-Ethidium
2-OH-Mito-E+	2-hydroxy-Mito-Ethidium

CHAPTER ONE

Introduction

1.1 Role of VSMC in arterial disease

The arterial wall consists of three layers. The tunica adventitia is the strong, outermost layer comprised mainly of irregularly arranged collagen fibres, fibroblasts and tiny blood vessels called the vasa vasorum, or 'vessels of the vessels' in larger arteries. The middle layer is termed the tunica media and is made up of smooth muscle cells intermixed with elastic fibres. These fibres enable the arteries to stretch and recoil to accommodate the pressure provided by blood flow. This layer is thicker in arteries than in veins, permitting the artery to withstand high pressure from the narrow lumen. The innermost layer is the tunica intima; composed of a thin layer of cells known as endothelium, anchored to the wall by a layer of connective tissue. The cells in this layer reduce the friction of blood flow through the vessel (Sutton & Fagan, 2008; Braunwald, 2012).

Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are important in the cardiovascular system. ECs line the tunica intima and not only act as a selective permeability barrier, but they also react with physical and chemical stimuli, regulating homeostasis, vasomotor tone and immune and inflammatory responses (Rudijanto, 2007; Braunwald, 2012). VSMCs on the other hand are the predominant cellular element of the vascular media. They are responsible for vasoconstriction and vasodilation in response to normal and pharmacological stimuli (Rudijanto, 2007). In normal mature blood vessels, VSMCs predominantly have the quiescent contractile phenotype, for which their major function is the regulation of vasoconstriction and vasodilation. The alternative phenotype is the synthetic migratory phenotype, which is present in response to vascular injury. Cells of the synthetic migratory phenotype have reduced expression of proteins required for normal regulation of contractile function, yet they do possess an increased capacity to synthesise extracellular matrix (ECM) components, consequently controlling vascular construction (Rudijanto, 2007).

VSMC are intimately involved in atherogenesis. Following vascular injury, VSMCs migrate to the tunica intima of an artery or vein, where they proliferate and synthesise ECM proteins; thus they are important elements of both normal vascular repair and pathologic processes including atherosclerosis (Wang & Bennett, 2012). The cellular component of a plaque

comprises inflammatory and immune cells as well as VSMCs and ECs, and the process of atherogenesis is outlined in **Figure 1.1**.



Figure 1.1: Schematic of atherogenesis and an unstable atherosclerotic plaque. *Adapted from* (Wang & Bennett, 2012)

After injury or EC dysfunction (1), ECs express increased surface adhesion molecules to promote the attachment of inflammatory cells recruited from the circulation (2). Monocytes then differentiate into the macrophages upon entry, engulf lipid to form foam cells (3) and release mediators such as pro-inflammatory cytokines and growth factors. The release of these factors stimulates the reverse phenotypic shift of VSMCs from the quiescent contractile state to the active synthetic state enabling their proliferation and migration (4) from the media into the intima where they synthesise ECM components to form a fibrous cap that encloses the growing lipid core. Eventually the VSMCs undergo senescence and release multiple cytokines as part of the senescence associated secretory phenotype (5); which augments inflammation. With time, the engagement of the death receptors on VSMCs with the death ligands on immune cells causes the gradual loss of VSMCs by apoptosis (6). As the atheroma progressively enlarges, it may grow to a size where it occludes the blood vessel and restricts blood flow through the coronary artery. Plaque rupture (7) or endothelial erosion exposes the pro-thrombotic material within the core to the blood which causes coronary thrombosis.

Plaque rupture can lead to myocardial infarction and in some instances, sudden cardiac death (Hansson, 2005; Braunwald, 2012). In some instances, plaque rupture is frequently subclinical due to the ability of VSMCs to proliferate and synthesise ECM components thus repairing the rupture and reorganising the associated thrombus. Complicated plaques show evidence of multiple ruptures and repairs (Wang & Bennett, 2012).

1.1.1 Ageing and Cardiovascular Disease

Whilst typically considered a disease of developed countries, the environmental factors we encounter in the present day through economic development, urbanisation's promotion of poor eating habits and low physical activity, mean that we now face an epidemic that reaches far beyond Western societies (Braunwald, 2012; Wang & Bennett, 2012). Apart from environmental influences; there are traditional CVD risk factors such as age, diabetes, hyperlipidemia, and hypertension (Tsirpanlis, 2008).

Ageing is a well-known risk factor in atherosclerotic disease. Coronary heart disease, stroke, and peripheral vascular disease incidence increase with age (Tsirpanlis, 2008; Wang & Bennett, 2012). Biomarkers of ageing include telomere shortening and cellular senescence; the characteristics of cell senescence are described below in sections **1.2** and **1.3**. In the human aorta, smooth muscle cells in atherosclerotic plaques show distinct morphological and biochemical features of senescence (Vasile *et al.*, 2001; Gorenne *et al.*, 2006; Minamino & Komuro, 2007). Plaques obtained from arteries of patients with ischaemic heart disease were found to contain ECs and VSMCs positive for SA β -galactosidase and expressing high levels of p16, p21 and p53 senescence markers (Munoz-Espin & Serrano, 2014; Minamino & Komuro, 2007). In advanced plaques, senescence-positive VSMCs also exhibit other functional abnormalities including increased expression of proinflammatory molecules highlighting the role senescence may play in the pathogenesis of vascular ageing (Minamino & Komuro, 2007).

A complicating aspect is that blood vessels undergo structural changes and their compliance decreases as the years progress, even in the absence of atherosclerosis (Tsirpanlis, 2008). Therefore, a complex relationship between normal arterial ageing and development of atherosclerosis is apparent.

Telomere attrition, apoptosis and senescence of vascular cells at sites of atheroma in man and experimental animals strongly implicate biological ageing as a key contributor to the development of CVD (Bennett *et al.*, 1998; Minamino *et al.*, 2002; Herbert *et al.*, 2008). Therefore, there is great interest in understanding how these processes work and to develop better mechanistic biomarkers of ageing arteries, in order to enable the development of therapies that can help prevent the onset of these diseases or reduce the morbidity and mortality.

1.2 Cellular Senescence and Telomeres as Markers of Biological Ageing in CVD

1.2.1 Senescence

There are many environmental and lifestyle factors that have the potential to be damaging to the cells of an organism, including smoking, sunlight, diet as well as exercise (Anand *et al.*, 2008). As the exposure to these factors is prolonged, the cells are subjected to a process termed as cellular senescence. Senescence is defined as a terminal differentiation state in which cells have undergone irreversible proliferation arrest and they display altered gene and protein expression profiles compared to those still proliferating (Min *et al.*, 2009). These cells are not only resistant to growth factors and other signals that induce cell proliferation, but they are also characterised by distinctive morphological and other phenotypic changes including the activation of the guardian of the genome, p53; increased expressions of tumour suppressor proteins p21 and p16; increased activity for the enzyme, Senescence-Associated β -galactosidase (SA- β -galactosidase); increased formation of senescence-associated heterochromatin foci (SAHF); permanent DNA damage, chromosomal instability and an inflammatory secretome termed the Senescence Associated Secretory Phenotype (SASP) (reviewed in Sikora *et al.*, 2011). In some cases, oxidative damage can also be observed (Wang & Bennett, 2012; Zhang *et al.*, 2015).

As a direct consequence of permanent growth arrest, cellular senescence impairs the selfrenewal potential of organs such as the liver. Due to cell-type specific changes in gene expression, the functional properties of senescent cells may be affected; in some cases, these cells become more resistant to apoptosis (Erusalimsky & Kurz, 2005). Despite these various

changes, senescent cells are still metabolically active, allowing them to be maintained in culture for several years (Shay & Roninson, 2004).

The phenomenon of senescence was first observed by Leonard Hayflick in the early 1960s (Shay & Wright, 2000). Hayflick intended to expose normal cells to cancer-cell extracts, in the hope of observing cancer-like changes in the cells (Shay & Wright, 2000). However, what he found rather was that after a finite number of cell divisions, the primary human fibroblasts succumbed to an irreversible proliferation arrest (Adams, 2009). He proposed a theory for his observations, which stated that normal cells cannot divide indefinitely because they are programmed for a set proliferative lifespan (Mathon & Lloyd, 2001). The postulated evolutionary benefit of Hayflick's observations was that the halt in cell division blocked immortalisation and provided a simple mechanism to stop the transmission of accumulated mutations from mother to daughter cells (Colavitti & Finkel, 2005). Beyond halting cells before they potentially become tumorigenic, recent findings have revealed the importance of a scheduled and programmed process of senescence in development and physiology. Senescence occurs during embryonic development however this developmental senescence, despite having the usual distinctive features of senescence such as positive staining for SA β galactosidase; increased heterochromatin markers; and increased expression of cell cycle inhibitors; there is a lack of p53 activation as well as the DNA damage markers observed with damage-induced senescence (Munoz-Espin & Serrano, 2014; van Deursen, 2014).

Senescence can occur by 'Replicative Senescence' or by 'Stress-Induced Premature Senescence' and **Figure 1.2** shows how these processes converge to activate downstream messengers that lead to the induction of senescence.



Figure 1.2: Established Triggers and Effectors of Cellular Senescence. Adapted from (Adams, 2009)

A variety of intrinsic and extrinsic factors are able to induce senescence. Factors including shortened telomeres and oxidative stress stimulate cellular signalling pathways that result in the activation of p53, increased expression of p21 and subsequent inhibition of Rb phosphorylation, leading to the induction of senescence. The activation of oncogenes also causes p53 activation via ARF, a protein that sequesters a p53 antagonist named mdm2. p16 is also believed to be activated through a variety of factors, causing subsequent pRb activation. The dashed lines represent links that are poorly defined mechanistically. The senescent phenotype is indistinguishable, irrespective of the inducer.

1.2.2 Telomeres as markers of biological ageing in replicative

senescence

Replicative senescence is the term used to describe senescence induced as a result of the countdown of an intrinsic mitotic counter; known as telomere attrition (Mathon & Lloyd, 2001). Telomeres are repetitive DNA sequences, TTAGGG in humans, which cap the ends of linear chromosomes and prevent their fusion by cellular DNA repair processes. Because

functional telomeres maintain the integrity and stability of the genome, they suppress the development of cancer (Campisi, 2005). At birth, telomere lengths in most tissues from the same individual are similar (Wang & Bennett, 2012). However, with each cell division, the telomeres shorten due to incomplete duplication known as the 'end replication problem'. This incomplete duplication occurs as DNA polymerases are unidirectional and cannot prime a new DNA strand; resulting in loss of DNA near the end of the chromosome (Hanahan & Weinberg, 2000). As a consequence, telomere length can be used to track a tissue's replication history and has been used as a surrogate of 'biological' age (Rodier & Campisi, 2011; Wang & Bennett, 2012). Apart from the end replication or dysfunction of the proteins involved in telomere maintenance. As telomeres shorten, the senescence response ensures that cells with dysfunctional telomeres are withdrawn from the cell cycle on a permanent basis, rendering them incapable of forming a tumour (Campisi, 2005).

Telomerase enables cells to elongate and maintain their telomeres, leading to immortalisation (Passos & Von Zglinicki, 2006). Once differentiated, most human somatic cells do not express telomerase, and so would be predicted to have a limited replicative lifespan as stated by Hayflick (Mathon & Lloyd, 2001). In certain specialised cells such as germ cells and stem cells however, the expression of Telomerase Reverse Transcriptase (TERT), the catalytic subunit of the enzyme Telomerase, allows these cells to proliferate indefinitely. Some cancer cells overexpress telomerase, giving them the opportunity to be immortal (Tsirpanlis, 2008); and the ectopic expression of TERT can also yield immortal cell lines as *in vitro* culture models, with non-transformed, non-tumourigenic phenotypes (McKee *et al.*, 2003; Poh *et al.*, 2005; Rensen *et al.*, 2007).

A relationship between telomeres and atherosclerotic disease has been described in many studies. A 2003 study (Brouilette *et al.*) investigated 383 subjects who had suffered from myocardial infarction before the age of 50. They found a highly significant and strong association between mean leukocyte telomere restriction fragment (TRF) length and premature myocardial infarction. Their results showed that a mean TRF length shorter than the average increased the risk of myocardial infarction by approximately 3fold. The authors suggested that there may be a genetic basis for the difference in the results observed between the controls and the cases and proposed that individuals born with relatively shorter

telomeres may be at a higher risk of developing premature coronary heart disease (Brouilette *et al.*, 2003).

A later and larger study by the same group containing 1542 subjects confirmed their previous suggestions. The results from this study found that individuals with a leukocyte telomere length in the lowest or middle tertile were at an increased risk of developing coronary heart disease compared to individuals with the longest telomeres (Brouilette *et al.*, 2007). Following these studies, many studies into various diseases have been conducted and reviewed (Saliques *et al.*, 2010) with the hypothesis that telomere shortening could be involved in the onset and development of CAD and increased mortality. Recent studies have now established causality – a significant inverse association between telomere length and coronary heart disease was found (Haycock *et al.*, 2014; Smeets *et al.*, 2015).

1.2.3 Stress Induced Premature Senescence

Stress-Induced Premature Senescence (SIPS) is the term used to describe senescence induced by factors extrinsic to the cell (Mathon & Lloyd, 2001). Such factors include oxidative stress, protein aggregation and DNA damage. Telomeres can also be involved in SIPS, however not due to telomere attrition, but rather due to changes in the structure of the telomeres (Tsirpanlis, 2008; Tchkonia *et al.*, 2013). Cells are capable of undergoing senescence following exposure to strong mitogenic signals such as the oncogenic Ras protein, which induces both p53 and p16. Constitutive activation of MEK, a component of the mitogen activated protein kinase cascade, is required for this senescence induction (Lin *et al.*, 1998). Other less studied stresses capable of inducing senescence include epigenetic, nucleolar and mitotic spindle stresses (van Deursen, 2014). For example, genome-wide chromatin decompression by exposure to inhibitors of histone modifying enzymes is capable of inducing senescence in a manner dependent on p21 (Romanov *et al.*, 2010).

SIPS and replicative senescence share common molecular signals which usually involve stabilisation of the tumour suppressor protein, p53; increased expression of a cyclin-dependent kinase (CDK) inhibitor, p21; and subsequent hypophosphorylation of the retinoblastoma protein, pRb, which mediates growth arrest through its action on the cell cycle (Toussaint *et al.*, 2000; Herbert *et al.*, 2008). Another tumour suppressor protein, p16, also

acts as a CDK inhibitor playing a role in mediating growth arrest for some stressors (Toussaint *et al.*, 2000) (**Figure 1.2**).

1.3 The Senescence Associated Secretory Phenotype

The Senescence Associated Secretory Phenotype (SASP), also known as the Senescence Messaging Secretome, is an inflammatory secretome that senescent cells acquire once they have undergone widespread alterations in their genetic and proteomic profiles (Coppe *et al.*, 2010). The SASP is mediated by NF- κ B and CCAAT/enhancer-binding protein- β (CEBP β) transcription factors (Munoz-Espin & Serrano, 2014). These regulate the expression and secretion of a large array of factors including proinflammatory cytokines, chemokines, growth factors, proteases, ECM components, and oxidant species. The SASP also creates a positive feedback loop, reinforcing the proliferation arrest of the senescent cells (Coppe *et al.*, 2010; Sikora *et al.*, 2011).

Regardless of the secreting cell type, the composition of SASP may vary with time and the proteins secreted and their amounts may depend on the inducing stress (Tchkonia *et al.*, 2013). Nevertheless, the senescence-associated changes in gene and protein expression are believed to be specific and mostly conserved within individual cell types (Coppe *et al.*, 2010). This is important as there is unlikely to be one 'singular SASP concoction' that all senescent cells uniformly secrete, so the components of SASP derived from different cell types are particularly fascinating as we attempt to probe certain diseases at the molecular level.

The main families of the different secreted components are described below and shown in **Table 1.1**.

Factor	Secretory	Cell Type	Reference
	profile of		
	senescent		
	selle		
	cens		
Soluble signalling factors – chemokines, cytokines and growth factors			
IL-6	↑	HUVECs, HDFs (fibroblasts), Keratinocytes, melanocytes,	(Coppe <i>et al.</i> , 2010; Rodier & Campisi, 2011)
		epithelial cells	2011)
IL-8	↑	Fibroblasts	(Coppe <i>et al.</i> , 2010; Rodier & Campisi, 2011)
IL-1α and IL-1β	^	ECs, fibroblasts	(Coppe <i>et al.,</i> 2010)
GRO-α and GRO-β	↑	Fibroblasts	(Coppe <i>et al.,</i> 2010; Freund <i>et al.,</i> 2010)
IGFBP2, 3, 4, 5, 6, 7	^	Mesenchymal stem cells, melanocytes, HUVECs, epithelial cells, fibroblasts	(Kim <i>et al.</i> , 2007; Severino <i>et al.</i> , 2013; Tchkonia <i>et al.</i> , 2013; Wajapeyee <i>et al.</i> , 2008)
VEGF	↑	ECs, fibroblasts, mesothelial cells	(Lee & Lee, 2014; Coppe <i>et al.</i> , 2010; Rodier & Campisi, 2011)
Soluble signalling factors – Proteases and regulators			
MMP1, 3, 10, 12	^	Fibroblasts, keratinocytes	(Rodier & Campisi, 2011; Hassona <i>et al.,</i> 2014)
PAI1 and 2	^	VSMC, ECs, fibroblasts, progeroid mice	(Najjar <i>et al.,</i> 2005; Yamamoto <i>et al.,</i> 2005; Tchkonia <i>et al.,</i> 2013)
uPA/tPA	↑	Fibroblasts	(Coppe <i>et al.,</i> 2010;
	Soluble on che	d nagantang an liggn da	Freund <i>et al.,</i> 2010)
Octoon rot or of sile			
Osteoprotegerm	Т	FIDIODIASIS	Freund <i>et al.</i> , 2010,
PAI1	^	Fibroblasts	(Kortlever & Bernards, 2006; Coppe <i>et al.</i> , 2010)
	ЕСМ	l components	,
Fibronectin	↑	ECs, fibroblasts	(Kumazaki <i>et al.,</i> 1993; Coppe <i>et al.,</i> 2010)
Collagens	Altered	Fibroblasts	(Coppe <i>et al.,</i> 2010)
Laminin	Altered	Fibroblasts	(Coppe <i>et al.,</i> 2010)
Non-protein soluble factors			
NO	↑	ECs, fibroblasts	(Coppe <i>et al.,</i> 2010)
ROS	Altered	ECs, fibroblasts	(Coppe <i>et al.,</i> 2010)
Table 1.1: SASP Factors previously identified and compiled into published reviews			

Factors significantly altered between the pre-senescent and senescent state.

1.3.1 Soluble signalling factors

1.3.1.1 Cytokines

The most prominent reported cytokine secreted by senescent cells is Interleukin-6 (IL-6), which increases significantly after DNA damage and oncogene-induced senescence in various mouse and human cell types (Davalos *et al.*, 2010). IL-6 plays a central role in diverse host defence mechanisms. *In vivo* studies using IL-6 knockout mice show impaired immune and acute phase responses (Simpson *et al.*, 1997). In addition to its role in the acute phase response, it has diverse roles driving chronic inflammation, autoimmunity, endothelial dysfunction and fibrogenesis, and has thus been implicated in many diseases (Simpson *et al.*, 1997; Barnes *et al.*, 2011). Downstream effects of endothelial cell activation includes increased expression of adhesion molecules, release of chemokines and release of more IL-6; promoting leukocyte accumulation (Barnes *et al.*, 2011). By secreting IL-6, senescent cells are able to directly affect neighbouring cells that express the IL-6 receptor (IL-6R) and the gp130 signalling complex at their surface (Coppe *et al.*, 2010).

On average, there is a 2-4 fold increase in serum levels of IL-6 in aged individuals compared with younger individuals (Bruunsgaard, 2006; Freund *et al.*, 2010). Aged rodent aortas show increased IL-6 levels and aged aortic VSMCs have a higher basal secretion of IL-6 than young VSMCs (Song *et al.*, 2012). Administration of IL-6 and the soluble IL-6 receptor to young human fibroblasts caused growth arrest and other phenotypes of senescence including SA β galactosidase activity (Kojima *et al.*, 2013). From this study, it can be hypothesised that as a SASP component, IL-6 in the environment may not simply facilitate immune cell infiltration, but it may contribute to the tissue ageing process by causing senescence as well as reinforcing growth arrest (Freund *et al.*, 2010; Kojima *et al.*, 2013). It must be noted, however, that in contrast to cell types that show increased IL-6 expression as part of their SASP, senescent human fibroblastoid keratocytes in the eye show a decreased IL-6 expression profile compared to their young counterparts highlighting the difference of SASP components in different cell types (Tchkonia *et al.*, 2013; Kipling *et al.*, 2009).

Both IL-1 α and IL-1 β have been found to be secreted by senescent cells in many studies (Coppe *et al.*, 2010). These cytokines affect neighbouring cells that express the IL-1 receptor and receptors originating from the Toll-like receptor superfamily, which act primarily to trigger the NF- κ B and AP1 signalling pathways (Davalos *et al.*, 2010; Coppe *et al.*, 2010). IL-1 α regulates its

own synthesis in an autocrine receptor-mediated, positive-feedback loop dependent on NF-κB. The senescence-induced increase in IL-1α expression causes the membrane-bound form of IL-1α to activate its receptor, IL-1R in a juxtacrine manner, triggering the production of more IL-1α. The positive feedback loop also allows for the expression of other NF-κB targets such as key SASP components, IL-6 and IL-8 which has been observed in senescent human cells in culture (Laberge *et al.*, 2012; Lee & Lee, 2014; Freund *et al.*, 2010). Loss of IL-1α signalling in senescent cells by interfering with expression or receptor activity reduces IL-6 and IL-8 levels (Orjalo *et al.*, 2009). This positive feedback loop sustains the SASP and reinforces senescence growth arrest.

1.3.1.2 Chemokines

IL-8 is a proinflammatory chemokine, primarily regulated by transcription factors AP-1 and NF- κ B (Waugh & Wilson, 2008) and secreted by multiple cell types including monocytes, fibroblasts and ECs in response to inflammatory stimuli (Qazi *et al.*, 2011). Its biological effects are mediated through binding to specific cell surface G-protein coupled receptors; and its downstream effects include stimulating angiogenesis, disrupting cell-cell communication, inducing innate immune responses, and promoting epithelial and endothelial cell migration and invasion (Waugh & Wilson, 2008; Tchkonia *et al.*, 2013). IL-8 plays a role in inflammatory cells. IL-8 also acts as a chemotactic for fibroblasts, accelerating their migration and stimulating deposition of fibronectin and collagen during wound healing *in vivo* (Qazi *et al.*, 2011). Most senescent cells, reported to date, overexpress IL-8, along with other chemokines, GRO- α and GRO- β (Coppe *et al.*, 2010). Malignant melanocytes express high levels of GRO- α and IL-8 receptors, suggesting that the senescent microenvironment may enable melanoma development (Lee & Lee, 2014).

1.3.1.3 Growth Factors

Senescent cells have been reported to express high levels of almost all the Insulin-like Growth Factor-binding proteins (IGFBP) and their regulators (Coppe *et al.*, 2010). Activation of the BRAF oncogene in primary fibroblasts leads to the secretion of IGFBP7, which acted through autocrine and paracrine signalling to induce senescence and apoptosis in neighbouring cells (Wajapeyee *et al.*, 2008; Coppe *et al.*, 2010). In Human Umbilical Vein Endothelial Cells (HUVECs), IGFBP5 has been shown to play a role in replicative senescence; and is able to induce premature senescence (Kojima *et al.*, 2013; Kim *et al.*, 2007) and IL-6 and IGFBP2 expression were found to be much higher in p16-positive senescent cells isolated directly from adipose tissue of older progeroid mice compared with non-senescent cells from the same tissue (Tchkonia *et al.*, 2013). Taken together, these studies highlight a role for various IGF binding proteins in senescence.

1.3.1.4 Other soluble factors in SASP

Osteoprotegerin, a secreted decoy receptor for TNF- α is present at high levels in the extracellular environment of senescent fibroblasts (Coppe *et al.*, 2010). Osteoprotegerin competes for the binding of death ligands to block the apoptotic pathway that would be triggered upon activation of functional death receptors and trimerisation of the functional death domains (Jin & El-Deiry, 2005; Fesik, 2005; Coppe *et al.*, 2010). The significance of the soluble decoy receptor may be to prevent the death of dysfunctional cells in the microenvironment and in this way osteoprotegerin may facilitate tumourigenesis.

1.3.2 Secreted proteases

The major secreted proteases include matrix metalloproteinases (MMP) -1, -3, -10, -12; Plasminogen Activator Inhibitor (PAI) -1 and -2 and Cathepsin B (Coppe *et al.*, 2010). SASP proteases have 3 major effects: shedding of membrane-associated proteins, resulting in soluble versions of membrane-bound receptors; cleavage or degradation of signalling molecules; and degradation or processing of the ECM. All of these effects enable senescent cells to modify their microenvironment (Liu & Hornsby, 2007; Coppe *et al.*, 2010; Tchkonia *et al.*, 2013; Hassona *et al.*, 2014).

The plasminogen activation system plays a role in various processes, including vascular and tissue remodelling (Deryugina & Quigley, 2012). Serine proteases are a family of proteases that are plasminogen activators, but also found in high abundance in SASP (Herbert *et al.*, 1997; Coppe *et al.*, 2010). Members of this family include urokinase- or tissue-type plasminogen activators (uPA or tPA, respectively), the uPA receptor (uPAR), as well as inhibitors of these serine proteases, PAI1 and 2 (Blasi & Carmeliet, 2002; Coppe *et al.*, 2010; Deryugina & Quigley, 2012). uPA is secreted by tumour and stromal cells as an inactive pro-enzyme (pro-uPA), which

binds the cell surface receptor uPAR (Goretzki et al., 1992). The binding of pro-uPA to uPAR facilitates its activation to uPA, allowing it to convert plasminogen to plasmin; a serine protease that degrades fibrin and other ECM components (Blasi & Carmeliet, 2002). pro-UPA activation enables it to have mitogenic and chemotactic effects on several cell types including VSMCs (Korner et al., 1993; Coppe et al., 2010; Deryugina & Quigley, 2012). uPA and tPA produced by ECs was found sequestered in an active form by the subendothelial extracellular matrix, suggesting they may play a role in sequential matrix degradation, and may also function in the release of ECM-bound growth factors which stimulates VSMC growth; a crucial step in atherogenesis (Korner et al., 1993; Herbert et al., 1997). In the context of senescence, a 50 fold increase in total plasminogen activator activity has been reported in senescent lung and skin fibroblasts (West et al., 1996). The inhibitor, PAI1, is also a known marker of senescence in fibroblasts, both in vivo and in vitro (Mu & Higgins, 1995; Martens et al., 2003; Kortlever & Bernards, 2006). PAI1 has also been found to be upregulated in ECs and fibroblasts obtained from human breast cancer tissue (Comi et al., 1995; Martens et al., 2003). Like IL-6 and IGFB2, PAI1 expression was found to be much higher in p16-positive senescent cells isolated from adipose tissue of older progeroid mice compared with non-senescent cells from the same tissue (Baker et al., 2011; Tchkonia et al., 2013). The alterations in plasminogen activator and inhibitor expression upon senescence suggest that these SASP components may facilitate cell migration and tissue remodelling, possibly promoting metastasis of tumourigenic cells in the process.

1.3.3 Other components of the Senescence Associated Secretory Phenotype

The major ECM protein secreted by senescent cells is fibronectin (Coppe *et al.*, 2010). Fibronectins are large dimeric glycoproteins present in the ECM and in the blood. These molecules affect cell morphology, adhesion, migration and differentiation by binding to cell surface integrins, and triggering a downstream signalling cascade (Egeblad & Werb, 2002). Additionally, fibronectins also interact with components of the cytoskeleton and other ECM molecules. ECs aged both *in vitro* and *in vivo*, human skin fibroblasts aged *in vivo* and foetal lung fibroblasts aged *in vitro* showed increased fibronectin expression (Kumazaki *et al.*, 1993; Coppe *et al.*, 2010). *In vivo* ageing was achieved by obtaining cells from donors of different ages.

Other factors secreted include Nitric Oxide (NO), Reactive Oxygen Species (ROS) and transported ions. Senescent cells have been shown to release NO and ROS due to changes in the activities of inducible and endothelial nitric oxide synthase and superoxide dismutase (SOD) (Lee *et al.*, 1999; van der Loo *et al.*, 2000; Macip *et al.*, 2002; Coppe *et al.*, 2010).

Studies have shown elevated oxidant concentrations in cells and tissues obtained from aged organisms (Zhang *et al.*, 2015). Aged VSMCs derived from mouse aortas also contain higher levels of oxidant species (Moon *et al.*, 2001; Wang & Bennett, 2012). Ras induced senescence caused increases in mitochondrial hydrogen peroxide (H₂O₂) in primary human fibroblasts. Scavenging of ROS with N-acetyl cysteine (NAC) and a superoxide dismutase (SOD) mimetic prevented the induction of senescence indicating superoxide and H₂O₂ involvement in the Ras signalling mechanism (Lee *et al.*, 1999). Retroviral p21 expression caused senescence and increased ROS levels in human fibroblasts; which was also prevented with NAC exposure (Macip *et al.*, 2002). ROS are also capable of damaging DNA by introducing DNA single-stranded breaks and inducing mutagenic oxidised guanine bases; and also target other biomolecules such as lipids and proteins (Sokolov *et al.*, 2007; Wang & Bennett, 2012; Kawanishi & Oikawa, 2004).

NO is a small lipophilic molecule, and is capable of diffusing freely into cells where it can damage DNA in a number of ways. Such alterations include base aminations leading to point mutations; nitration of DNA repair proteins which can alter their function; and lipid peroxidation which may activate the arachidonic acid signalling cascade, producing DNA damaging agents such as 4-hydroxynonenol (Sokolov *et al.*, 2007). Studies in excised aortic tissue from rats found that NO levels decreased with age despite increased endothelial nitric oxide synthase expression; however, superoxide levels were reportedly 3 fold higher than in the young aortas. The authors proposed that the superoxide was reacting rapidly with the NO, forming peroxynitrite; which was reflected by tyrosine nitration in proteins such as mitochondrial SOD (van der Loo *et al.*, 2000). Peroxynitrite reacts slowly with most biological molecules, making it a selective oxidant (Beckman & Koppenol, 1996). However, as it is stable, it is able to diffuse through a cell where it is able to modify tyrosine residues in proteins, creating nitrotyrosines. As the nitration of tyrosines changes a normally hydrophobic residue into a negatively charged hydrophilic residue, peroxynitrite damages cellular processes by altering protein structure and subsequent function (Beckman & Koppenol, 1996).

1.4 Cell Senescence, Ageing and Cardiovascular Disease

Since senescent cells acquire characteristics that may compromise normal tissue function such as renewal and repair (as those described above), the accumulation of these non-dividing cells in later life has been postulated to contribute to the ageing process and to the development of age-related diseases (Campisi, 2005).

Evolutionary theory does not support a primary role for senescence in driving tissue dysfunction with age, because this would provide the process with no selective advantage. Instead, the contribution of senescence to ageing has been explained by the principle of 'Antagonistic Pleiotropy'. The concept of antagonistic pleiotropy (**Figure 1.3**) is that when properly controlled, senescence has positive functions, and these functions provide the selective pressure for its evolution. However, uncontrolled senescence, plus the accumulation of controlled senescent cells, comes at a cost for the organism (Campisi, 2003; Adams, 2009).



Figure 1.3: Antagonistic Pleiotropy, an Important Evolutionary Theory of Ageing

A process can be beneficial to young organisms but yet harmful to older organisms. Crucially, the balance is set so that the advantages are dominant early in life prior to the age of reproduction. Beyond this, as we age, the selective pressure for senescence to be beneficial is gone and cumulative environmental and cell autonomous stresses reveal the detrimental side of the process (Adams, 2009).

According to the review written by Rodier and Campisi (2011), there are three possible scenarios by which senescent cells may drive ageing. Firstly, senescence may deplete the tissues of stem or progenitor cells which in turn will compromise tissue repair, regeneration, and normal turnover. Secondly, the factors senescent cells secret may affect vital processes that are usually under tight regulation. Thirdly, the SASP includes several potent inflammatory cytokines and the resulting low-level, chronic inflammation is a hallmark of ageing (sometimes known as 'inflamm-ageing') that initiates, or promotes most age-related diseases (Rodier & Campisi, 2011).

The accumulation of senescent cells is believed to cause some tissue damage as well as hinder tissue renewal capabilities of surrounding cells due to the high levels of the proteins secreted into the local extracellular environment (**Figure 1.4**) (Sikora *et al.*, 2011). The low grade inflammation or sterile inflammation (termed sterile to indicate the absence of detectable pathogens) provided by the senescent cells can drive pathology and contribute to age-related diseases (Sikora *et al.*, 2011; Tchkonia *et al.*, 2013). In CVD, inflammation is also considered a critical initial step in the development of atherosclerosis (Sikora *et al.*, 2011) and accumulating evidence suggests a role for senescent vascular cells in atherogenesis. VSMC from human atherosclerotic plaques undergo senescence earlier than cells from normal vessels and they also exhibit the morphological features of senescence (Vasile *et al.*, 2001; Gorenne *et al.*, 2006; Minamino & Komuro, 2007; Sikora *et al.*, 2011).

Nelson et al., (2012), hypothesised that pro-oxidant and pro-inflammatory signals from primary senescent cells may trigger DNA damage and premature senescence in surrounding cells. They investigated their hypothesis using fibroblasts – they co-cultured senescent fibroblasts (termed founder cells) with young fibroblasts (termed recipient/bystander cells) *in vitro* (Nelson *et al.*, 2012). Their results showed that the rates of DNA damage foci production in recipient cells were significantly increased after a 2 day exposure to the senescent cells and remained elevated during prolonged co-culture. This indicated that senescent human fibroblasts induced a DDR as a bystander effect in the surrounding proliferating cells. Additionally, there was an increase in cells positive for SA β -galactosidase activity and other markers of cell senescence after co-culture for 15 days or more. Further investigations found that blocking gap junction-mediated cell-cell contact or scavenging extracellular ROS with the use of the antioxidant catalase (CAT) blocked the increase in foci formation rate in the

bystander cells. The removal of the founder cells did not reverse the effect, showing that the bystander effect had induced permanent senescence (Nelson *et al.*, 2012).



Figure 1.4: Senescence Factors that contribute to the induction and maintenance of senescence in neighbouring cells. *Adapted from* (Kuilman & Peeper, 2009)

The induction of senescence in neighbouring cells has been found to depend on a variety of secreted factors including members of the IGFBP and Interleukin families, PAI1, ROS/RNS, and MMPs. TGF- β , and NF- κ B mediate the expression of many SASP factors.

Orange cells – normal; pink cells – dysfunctional; red cell – senescent.

1.4.1 Why should senescence be involved in ageing?

There is now substantial evidence that cellular senescence is indeed a potent tumoursuppressor mechanism, and there is also mounting, but still largely circumstantial, evidence that cellular senescence also promotes ageing (Campisi & di Fagagna, 2007). An example of such evidence was provided (Baker *et al.*, 2011) using a mouse model where elimination of cells positive for the p16 tumour suppressor protein delayed the acquisition of age-related pathologies (Baker *et al.*, 2011). In the study, two types of mice were used – BubR1-insuffient mice and INK-ATTAC transgenic mice. BubR1-insufficient mice suffer from age-related disorders prematurely and exhibit a variety of age-related phenotypes including cataracts, cardiac arrhythmias and arterial stiffening. It is thought that these mice age prematurely due to the factors secreted by the accumulating p16-positive cells which subsequently cause cellular and tissue dysfunction (Peeper, 2011). The INK-ATTAC mice are engineered transgenic mice whose p16-expressing cells could be killed selectively using the synthetic drug, AP20187; by a drug-inducible caspase enzyme (Peeper, 2011; Baker *et al.*, 2011). The resulting intercrossing of these two mouse types is shown in **Figure 1.5**.



Figure 1.5: Delaying the ageing process (Peeper, 2011)

Mice whose senescent cells could be selectively killed by drug treatment were intercrossed with mice that express low levels of the BubR1 mitotic checkpoint protein. When the authors eliminated p16-positive cells in the offspring of this intercross, the onset of age-associated deficits was delayed, causing improved cellular and tissue dysfunction. Compared with control mice, there was a profound delay of age-related deficits such as hunchback and cataracts and their muscle fibres were thicker and their exercise fitness significantly greater. All this coincided with signs of clearance of senescent cells, such as reduced β -galactosidase activity and decreased expression across different tissues of SASP including IL-6 and MMPs.

In young offspring that were yet to show the senescent phenotype, lifelong treatment every 3rd day from weaning onwards resulted in the elimination of the p16-positive cells and delayed the onset of age-associated deficits such as hunchback, skeletal muscle deterioration and cataracts. In the long-term, the loss of p16-positive cells resulted in larger fat deposits – an indication of the attenuation of ageing; all of this correlating with the reduction of senescent cell markers. To investigate the effect of the late-life clearance, they used 5 month old offspring that had begun to show the senescent traits rather than the weaning age. The results showed that 5 months after treatment, there was an attenuation of the progression of already established age-related disorders in skeletal muscle, fat tissues, and in the eye in these now 10month old mice. However, neither lifelong nor late-life treatment could prolong lifespan as the features of ageing that were p16-independent remained unchanged such as accelerated cardiovascular dysfunction. Their data suggest that the elimination of p16-positive cells can delay tissue dysfunction and extend health span and it could provide some possible therapeutic ideas for the treatment or delay of age-related diseases (Baker *et al.*, 2011; Naylor *et al.*, 2013).

1.5 Reactive Oxygen Species

One of the mechanisms for the induction of senescence is oxidative stress, which is defined as a disturbance in the balance between the production of oxidant species and antioxidant defences (Betteridge, 2000). ROS are a family of molecules that consist of highly reactive free oxygen radicals such as the superoxide anion $(O_2^{\bullet-})$ and the hydroxyl radical ($^{\bullet}OH$) as well as the more stable 'diffusable' non-radical oxidants such as hydrogen peroxide (H_2O_2) (Gough & Cotter, 2011). Dismutation of superoxide, either spontaneously or through a SOD catalysed reaction, produces H_2O_2 , which can subsequently be reduced to water by glutathione peroxidase (GPX) or CAT; or partially reduced to the *OH, one of the strongest oxidants in nature. The latter process is a chain reaction termed the Fenton reaction; catalysed by the reduced transition metals, iron (Fe²⁺) and ferric iron (Fe³⁺), producing *OH, the hydroxyl anion (OH) and O₂^{•-} (Pacher et al., 2007; Das et al., 2015). The net reaction is known as the ironcatalysed Haber-Weiss reaction which makes use of Fenton chemistry, although other transition metal ions are capable of catalysing this reaction (Kehrer, 2000; Pacher et al., 2007; Lemire et al., 2013). The Haber-Weiss reaction is now considered to be the major mechanism by which the highly reactive 'OH is generated in biological systems (Kehrer, 2000). 'OH also happens to be one of the most potent inducers of lipid peroxidation (Orrenius et al., 2006), a process in which free radicals abstract a hydrogen atom from a double bond within a polyunsaturated lipid; yielding a new radical species that can readily interact with molecular oxygen (Kehrer, 2000). The resultant lipid peroxyl radical can abstract a hydrogen atom from another lipid, establishing a chain reaction. As the reaction progresses, ion channels may be affected, membrane transport proteins or enzymes may be inactivated or the lipid bilayer may become more permeable disrupting ion homeostasis (Kehrer, 2000).
There are various endogenous sources of ROS, such as mitochondria, lipoxygenase and myeloperoxidase. However, all of these fail to show the diverse physiological functions attributed to the NOX family of enzymes (Gough & Cotter, 2011). The NAD(P)H oxidases (NOX) are a family of haem-containing proteins that transfer electrons from NAD(P)H to molecular oxygen to form ROS. Although their biological functions are still largely unknown, evidence accumulated over recent years show that the NOX family enzymes are key contributors to ROS generation in phagocytic and non-phagocytic cells (Liu *et al.*, 2010).

1.5.1 Reactive Oxygen Species signalling

ROS operate as signalling molecules, a function that has been widely documented however still remains controversial. The controversy arises from the paradox between the specificity required for cellular signalling molecules and the reactive nature of ROS that renders them indiscriminate and potentially lethal oxidants (D'Autreaux & Toledano, 2007). ROS play a role in signalling through chemical reactions with specific atoms of target proteins, leading to covalent protein modification. This means that ROS molecular recognition occurs at the atomic level and the amino acid building blocks of numerous proteins are their atomic targets (Turrens, 2003). Direct oxidation of amino acids such as lysine, proline, arginine and threonine promotes the formation of protein carbonyls. These carbonyls alter the tertiary structure of a protein, resulting in partial, or at times, complete unfolding; causing a loss in normal protein functions and enhanced susceptibility to proteolytic degradation (Orrenius *et al.*, 2006).

A study has investigated the mechanism of ROS signalling and found H_2O_2 to act as an intracellular messenger in signal transduction (Sobotta *et al.*, 2015). H_2O_2 modulates protein function by inducing the transient oxidation state of protein cysteinyl thiols, including the formation of disulphide bonds. In this study, the antioxidant, peroxiredoxin 2 (PRX2), acted as a H_2O_2 signal receptor, and formed a redox relay with the transcription factor, STAT3, allowing oxidative equivalents flow from PRX2 to STAT3; which subsequently attenuated STAT3 transcriptional activity (Sobotta *et al.*, 2015). The study explains a mechanism for oxidative equivalents to be eliminated once they have been rerouted through other proteins for signalling purposes.

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1.5.2 Reactive Oxygen Species and Senescence

Oxidative DNA damage occurs in both mitochondrial and nuclear DNA and in both telomeric and non-telomeric regions (Wang & Bennett, 2012). As mentioned earlier, telomeres contain the repeat sequence TTAGGG. Due to their high content of guanines, telomeres have previously been demonstrated highly sensitive to damage by oxidative stress (Houben et al., 2010). Kawanishi and Oikawa (2004) showed that oxidative stress efficiently induced DNA damage at the 5' site of 5'-GGG-3' in the telomere sequence by using H_2O_2 and reduced copper to cause predominant DNA cleavage. They also found that photosensitisation induced the formation of the mutagenic oxidised guanine base (8-oxodG), specifically at the central guanine of the telomere sequence. Their results led them to speculate that GGG-specific DNA damage in the telomere sequence may play an important role in increasing the rate of telomere shortening (Kawanishi & Oikawa, 2004). An earlier study found that senescent cells contained 35% more 8-oxodG in their DNA compared to young cells, which indicates an accumulation of oxidative damage with age (Chen et al., 1995). Leukocyte telomere length has been associated with chronological age and many diseases including CVD (Zierer et al., 2016). A recent study aimed to identify novel pathways regulating leukocyte telomere length using a metabolomics approach, as alterations in metabolite concentrations can reflect physiological functions and can indicate early stages of diseases. The identified metabolites indicate an involvement of increased oxidative stress due to alterations in the GSH metabolism, which has been previously related to LTL and aging phenotypes (Zierer et al., 2016). This highlights the importance of detoxification, particularly of ROS, in biological aging.

Beyond telomeric and DNA damage, many *in vitro* studies have looked into the role ROS play in senescence. As mentioned earlier, retroviral p21 expression caused premature senescence and increased ROS levels in human fibroblasts. NAC exposure abolished this, indicating that ROS elevation is necessary for the induction of senescence (Macip *et al.*, 2002). Exogenous H_2O_2 can also induce prolonged growth arrest in human fibroblasts, with a sustained elevation of p21 (Chen *et al.*, 1998). Tert-butylhydroperoxide (tBHP) exposure also induced senescence in human fibroblasts with an overexpression of p21 and inability to hyperphosphorylate pRB, preventing cell cycle progression. This growth arrest was accompanied with increased gene expression of a variety of proteins including the SASP factor, fibronectin (Dumont *et al.*, 2000). The choice between the induction of growth arrest or apoptosis appears to be dependent on the concentration of H_2O_2 as well as the length of exposure (Chen *et al.*, 1998; Colavitti &

Finkel, 2005). Mitochondrial ETC inhibitor, Antimycin A (AA), was shown to cause increased SA β -galactosidase activity indicating the ability of mitochondria-derived ROS to also induce senescence (Panieri *et al.*, 2013). Finally, the Angtiotensin II (AngII) induced senescence pathway also appears to depend on the generation of ROS as exposure to the hormone caused increased mitochondrial superoxide generation and senescence; whilst CAT, NAC, SOD and a mitochondria-targeted antioxidant, MitoTEMPO, were all able to prevent this premature senescence (Mistry *et al.*, 2013). Together, the studies indicate that irrespective of the inducer, oxidant species play a vital role in the pathway leading to senescence induction.

A role for ROS in atherosclerosis has been summarised based on the presence of damaged biomolecules in the plaque; including an elevation in 8-oxodG in VSMCs and macrophages in plaques (Martinet *et al.*, 2001; Malik & Herbert, 2012), and lipid peroxidation products and the oxidative modification of proteins (Suematsu *et al.*, 2003; Najjar *et al.*, 2005; Kang *et al.*, 2011). Increased ROS production has been proposed to be a marker of unstable plaques due to the higher proportion of active macrophages present as well as lipid laden macrophages, or foam cells; which increase ROS levels in the lesion (Channon, 2002; Park & Oh, 2011). Some studies have shown an elevation of oxidant concentrations in cells and tissues obtained from aged organisms (Zhang *et al.*, 2015). The increased oxidative stress-induced damage in these models may likely be due to a combination of higher ROS generation alongside an impaired antioxidant defence (Wang & Bennett, 2012).

1.6 Mitochondrial Reactive Oxygen Species

The 'mitochondrial theory of ageing' suggests a critical role for mitochondrial dysfunction and subsequent increased ROS production as an inducer of ageing and premature senescence (Lener *et al.*, 2009). The resulting unrepaired and therefore accumulating oxidative damage to proteins and lipids leads to the initiation of degenerative processes, causing organ function deterioration and crucially determines health and lifespan (de Cavanagh *et al.*, 2011). The theory is an attractive hypothesis with evidence to support it. Many age-dependent human pathologies are associated with oxidative damage to protein, DNA and lipid. In addition, the maximum lifespan of higher mammals and birds inversely correlates with the rate of mitochondrial ROS generation as well as oxidative damage to mitochondrial DNA (mtDNA) but

not nuclear DNA (James *et al.*, 2005). Many of these modifications are mutagenic and although some can be reversed by DNA repair enzymes, there is a possibility that others go unrepaired.

Mitochondria represent the major source of free radical species in all cells except those specialised to generate free radicals as part of their normal functions such as phagocytic cells of the immune system (Duchen, 2004). Mitochondria utilise more than 90% of cellular oxygen and while most of this is transformed to water at complex IV of the mitochondria electron transport chain (mETC) (**Figure 1.6**), an estimated approximately 1-2% of the consumed oxygen results in $O_2^{\bullet-}$ production (de Cavanagh *et al.*, 2007; West *et al.*, 2011).





The mETC carries out the process of oxidative phosphorylation. The chain is composed of 5 multisubunit protein complexes embedded on the inner membrane of the organelle. Electrons are transferred from electron donors to electron acceptors via redox reactions, a process coupled with the transfer of protons across the inner membrane. These processes create an electrochemical proton gradient that drives the synthesis of ATP. Superoxide can be converted into H_2O_2 by superoxide dismutases 1 and 2 (SOD1 and SOD2). H_2O_2 can be converted into water and oxygen by CAT or GPX depending on the location. Unlike superoxide, H_2O_2 can freely pass across the outer membrane where it goes on to act as a signalling molecule in a variety of pathways. Rotenone and Antimycin A are inhibitors of the mETC at complexes I and III respectively.

The mETC contains several redox centres that are able to leak electrons to molecular oxygen, thus forming the superoxide anion, $O_2^{\bullet-}$. Only a small proportion of mitochondrial electron carriers with the thermodynamic potential to reduce molecular oxygen to superoxide do so. Under most circumstances, small-molecule electron carriers such as NADH, NADPH and reduced Coenzyme Q (CoQH₂) do not react with oxygen to generate superoxide. Instead, mitochondrial superoxide production takes place at redox-active prosthetic groups within proteins, or when electron carriers such as CoQH₂ are bound to proteins (Murphy, 2009). The major sites of superoxide generation within the mitochondria are at complexes I and III (West *et al.*, 2011). The inner membrane of mitochondria is impermeable to superoxide, making the mitochondria a separate and distinct compartment for superoxide anions within a cell (Robinson *et al.*, 2008). As well as the ETC, other possible sources of mitochondrial ROS include matrix enzymes, outer membrane monoaminoxidases, and uncoupled mitochondrial nitric oxide synthase (de Cavanagh *et al.*, 2007).

Besides superoxide produced as a consequence of mitochondrial respiration, other ROS such as H_2O_2 may also be present in reasonably high concentrations, posing a risk of lipid peroxidation and damage to cell membranes and DNA. Oxidative phosphorylation is the most immediate target of ROS attack because the biomolecules essential for oxidative phosphorylation activity such as mitochondrial DNA and iron-sulphur proteins are in close proximity to the source of superoxide. Unlike nuclear DNA, mtDNA has no histones associated and therefore is less protected from oxidative damage (Duchen, 2004). To prevent the occurrence of such damage, mitochondria are equipped with an exhaustive list of antioxidant defences. These defences include the tripeptide, glutathione (GSH), which is imported from the cytosol, along with a collection of enzymes such as a variant of superoxide dismutase, known as SOD2 (MnSOD or mtSOD); and peroxiredoxins (PRXs) III and V to reduce H_2O_2 ; many of which are under the regulation of Peroxisome Proliferator Activated Receptor- γ Coactivator 1- α (Duchen, 2004; Valle *et al.*, 2005; Rhee *et al.*, 2012).

The presence of enzymes such as SOD2 highlights the possibility of ROS having a role in signalling. It could be argued that during evolution, ROS were an unavoidable consequence of electron transport that uses oxygen as the terminal electron acceptor. Once cells learned to cope with ROS toxicity through the acquisition of various antioxidants and detoxifying enzymes, a role for ROS was then established; utilising them as signalling molecules. There are various advantages for adopting ROS as signalling molecules which include the capacity for the

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cell to alter the levels of ROS rapidly as well as the tight control over subcellular ROS localisation within cells (Mittler *et al.*, 2011).

1.7 Mitochondria-targeted antioxidants

1.7.1 MitoQ

Antioxidants have had limited success in preventing the progression of diseases involving mitochondrial oxidative damage. A possible explanation may be that most small molecule antioxidants distribute around the body, with a small fraction being taken up by the mitochondria (Murphy, 2008). This creates a need for stable small molecule antioxidants that can selectively be taken up by the mitochondria within organs most affected by mitochondrial oxidative damage. One approach to satisfy this criteria is to selectively target antioxidants to the mitochondria by conjugation to lipophilic cations such as triphenyl phosphonium (TPP⁺) which easily pass through lipid bilayers as their charge is dispersed over a large surface area (Murphy & Smith, 2007; Murphy, 2008).

A well-studied example of these lipophilic cation conjugated antioxidants is known as MitoQ; the targeted version of ubiquinol. Its structure is shown in **Figure 1.7** along with the TPP⁺ moiety without the antioxidant (Murphy & Smith, 2011). The large membrane potential across the inner mitochondrial membrane causes MitoQ to accumulate several hundredfold within mitochondria and causes subsequent adsorption to the matrix surface of the inner membrane (Smith *et al.*, 2004; Murphy & Smith, 2007).



Figure 1.7: Structure of MitoQ (Ross et al., 2008)

MitoQ consists of a ubiquinone moiety linked to a TPP⁺ moiety by a ten-carbon alkyl chain (decylTPP⁺). The TPP⁺ cation enables rapid MitoQ uptake through the plasma and mitochondrial membranes without the requirement of a carrier.

Strong evidence suggests that MitoQ is predominantly accumulated within the mitochondria through the action of the TPP⁺ moiety; however it is currently difficult to quantify the amount of MitoQ present throughout the cell (Murphy & Smith, 2007). It is therefore assumed that relatively insignificant amounts of MitoQ may be present in the cytosol and other nonmitochondrial cell compartments. Upon entry into the mitochondria, MitoQ is reduced to the active ubiquinol form by complex II of the ETC. When the ubiquinol form acts as an antioxidant, it becomes oxidised to the ubiquinone form, which is then subsequently rapidly reduced by complex II, restoring the active ubiquinol antioxidant; enabling the continuous recycling of the targeted antioxidant (Murphy, 2008).

MitoQ is reportedly several hundredfold more potent at preventing mitochondrial oxidative damage than an untargeted antioxidant (Smith *et al.*, 2004; Lowes *et al.*, 2008). MitoQ has been shown to protect against peroxynitrite (ONOO⁻), formed by an extremely rapid reaction between superoxide and nitric oxide; and the ubiquinone form may also react directly with superoxide (Murphy & Smith, 2011).

1.7.1.1 *MitoQ in vitro – effects in cells*

In 2003 (Jauslin *et al.*), MitoQ was used on fibroblasts that were isolated from Friederich Ataxia (FRDA) patients. FRDA is caused by an intronic GAA triplet expansion in the gene for the mitochondrial protein, frataxin, causing a significant decrease in the amount of the protein produced. This mutation leads to mitochondrial dysfunction and increased oxidative stress in FRDA patients. In the study, isolated fibroblasts were exposed to GSH synthesis inhibitor L-buthionine-S,R-sulfoximine (BSO). Previously, it had been shown that BSO treatment led to a 70% decrease in the antioxidant GSH content of FRDA and control cells, however only the FRDA cells died. The difference in susceptibility to GSH depletion was thought to be due to elevated endogenous oxidative stress in the FRDA cells, mimicking the cause of cell death within the cells of FRDA patients. MitoQ blocked death of FRDA fibroblasts, highlighting the fact that increased mitochondrial oxidative stress in FRDA cells could be blocked by antioxidants. The authors also abolished the mitochondrial membrane potential with the uncoupler FCCP, preventing the selective uptake of MitoQ by ~25 fold, resulting in an antioxidant potency similar to that of an untargeted antioxidant (Jauslin *et al.*, 2003).

In a second study, MitoQ was proposed to be an attractive candidate for reducing oxidative damage in sepsis. Growing evidence suggests that mitochondrial oxidative damage may disrupt mitochondrial ATP production and that this damage is a major mechanism by which cell death and organ failure occur during sepsis. HUVECs were incubated with lipopolysaccharide and peptidoglycan, which mimic the events observed in sepsis. The rate of ROS production in MitoQ-treated cells was lower than the control cells and mitochondrial membrane potential was maintained suggesting the use of MitoQ as a protective agent was a potential viable strategy for the treatment of sepsis (Lowes *et al.*, 2008).

1.7.1.2 *MitoQ in vivo – effects in animals*

Smith and Murphy (2010) measured oral toxicity in mice by administering MitoQ in their drinking water and found that the compound was well tolerated up to7.5 µmol/kg. MitoQ was excreted in the urine and bile as unmodified MitoQ and also with modifications such as sulfation and glucuronidation of the ubiquinol ring (Murphy & Smith, 2011; Smith & Murphy, 2010).

An investigation by Rodriguez-Cuena's group (2010) looked into the effects of long-term orally administered MitoQ on healthy, young mice. Their results showed that the long term

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administration of MitoQ had no effect on the accumulation of mitochondrial oxidative damage markers, such as protein carbonyl formation, mtDNA damage, and oxidation of the phospholipid unique to mitochondria, cardiolipin. They also found that long term exposure to MitoQ had no effect on the expression of antioxidant genes in the heart or the liver. For example, the mtSOD encoded by the *SOD2* gene is sensitively upregulated in response to increased mitochondrial ROS production, however its expression level was unaffected by the administration of MitoQ. This lack of an increase in oxidative damage and the unaltered gene expression levels of mitochondrial antioxidant defence enzymes upon MitoQ exposure in highly oxidative tissues indicate that MitoQ does not increase oxidative stress or the flux of ROS within mitochondria *in vivo*. The team concluded that MitoQ could be administered safely (Rodriguez-Cuenca *et al.*, 2010).

1.7.1.3 MitoQ in vivo – effects in man

Human clinical trials have been carried out in patients with Parkinson's disease as well as chronic Hepatitis C patients. Newly diagnosed untreated patients with Parkinson's disease were treated with two doses of MitoQ a day and compared with a placebo over a 12 month period to see whether MitoQ would slow the disease progression. Results showed no difference between the treatment groups (MitoQ versus placebo) on any measure of disease progression. Their suggestions for the lack of therapeutic efficacy were that there could have been insufficient brain penetration; or possibly that at the time of diagnosis, the disease could have progressed so far that MitoQ could not have had any protective effects. However, the trial did confirm that the compound could be safely administered in patients for up to a year as no adverse events were reported (Snow *et al.*, 2010).

The second human trial involved patients who were unresponsive to conventional hepatitis C virus treatments. These patients also displayed evidence of increased oxidative stress and mitochondrial damage, which played a role in their liver damage. Results showed that although there was no effect of MitoQ on viral load indicating that MitoQ did not have an effect on viral replication, the compound did reduce the liver damage. This trial was the first report of a clinical benefit from the use of a mitochondria-targeted antioxidant in humans. No severe side effect was reported; the most common problem was simply mild-nausea which was dosage-dependent (Gane *et al.*, 2010).

Whilst being described as an 'antioxidant', the success of MitoQ may not necessarily always be due to its ability to act as an antioxidant. It is crucial to note that many studies do not observe

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any reduction in ROS levels following MitoQ administration, however the compound may still provide overall beneficial effects and thus it makes it worth studying as a therapeutic strategy for diseases associated with increased oxidative stress (Ng *et al.*, 2014; Smith & Murphy, 2010).

1.7.2 MitoTEMPO

Nitroxide compounds are low molecular weight, membrane permeable, stable free radicals that have been used in various applications including paramagnetic contrast agents in NMR imaging as probes for membrane structure and oxygen sensors in biological systems (Suy *et al.*, 1998). Nitroxides have been shown to possess antioxidant activity and protect against a variety of oxidants including superoxide, H₂O₂, and ionising radiation. Numerous mechanisms for nitroxide antioxidant activity have been proposed, including SOD or CAT mimicry, detoxification of secondary organic radicals or oxidation of reduced metals that would otherwise catalyse the formation of hydroxyl radicals (Suy *et al.*, 1998). MitoTEMPO, shown in **Figure 1.8**, is a derivative of the stable free radical nitroxide, Tempo, with a conjugated TPP⁺ moiety causing accumulation within the mitochondria. It is believed to act as a mitochondria-targeted superoxide scavenger and can therefore be defined as a SOD mimetic (Nazarewicz *et al.*, 2013).



Figure 1.8: Structure of MitoTEMPO (Nazarewicz et al., 2013)

The stable free radical nitroxide, Tempo, was conjugated to a TPP⁺ moiety enabling accumulation of the SOD mimetic within the mitochondrial compartment.

1.7.2.1 MitoTEMPO in vitro – effects in cells

Previous work from this group has demonstrated the effectiveness of MitoTEMPO to completely prevent AngII induced senescence in human VSMC, strongly implicating mitochondrial function and specifically superoxide in the rapid senescence response. The study did not investigate the action of MitoTEMPO on ROS within the cell however it provided evidence of a cross talk between the mitochondria and the AngII-activated NADPH oxidase enzymes (Mistry *et al.*, 2013).

Another study found that treatment with MitoTEMPO specifically inhibited mouse melanoma cell growth and induced apoptosis but did not affect non-malignant skin fibroblasts. MitoTEMPO significantly diminished mitochondrial superoxide levels, detected by MitoSOX[™] and H₂O₂ levels detected by Amplex Red assay; inhibited redox-dependent Akt and Erk activity; and restored activity of mitochondrial pyruvate dehydrogenase, which would otherwise limit mitochondrial metabolism. These changes in signalling events caused a metabolic switch from glycolysis to mitochondrial metabolism. The data support the hypothesis that the anti-cancer activity of mitochondrial-targeted antioxidants is mediated by inhibition of ROS-sensitive signalling and metabolic changes leading to reduced cancer cell survival (Nazarewicz *et al.*, 2013). The authors also speculate that their success may be due to nanomolar concentrations of MitoTEMPO, and the potential that high concentrations may fail to inhibit redox signalling due to the potential oxidation of components within the ETC, proteins within the mitochondrial matrix and other possible off-target effects.

1.7.2.2 MitoTEMPO in vivo – effects in animals

Nazarewicz et al., (2013) tested the effects of MitoTEMPO on A375 human cell tumour growth in a nude mouse model (Nazarewicz *et al.*, 2013). The melanoma cells were subcutaneously injected into the nude mice to induce a tumour. Once established, pumps with MitoTEMPO were implanted, with saline being used as a vehicle control. The results showed that 12day administration of the mitochondria-targeted antioxidant led to a significant attenuation of tumour growth. The mouse study provided evidence of mitochondrial superoxide scavenging having the ability to impair tumour progression *in vivo* (Nazarewicz *et al.*, 2013).

In another study, MitoTEMPO was found to diminish mitochondrial ROS and inhibit AngIIstimulated endothelial superoxide (Dikalova *et al.*, 2010). The authors found treatment of MitoTEMPO *in vivo* significantly attenuated AngII-induced hypertension in a dose dependent manner when given at the onset of AngII infusion; and reduced blood pressure after AngII- induced hypertension had been established. MitoTEMPO also inhibited the increase in vascular superoxide levels following AngII infusion *in vivo*, whilst the untargeted TEMPOL failed to have the same effects at the same dose (Dikalova *et al.*, 2010). The study demonstrated the interplay between AngII-induced NADPH oxidase activation and mitochondrial-derived superoxide production; and also highlighted the ability of mitochondrial superoxide scavenging to disrupt this signalling process.

To date, MitoTEMPO has not yet been administered in human trials.

1.7.3 Other mitochondrial-targeted antioxidants

There are other mitochondria-targeted antioxidant alternatives including SkQ1. SkQ1 is TPP⁺ linked with a plastoquinone moiety rather than ubiquinone. SkQ1 is believed to be a more effective antioxidant than MitoQ as it is uncomplicated by the danger of pro-oxidant effects within a larger range of concentration than MitoQ. SkQ1 has been found to prolong the lifespan of various laboratory animals ranging from flies to mice (Skulachev *et al.*, 2009).

Other neutral antioxidants have been subjected to mitochondrial targeting with the conjugation of the lipophilic TPP⁺ cation. Such antioxidants include MitoSOD which degrades superoxide; MitoPeroxidase which is a targeted version of the peroxidase mimetic, ebselen; and MitoE which decreases lipid peroxidation; and another targeted nitroxide, MitoTEMPOL which acts as a SOD mimetic like MitoTEMPO, and also promotes the detoxification of ferrous iron (Smith & Murphy, 2010). Work with these antioxidants is ongoing; however the ultimate aim is to create a suite of mitochondria-targeted antioxidants that can be used to intervene at several stages of the mitochondrial ROS cascade (Smith & Murphy, 2010).

1.8 Aims

Published data from this research group suggests that inhibition of mitochondrial oxidant production completely prevented the pro-ageing effects of AngII (Mistry *et al.*, 2013). The hypothesis is that mitochondrial oxidants mediate the induction of SIPS in VSMC.

The hypothesis will be investigated using three studies, firstly, to provide evidence that targeting mitochondrial ROS, in general, prevents the induction of premature senescence. This aim will be met by stimulating mitochondrial ROS production with mETC inhibitors, or a mitochondrial redox cycler; and determining if mitochondrial ROS induction is able to cause premature senescence in VSMCs. Subsequently, the aim will be to determine if it is possible to modulate this generated ROS with mitochondria-targeted and non-targeted antioxidants. Finally, further investigation of the ability of AngII to induce mitochondrial ROS generation and cause senescence in VSMC will be undertaken. By being able to prove mitochondrial ROS involvement in these studies, we can begin to build better links in the senescence signalling pathway, allowing us to suggest new ways of specifically and potently modifying premature senescence of VSMC.

The final part of this thesis involves establishing a model of replicative senescence in human VSMC. Once established, the aim will be to derive conditioned media from the senescent VSMC and use shotgun proteomics to profile the proteins within the secretome in order to identify potential new biomarkers of the ageing vasculature; the application of bioinformatics will aim to facilitate our understanding of the mechanisms of VSMC senescence.

CHAPTER TWO

Materials and Methods

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2.1 Materials

2.1.1 Cell Lines

Human coronary artery smooth muscle cells (hCASMC) were purchased from Promocell, Heidelberg, Germany. Cells were used until Passage 20 when they were defined as senescent. For experiments using 'young' hCASMC, the cells were used until passage 15.

Human aortic smooth muscle cells from a 17 year old donor were transfected with the catalytic subunit of the human enzyme telomerase (hTERT) producing hTERT-hASMC; a gift from Professor Laura Niklason, Department of Anaesthesia and Biomedical Engineering, Yale University, Connecticut, USA. hTERT hASMC were used at all passages as the cells did not undergo replicative senescence.

Human Dermal Fibroblasts (HDF) were purchased from Cellworks, Caltag Medsystems Ltd, Buckingham, Buckinghamshire, UK. Cells were used until Passage 12 when they were defined as senescent. For experiments using 'young' HDF, the cells were used up until passage 8.

2.1.2 Cell culture consumables

96 well deep well plates, Nunc 100 mm cell culture dishes, 15 ml and 50 ml centrifuge tubes were purchased from Corning Life Sciences, Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK. The stripettes were obtained from Costar, Sigma-Aldrich Company Ltd, Poole, Dorset, UK. The clear-bottom 96 well plates came from Corning, Sigma-Aldrich Company Ltd, Poole, Dorset, UK. T75 culture flasks, 96 well cell culture plates, 6 well cell culture plates and CellView[™] cell culture 4 compartment dishes were purchased from Greiner Bio-One, Gloucestershire, UK.

2.1.3 Cell Culture Media

2.1.3.1 Basal media

Smooth Muscle Cell Growth Medium 2 (Ready-to-use) was purchased from Promocell, Heidelberg, Germany. The basal medium RPMI 1640 with L-glutamine and phenol red and Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (containing 4500 mg/L glucose, 110 mg/L sodium pyruvate and 4 mmol/L L-glutamine) were from Gibco, Life Technologies Ltd, Paisley, Scotland, UK.

2.1.3.2 Cell media additives and supplements

Additional items purchased from Life Technologies include Foetal Bovine Serum (Heat Inactivated EU Approved South American origin), Dulbecco's phosphate buffered saline (DPBS) with calcium and magnesium, 50 mg/mL Hygromycin B, 0.25% Trypsin/Diaminoethanetetraacetic acid (EDTA) (1X) and 1 mol/L HEPES buffer solution. The SmGM[™]-2 SingleQuot Kit Supplements & Growth Factors were purchased from Lonza, Slough, Berkshire, UK. Penicillin/Streptomycin (1000 units/1000 µg) and Foetal bovine serum (Gold EU Approved) was obtained from PAA, Yeovil, Somerset, UK. Human smooth muscle cell growth supplement with serum (100X) was purchased from Sciencell, Caltag Medsystems Ltd, Buckingham, Buckinghamshire, UK. DetachKit and Cryo-SFM were purchased from Promocell.

2.1.4 Cell treatment reagents

Trypan Blue solution (0.4%), Dimethyl Sulphoxide (DMSO), hydrogen peroxide (H₂O₂), tert-Butyl hydroperoxide solution (t-BHP), N-acetyl-L-cysteine (NAC), rotenone (Rot), Antimycin A (AA), Angiotensin II (AngII) and Cell-Counting Kit 8-(CCK-8) were purchased from Sigma Aldrich. MitoPQ and MitoQ were kindly supplied by Dr Michael Murphy (University of Cambridge, Cambridgeshire, England, UK) and MitoTEMPO was purchased from Enzo Life Sciences. Dihydroethidium (DHE), MitoSOX[™] Red Mitochondrial Superoxide Indicator and the ATP Determination Kit were purchased from Life Technologies.

2.1.5 Amplex Red Assay

Ampliflu™ Red 5 mg DMSO As Paquired
DMSO As Paquirad
DWSO As required
Peroxidase type II from Horse Radish0.05 mg
(188 U/mg)
Hydrogen Peroxide As Required
PBS As Required
(+ CaCl ₂ and MgCl ₂)
Menadione 50 mM

 Table 2.1: The reagents individually provided by Sigma Aldrich required for the detection of

 hydrogen peroxide using the Amplex Red assay

A 10mM Ampliflu[™] Red stock solution was made by adding 1.943 ml of DMSO to 5 mg Ampliflu[™] Red. The stock solution was then aliquoted into 100 µl stocks and stored at -20°C until required.

2.1.6 Senescence Cells Histochemical Staining Kit

The Senescence Cells Histochemical Staining kit was purchased from Sigma Aldrich. The kit contained the following reagents:

Reagent	Volume Supplied (ml)
······	
10x Fixation Buffer (containing 20% formaldehyde, 2% glutaraldehyde,	15 ml
70.4 mM Na ₂ HPO ₄ , 14.7 mM KH ₂ PO ₄ , 1.37 M NaCl, and 26.8 mM KCl)	
Reagent B – 400 mM Potassium Ferricyanide	1.5 ml
Reagent C – 400 mM Potassium Ferrocyanide	1.5 ml
X-gal solution 40 mg/ml	4 ml
10x Staining Solution	15 ml
10x Phosphate Buffered Saline (PBS)	60 ml

 Table 2.2: The reagents provided by Sigma Aldrich required for the detection of senescent cells

The parafilm used to seal the plate was purchased from Sigma Aldrich.

2.1.7 RNA Extraction

The extraction of RNA from the cells required the RNeasy Plus Kit and the RNase Free DNase kit, both purchased from Qiagen, Manchester, Lancashire, England, UK. The Trizol[®] Reagent was purchased from Invitrogen and the QIAshredder spin column was purchased from Qiagen. The RNeasy Plus kit contained the following reagents:

Component/Reagents	Volume Supplied (ml) / Number
gDNA Eliminator Mini Spin Columns	50
RNeasy Mini Spin Columns	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
Buffer RLT Plus	45 ml
Bufer RW1	45 ml
Buffer RPE (concentrate)	11 ml
RNase-Free Water	10 ml

Table 2.3: The components provided by Qiagen required for RNA extraction from cells

4 volumes of 96-100% ethanol were added to the Buffer RPE concentrate to obtain a working solution.

When the QIAshredder spin columns were used, 10 μ l of β -mercaptoethanol were added per 1 ml of Buffer RLT Plus.

The RNase Free DNase I set contains:

Reagents	Volume Supplied (ml)	
DNase I, RNase-Free (lyophilized)	1500 Kunitz units	
Buffer RDD	2 x 2 ml	
RNase-Free Water	1.5 ml	
Table 2.4: The reagents provided by Qiagen for DNase treatment		

DNase I stock solution was prepared by injecting 550 μ l of RNase-Free water into the vial of lyophilized DNase using a needle and a syringe. This was to prevent loss of the DNase as the lyophilized enzyme can stick to the septum. The dissolved DNase was then removed using the needle and syringe and divided into 50 μ l or 10 μ l aliquots and stored at -20°C.

For use during RNA extraction, the DNase I was prepared by adding 10 µl of DNase stock solution to 70 µl of Buffer RDD per sample.

Other consumables used for the RNA extraction procedure include Kimtech Science Kimwipes purchased from Kimberley Clark Professional, West Malling, Kent, England, UK; 1.5 ml flip top tubes and RNase-free eppendorfs from Fisher Scientific; extra 2 ml collection tubes from Qiagen and the RNase Zap[™] was purchased from Sigma Aldrich.

2.1.8 Reverse Transcription

DEPC-treated water was purchased from Invitrogen. Collection tubes (2 ml) were purchased from Qiagen. cDNA was made from RNA with the use of the Applied Biosystems High Capacity cDNA Reverse Transcription kit purchased from Life Technologies Ltd, Paisley, Scotland, UK. The kit contained the following:

Reagent	Volume	
10x RT Buffer	1 x 1 ml	
25x dNTP Mix (100 mM)	1 x 0.2 ml	
10x RT Random Primers	1 x 1 ml	
Multiscribe™ Reverse Trasncriptase (50 U/μl)	2 x 0.2 ml	
Table 2.5: The components in the kit required to make cDNA from RNA		

2.1.9 Real time Polymerase Chain Reaction

The Taqman[®] Gene Expression Master Mix was purchased from Applied Biosystems, Life Technologies Ltd, Paisley, Scotland, UK. The Taqman[®] Gene Expression Assay probes were also purchased from Life Technologies Ltd. DEPC-treated water was purchased from Invitrogen. The 0.2 ml PCR tubes were purchased from Fisher Scientific and the MicroAmp[®] Optical 384-well reaction plates were purchased from ThermoFisher Scientific, Lutterworth, Leicestershire, UK.

2.1.10 Mass Spectrometry and Proteomic Analysis

The materials and reagents required for proteomics analysis were as follows:

Materials	Grade, Preparation	Source
3K Filter Units	Amicon Ultra 0.5 ml	Millipore UK Limited,
	centrifugal filter units	Livingston, West Lothian, UK
Glass Vials	Glass Screw Neck Vials	Waters Limited, Elstree,
		Hertfordshire, UK
50 mM Ammonium	0.79 g in 200 ml Ultrapure	Sigma Aldrich
Bicarbonate (AmBic), pH 7.6	water	
100 mM Dithioreitol (DTT)	3 mg into 200 μl of 50mM	Sigma Aldrich
	Ammonium Bicarbonate	
200 mM Iodoacetamide (IAA)	3.7 mg into 100 μl of	Sigma Aldrich
	50mM Ammonium	
	Bicarbonate	
Trypsin 1 μg/μl	20 μg or 25 μg vial. Add	Roche Diagnostics Ltd,
	20 μl or 25 μl 50mM	Burgess Hill, UK
	Ammonium Bicarbonate	
	to make 1 μg/μl	

Table 2.6: Components required for proteomic sample preparation

2.1.11 Bicinchoninic Acid Protein Micro Assay

Reagent [A]:

Weight (g) / Volume (ml)	Reagent	Company
0.8 g	Na Carbonate	Sigma Aldrich
	monohydrate/anhydrous	
0.16 g	Tartaric acid	Sigma Aldrich
10 ml	Water	-
Table 2.7: The components required to make Reagent A at a pH of 11.25 with 1 M NaOH		

Reagent [B]:

Weight (g) / Volume (ml)	Reagent	Company
1 g	Bicinchoninic Acid	Sigma Aldrich
25 ml	Water	-
Table 2.8: The components required to make Reagent B		

Reagent [C]:

Weight (g) / Volume (ml)	Reagent	Company
0.4 g	CuSO ₄ .5H ₂ O	Sigma Aldrich
10 ml	Water	-
Table 2.9: The components required to make Reagent C		

The Working Solution was freshly made when required:

50 μI [C] was mixed with 1.25 ml [B], and then 1.35 ml [A] was added.

2.1.12 Plasmid Propagation

pHyPer-cyto and pHyPer-dMito vectors were purchased from BioCat, Heidelberg, Germany. The α-Select Chemically Competent Cells (bronze efficiency) were purchased from Bioline Reagents Limited, London, England, UK and the EndoFree® Plasmid Maxi kit was purchased from Qiagen. 50 mg/ml Kanamycin antibiotic solution from *Stretomyces kanamyceticus* (1000X) and molecular biology grade 2-Propanol were purchased from Sigma Aldrich.

LB Media:

Component	Quantity	Company
LB Broth	10 g/400 ml or 25 g/Litre	Sigma Aldrich
MilliQ Water	As Required	-
Table 2.10: The components required to make LB Media		

LB Agar:

Component	Quantity	Company
LB Broth	10 g/400 ml or 25 g/Litre	Sigma Aldrich
Difco™ Agar Solidifying Agent	1.5 g/100 ml or 6 g/400 ml	Sigma Aldrich
MilliQ Water	As Required	-
Table 2.11: The components required to make LB Agar		

2.1.13 Equipment

For tissue culture studies, the equipment used included the Panasonic MCO-19AIC-PE CO₂ incubator; the Beckman Coulter Allegra X-30R centrifuge and the ScanLaf Mars 1200 Class 2 safety cabinet.

For nucleic acid work, the NanoDrop ND-800 from Labtech; the Power Gen 125 Homogeniser from Fisher Scientific; the microcentrifuge 5424R from Eppendorf; the AirScience Pure Air P20XT hood; the Techne Sample Concentrator; the Applied Biosystems Viia™7 Real-Time PCR System by Life Technologies and the Gstorm GS4 Multi Block Thermal Cycler were used.

For the transformation studies, the equipment included the Panasonic MIR-154-PE Cooled incubator; the Panasonic MIR-262-PE Heated incubator; the Panasonic MIR-S100-PE orbital shaker; the Beckman Coulter Allegra X-30 Centrifuge; the SLS Integra Fireboy and the Autoclave 2100 Classic from Prestige Medical. The transfection was carried out with NEPA GENE products; the NEPA21 Electroporator; the EC-002S electroporation cuvettes and the CU500 Cuvette Chamber.

The microscopes used include the EVOS[®] XL Core microscope and the fluorescent AMG Evos[®] Fl microscope from Life Technologies.

The R1010 SpeedVac[™] system and the Q Exactive[™] Hybrid Quadruple-Orbitrap Mass Spectrometer from Thermo Scientific were used for proteomic analysis.

Other equipment used included the NOVOstar plate reader from BMG Labtech; the ELx800 Absorbance Microplate Reader from BioTek; the CyAn[™] ADP Analyzer from Beckman Coulter, and the Wesbart IS89 Plate Shaker Heater.

2.2 Methods

2.2.1 Tissue Culture

2.2.1.1 Sub-culturing of cells

The hTERT hASMC were cultured in DMEM with 4.5 g/L glucose containing 20% FBS, 100 µg/ml penicillin/streptomycin, 60 µg/ml Hygromycin B and SmGM[™]-2 SingleQuots (hFGF-B, hEGF and insulin).

hCASMC were purchased from Promocell and cultured in Promocell Smooth Muscle Growth Medium 2 to which a vial of SupplementMix containing 0.05 ml/ml FCS, 0.5 ng/ml recombinant human EGF, 2 ng/ml recombinant human FGF-B and recombinant human insulin was added.

The HDF were cultured in DMEM with 4.5 g/L glucose containing 10% FBS.

All the cells were maintained in a $37^{\circ}C/5\%$ CO₂ incubator and passaged when they were approximately 80-90% confluent.

The hTERT hASMC and HDF cells were washed with pre-warmed DPBS then incubated with 5ml of pre-warmed 0.25% trypsin-EDTA solution for 5 minutes in the 37°C/5% CO₂ incubator. Upon release of adherent cells, the trypsin-EDTA solution was neutralised with cell media and the aspirated cell suspension was centrifuged at 200g for 5 minutes. Upon discarding the supernatant, cells were resuspended in fresh media and divided into sterile T75 flasks or multi-well plates for subsequent assays.

The hCASMC were passaged using the DetachKit purchased from Promocell. Cells were washed with 3 ml of HEPES-BSS, and then incubated at room temperature with 3 ml of pre-warmed trypsin-EDTA solution. Once adherent cells were released, 3 ml of Trypsin Neutralising Solution was added to neutralise the effect of the trypsin-EDTA solution and the cell suspension was centrifuged at 220g for 3 minutes. Upon discarding the supernatant, the cells were resuspended in fresh media and divided into sterile T25 or T75 flasks or multi-well plates.

2.2.1.2 Cell Count using the Trypan Blue exclusion assay

This assay is used to rapidly distinguish live cells from dead cells using a Trypan Blue dye which is only able to penetrate the membrane of dead cells, giving them a distinctive blue colour under a microscope.

Cells were grown in T75 flasks until confluent. The cells were then passaged as described in **2.2.1.1** and once the cell suspension was centrifuged at 200g for 5 minutes, the supernatant was discarded and 1 ml of fresh media was added to the cell pellet.

0.4% ("/ $_{v}$) Trypan Blue dye solution (20 µl) was pipetted into a 0.5 ml eppendorf tube containing a 20 µl aliquot of the cell suspension. The cells were left to stand for a minute to allow the dye to penetrate into dead cells then 10 µl of cell suspension-trypan blue mixture was applied to a haemocytometer and the number of viable cells was counted under a light microscope. Cells were counted within two 1 mm² regions of the haemocytometer and the mean value was used to calculate the percentage of viable cells using the equation below:

Cell concentration per ml = Average number of viable cells $x 10^4 x 2$

'10^{4'} represents the conversion factor to convert the volume of one large square in the haemocytometer, 10^{-4} ml to the 1 ml volume of the cell suspension.

'2' represents the 1:1 dilution factor from the addition of trypan blue.

2.2.1.3 Liquid Nitrogen storage

The cryopreservation of cells enables long term storage in liquid nitrogen. Cells were grown until confluent and then passaged as described by **2.2.1.1**. Once the cells had been centrifuged, a trypan blue cell count was carried out by adding 1 ml media to the pellet (**2.2.1.2**). After determining the number of cells, the cells were centrifuged again to remove the media and 1 ml of freeze mix was added to the resulting pellet. The freeze mix for the hTERT hASMC and HDF cells contained 50% serum, 40% Serum Free Media and 10% DMSO. For the hCASMC, the 'Cryo-SFM' freeze mix was purchased directly from Promocell. Once the pellet had been resuspended in freeze mix, the cell suspension was transferred into labelled cryovials and then placed into a CoolCell box to allow gradual freezing in the -80°C freezer. The cells were left in the -80°C freezer overnight and then transferred into liquid nitrogen for long term storage.

2.2.1.4 Resuscitation of frozen cells

A frozen vial of cells in a cryovial was taken out of liquid nitrogen and immediately placed into a sealed container for transport. The cryovial was placed into a 37°C water bath for 1 minute to allow the contents to thaw out and the cell suspension was added to a T75 flask containing 12 ml of pre-warmed media. The flask was then placed into the 37°C/5% CO₂ incubator. After 24 hours, the media in the flask was discarded and replaced with fresh pre-warmed media and the flask placed back into the incubator.

2.2.2 Determining cytotoxic effects using the CCK-8 Assay

To measure cell viability, the Cell Counting Kit-8 (CCK-8) was used. The assay contains a highly water soluble tetrazolium salt which allows for sensitive colorimetric assays for the determination of the number of viable cells in cytotoxicity assays. The salt, named as WST-8 is reduced by dehydrogenases in the cells, producing an orange coloured water-soluble formazan dye. The amount of formazan dye generated by the cellular dehydrogenases is directly proportional to the number of living cells.

The protocol provided from Sigma Aldrich was followed. Cells were seeded in 96-well plates at various densities as required. After treating the cells with the appropriate compound for the required treatment time, 5 μ l of the CCK-8 solution were added to each well and the plate was placed in the 37°C/5% CO₂ incubator for half an hour. The absorbance at 450 nm was then measured using the absorbance microplate reader. The cell viability results were recorded as a percentage of the vehicle controls. Experiments were carried out on separate days with 6 technical replicates.

2.2.3 Determining cytotoxic effects using the Trypan Blue Exclusion Method

Another method of recording cell death involved the use of the trypan blue exclusion method to determine compound cytotoxicity to the hTERT hASMC. Dead cells show a distinctive blue colour under a microscope, whilst live cells are impermeable to the trypan blue dye.

The hTERT hASMC were seeded in 6 well plates at a density of 2x10⁵ cells per well and treated with the required compound concentrations for the desired length of treatment time. Following treatment, the media was aspirated from each well and the cells were washed with 1 ml of pre-warmed DPBS. 1 ml of 0.25% trypsin-EDTA solution was added to each well and the plates were placed in the 37°C/5% CO₂ incubator for 5 minutes to allow the cells detach. Media was added to each well to deactivate the effect of the trypsin and the resulting suspension centrifuged at 200g for 5 minutes. After centrifugation, the supernatant was discarded and the cells resuspended in 1 ml of fresh media and cell counts were carried out using the trypan blue dye solution as directed in **2.2.1.2**. The resulting cell number was expressed as a percentage of the media control. Experiments were carried out on separate days with 3 technical replicates.

2.2.4 Determining cytotoxic effects using the ATP Assay

To quantitatively determine the ATP content in treated cells, the protocol from Invitrogen's ATP determination kit was followed. The assay relies on the recombinant firefly luciferase enzyme and its requirement for ATP in producing light. The luciferase substrate D-luciferin combines with ATP and cellular oxygen and with the action of luciferase, produces carbon dioxide, oxyluciferin, AMP, pyrophosphate and light; the latter which is measured using the NOVOstar plate reader.

Cells were seeded in clear-bottomed 96 well plates at a density of 1×10^3 cells per well and treated with the relevant compounds for appropriate treatment times. Standard reaction solution was prepared as instructed by the protocol and covered with foil to protect from light until use. At the end of the required treatment time, the treated media was removed from the wells using a multi-channel pipette and the wells were washed with 200 µl of DPBS, and then washed again with 100 µl of DPBS. After the second DPBS wash, 100 µl of the light-protected standard reaction solution were added to each well. The plate was then shaken for 1 minute on the plate shaker and the NOVOstar plate reader was used to measure the luminescence. The results from the ATP assay were recorded as a percentage of the vehicle controls. Experiments were carried out on separate days with 6 technical replicates.

2.2.5 Flow Cytometry and Reactive Oxygen Species detection

With the use of fluorogenic dyes, DHE and MitoSOX[™], cytosolic superoxide and mitochondrial superoxide respectively, can be detected in live cells. DHE exhibits blue fluorescence upon entering the cytosol, until it becomes oxidised by superoxide in the cell; causing it to intercalate into the cell's DNA and stain the nucleus bright red. MitoSOX[™] however is rapidly

and selectively targeted to the mitochondria with its cationic triphenylphosphonium moiety, making it more specific to superoxide produced from the mitochondrial compartment. Upon entering the organelle, MitoSOX[™] is readily oxidised by superoxide and exhibits red fluorescence. Like DHE, the oxidation product becomes highly fluorescent when bound to nucleic acid. Both dyes are excited by the blue 488 nm laser and can be detected by the FL2 detector for PE and the FL3 detector for PE-Texas Red (emission wavelengths 575 nm and 613 nm respectively).

Cells were seeded in 6 well plates at a density of 2x10⁵ cells per well for hTERT hASMC. Cells were grown to 70-80% confluency then treated with appropriate compounds for a desired time course.

After the required treatment time, either MitoSOX^m or DHE was added to the wells to give a final concentration of 5 μ M. The plate was covered with foil to prevent light exposure and incubated in a 37°C/5% CO₂ incubator for 30 minutes to allow loading of the dye. One control well was left without dye.

After the 30 minute loading period, the media was retained, the wells were washed with DPBS and trypsinised for 5 minutes then neutralised with fresh media and the cell suspension was added to the respective retained media and centrifuged for 5 minutes at 200g. The media was discarded, the pellet resuspended in 800 μ l of DPBS and centrifuged again at 200g for 5 minutes. The DPBS was discarded and the pellet resuspended into sterile FACS tubes containing 500 μ l of DPBS. The cells were stored on ice whilst the flow cytometry was performed. Cells in the media alone were used as a control. Experiments were carried out on separate days with 3 technical replicates.

2.2.6 MitoSOX™ microscopy and Reactive Oxygen Species detection

As an alternative to the quantitative flow cytometry method, mitochondrial superoxide was detected by fluorescence microscopy following staining with the targeted MitoSOX[™] dye. This was utilised when cell numbers were too low to carry out flow cytometry studies.

CellView[™] cell culture 4 compartment dishes were used and hCASMC were seeded at a density of 2.5x10⁴ into each compartment and allowed to adhere overnight. For short term treatments, MitoSOX[™] was loaded into the compartments giving a final concentration of 5 μM and the dishes were covered with foil to prevent light exposure and placed into the 37°C/5% CO₂ incubator for 10 minutes. One control well was left without the dye. The cells were then treated with the required compounds and placed back into the 37°C/5% CO₂ incubator for another 30 minutes. Following treatment, the cells were examined under the EVOS® fluorescence microscope. The microscope contained an RFP light cube with an excitation wavelength of 531±40 nm and an emission filter of 593±40 nm which was ideal for the MitoSOX[™] excitation/emission spectra of 510/580 nm. For longer treatments, the cells were first treated with the compounds for the desired treatment period. 30 minutes prior to the end of the treatment time, MitoSOX[™] was added at a final concentration of 5 µM within each compartment; covered with foil then placed back into the 37°C/5% CO₂ incubator for the final 30 minutes. The cells were then examined on the EVOS® fluorescence microscope.

To quantify the fluorescence, images were taken in 6 fields of view and ImageJ software was used to measure the Integrated Density. Integrated Density is the measurement for pixel intensity; and therefore the measurement for fluorescence intensity. The measurements for all the fields of view were averaged and presented in a quantitative manner.

2.2.7 The Amplex Red Assay and Reactive Oxygen Species detection

The Amplex Red assay is a sensitive, one-step assay that detects H_2O_2 produced by cells. In the presence of peroxidase, the Ampliflu^M Red reagent reacts with H_2O_2 to produce the red fluorescent oxidation product resorufin, with Ampliflu^M Red acting as the electron donor. Resorufin has excitation and emission maxima of approximately 571 nm and 585 nm respectively. Cells are impermeable to Ampliflu^M Red so the assay relies on the diffusion of H_2O_2 out of the cell and into the PBS.

Cells were seeded at a density of 5×10^3 per well in a clear-bottomed 96 well plate and left overnight in a 37° C/5% CO₂ incubator to allow the cells to adhere. The cells were then treated with the ROS inducer in a Class II biosafety cabinet, leaving some wells treatment-free for both the positive and negative controls. The plate was then incubated for the desired time course. An hour before the end of the treatment time, a 10 mM menadione stock solution was prepared (0.001 g of menadione dissolved into 581 µl DMSO). 100 µl of this 10 mM stock was added to 900 µl of PBS to give a 1 mM stock solution. The appropriate dilutions were then made into media and 100 μ l of the solution was added to each positive control well to give a 50 μ M menadione exposure to the cells.

An hour later (by the end of the desired treatment period), the media in all the wells was discarded and 20 μ l of PBS warmed to 37°C were added to each well. 20 μ l of each of the H₂O₂ standards (**Table 2.12**) were added into cell-free wells in triplicates.

H_2O_2 concentration (nM)	$1 \text{mM} \text{H}_2 \text{O}_2$ solution (µl)	PBS (µl)		
1 mM H ₂ O ₂ solution = 1 μl H ₂ O ₂ stock (9.8 M) + 9.8 ml PBS				
0	-	1000		
31.25	31.25 μl of 1 μM stock	968.75		
62.5	62.5 μl of 1 μM stock	937.5		
125	125 μl of 1 μM stock	875		
250	1	3999		
500	1	1999		
1000	2	1998		

The H₂O₂ standards were prepared as follows:

Table 2.12: H₂O₂ dilutions were prepared in order to establish a standard curve for the Amplex Red assay.

The Amplex Red assay reaction mixture was then prepared. Horseradish Peroxidase (0.0001 g) was dissolved in 2 ml PBS (10 U/ml) immediately before it was required. 100 µl of this was added to 100 µl of 10 mM Ampliflu[™] Red and 9800 µl PBS. This mixture was heated at 37°C for 10 minutes in the dark.

 $20 \ \mu$ l of the Amplex Red assay reaction mixture was then added to each of the wells and the plate was incubated at 37° C in the dark for at least 30 minutes prior to measurement of fluorescence.

Fluorescence at 590 nm was measured every 30 minutes for up to 2 hours with the NOVOstar plate reader using an excitation of 560 nm.

2.2.8 Senescence Detection using the Senescence Associated βgalactosidase staining kit

The Senescence Cells Histochemical Staining Kit is an assay based on a histochemical stain for β -galactosidase activity at pH 6. Unlike quiescent, immortal or tumour cells, there is increased activity of senescence associated β -galactosidase activity in senescent cells (Sikora *et al.*, 2011), making the assay an efficient method of detecting senescent cells in a population. The method relies on β -galactosidase hydrolysing X-gal into colourless galactose and 4-chloro-3-brom-indigo, forming an intense blue precipitate; signifying β -galactosidase and thus detecting senescent cells.

The assay was carried out as directed by the manufacturer (Sigma Aldrich). Following the desired treatments, cells were trypsinised, counted using a haemocytometer (**2.2.1.2**) and reseeded at a density of 1×10^4 per well of a 6 well plate and left overnight to adhere, with each treatment containing 3 technical replicates.

X-gal solution was warmed to 37° C for an hour prior to starting the assay to avoid formation of aggregates that could interfere with the visualisation of the stained cells. The growth medium was aspirated from the cells and the wells were washed twice with 1 ml of DPBS per well. The entire wash solution was removed by gentle aspiration and 1.5 ml of 1X Fixation buffer was added to each well and the plate was incubated for 7 minutes at room temperature. The wells were then rinsed 3 times with 1 ml of DPBS and 1 ml of sterile filtered Staining Mixture (1 ml of 10X Staining solution, 125 µl of Reagent B, 125 µl of Reagent C, 0.25 ml of X-gal solution and 8.5 ml of MilliQ water) was added to each well. The edges of the multiwall plate were then sealed with parafilm to prevent the plate from drying out and incubated in a 37°C incubator without CO₂ overnight. Senescent cells stain specifically at pH 6.0 therefore the cells needed to be incubated in an environment not rich in CO₂ as this may alter the pH of the solution.

After the incubation period, the cells were observed under an EVOS light microscope and the number of blue-stained and total number of cells was manually counted using 5 fields of view selected at random per well; allowing for the calculation of the percentage of cells expressing β -galactosidase. For long term storage at 4°C, the staining mixture was removed from each well and replaced with 1 ml of 70% glycerol per well.

Images were taken at x100 magnification using an EVOS light microscope.

2.2.9 Induction of Stress Induced Premature Senescence

2.2.9.1 Mitochondrial inhibitors

Stress Induced Premature Senescence (SIPS) can be induced in cells with the use of successive treatment over a number of days (Mistry, 2009).

Cells were seeded into a 6 well plate at a density of 1×10^5 per well and left to adhere overnight, in 3 technical replicates. The cells were then treated with the required compound for a period of 1-4 hours. Following the treatment, the media was removed; the wells rinsed with DPBS and then fresh media was added to each well to allow the cells to recover. This treatment was repeated the following 2 days enabling a 3 day successive treatment. After the third day, the cells were allowed to recover for 48 hours prior to SA β -galactosidase staining. This recovery period is important for *in vitro* SIPS measurement as it enables judgements independent from adaptive responses to be made (Mistry, 2009; Toussaint *et al.*, 2000). Following the recovery period, the cells were trypsinised, counted and reseeded into 6well plates at a density of 1×10^4 per well in complete media. The cells were then stained for SA- β galactosidase activity 24 hours after reseeding as detailed in section 2.2.8.

2.2.9.2 Angiotensin II

Alternative to successive treatment, SIPS can also be induced by a single hit with a stress inducer (Mistry, 2009). Cells were seeded into a 6 well plate at a density of 1×10^5 per well and left to adhere overnight, in 3 technical replicates. For treatments with AngII, the media was replaced with media containing 1% FBS minus smooth muscle cell growth supplement for the hTERT hASMC or media containing 0.5% FBS for the hCASMC for 24 hours. The absence of growth factors present in the FBS prevents the down-regulation of AT1R gene expression and synchronises the cells into the G₀ phase of the cell cycle (Mistry, 2009). The media of wells containing cells that would be treated with positive controls, t-BHP or AA were replaced with complete media.

Once rendered quiescent, the cells were incubated with 10^{-8} and 10^{-7} nM AngII for the desired time course. AngII was then removed; the wells rinsed with DPBS and then replaced with complete media to allow the cells recover for 48 hours. Treatment with 50 μ M t-BHP was carried out in the same manner but with media containing 20% FBS. Following the recovery period, the cells were trypsinised, counted and then reseeded into 6 well plates at a density of 1×10^4 per well in complete media.

The cells were then analysed for SA β -galactosidase activity 24 hours after reseeding as detailed in section **2.2.8**.

2.2.10 RNA Extraction

RNA was extracted from cells and stored at -80°C for future experiments. To extract the RNA, a modified protocol of the Qiagen RNeasy Plus Kit was followed with different protocols based on the different cell types.

2.2.10.1 Cell disruption with homogenisation

For the hTERT hASMC, a homogeniser was used initially to disrupt the cell membranes. The homogeniser was initially cleaned by wiping with 10% IMS. The cleaning process was continued by running the homogeniser with MilliQ water, RNase Zap, MilliQ water again and a final run with 100% Ethanol. The homogeniser was allowed to run dry to ensure all the material and solutions had been removed. Between disrupting each sample, the homogeniser was cleaned as described.

Cells were cultured in T75 flasks or seeded in 6 well plates for specific treatments. The medium was then aspirated and the cells gently washed with DPBS. The DPBS was removed thoroughly and trypsin was added to allow the cells to detach. The trypsin was then neutralised with pre-warmed media and the cells were centrifuged at 200g for 5 minutes. The media was discarded; the cells resuspended in 1 ml DPBS and the suspension was transferred into a cryovial on ice.

The samples were centrifuged in a microfuge at 9400g for 5 minutes and the supernatant carefully removed with a pipette. The pellet was resuspended in 350 μ l of Buffer RLT and the sample was homogenised until it was uniformly homogenous. The sample was then placed back on ice.

Once all the samples had been homogenised, 1 volume (350 μ l) of 70% ethanol was added to the lysate and the solution was mixed by pipetting. 700 μ l of sample were added to an RNeasy spin column and the modified QIAGEN RNeasy Protocol was continued (**2.2.10.4**).

2.2.10.2 Cell homogenisation with Trizol® Reagent

hCASMC numbers were generally much lower than hTERT hASMC so the homogeniser was not used. Trizol[®] Reagent was more appropriate as it was added to cell pellets following cell subculture.

hCASMC were sub-cultured as normal (**2.2.1.1**) and following centrifugation, the supernatant was removed, taking care not to disturb the pellet. 600 μ l of Trizol® Reagent were added to the pellet and mixed thoroughly by pipetting. The Trizol homogenate was added to a 1.5 ml eppendorf tube and incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex.

100 μ l of chloroform were added to the homogenate and capping the tube securely beforehand, the tubes were then shaken vigorously. The samples were left to incubate at room temperature for 2-3 minutes.

Following incubation, the samples were centrifuged at 18,400g for 10 minutes at 4°C. After centrifugation, there were 3 phases in each tube – a lower red phenol-chloroform phase, an interphase, and a colourless aqueous phase. As much of the aqueous phase as possible was removed by angling the tube at 45° and pipetting the solution out, taking care not to touch the interphase.

An equal volume of 70% ethanol was added to the aqueous phase in a new 1.5 ml eppendorf tube and this was mixed well by pipetting. This solution was added to an RNeasy spin column and the modified QIAGEN RNeasy protocol was continued (section **2.2.10.4**).

2.2.10.3 Cell disruption with RLT Buffer and the QIAshredder spin columns

For hCASMC with low starting material ($<3x10^5$ cells), this method of cell disruption was used to improve RNA yield and reduce contamination.

Prior to use, Buffer RLT Plus was prepared by adding 10 μ l of β -mercaptoethanol per 1 ml of Buffer in a fume hood.

When ready, the cells were sub-cultured as normal (**2.2.1.1**) and following centrifugation, the supernatant was completely aspirated, ensuring as much cell-culture medium as possible was removed from each sample. The pellets were then loosened thoroughly by flicking the tube and 350 μ l of Buffer RLT Plus were added to each pellet. The samples were then vortexed to mix and then homogenised by pipetting the sample directly into a QIAshredder spin column

placed into a 2 ml collection tube. The tubes were centrifuged for 2 minutes at maximum speed (21,100g).

1 volume (350 μ l) of 70% ethanol was added to the flow-through and mixed well by pipetting. This resulting 700 μ l of sample were then added to an RNeasy spin column and the modified QIAGEN RNeasy Protocol was continued (Section **2.2.10.4**).

2.2.10.4 Modified QIAGEN RNeasy RNA Extraction

Once the sample had been mixed with an equal volume of 70% ethanol and added to an RNeasy spin column, the samples were then centrifuged at 9,400g for 2 minutes. If the entire sample had not passed through the column, the column was centrifuged for an additional 2 minutes. The flow through was discarded and replaced with a fresh collection tube to prevent contamination of the sample with Buffer RLT.

350 μ l of RWI Buffer were added to each column, the columns were inverted and rolled 4-5 times and then spun at 9400g for 2 minutes. The flow through was discarded.

80 μ l of DNase I were added to each RNeasy column and the samples were incubated at room temperature for 30 minutes. After the incubation, 350 μ l of RWI buffer were added to each column, the columns inverted and rolled 4-5 times and then centrifuged at 9400g for 2 minutes. The flow through tubes were discarded and replaced with fresh collection tubes.

500 μ l of RPE buffer were added to each spin column; the columns were inverted and rolled and then left to incubate for 5 minutes at room temperature. The columns were then centrifuged at 9,400g for 2 minutes and the flow-through discarded.

Another 500 μ l of RPE buffer were added to each spin column; the columns were inverted and rolled and then left to incubate for 5 minutes at room temperature. The columns were then centrifuged for 4 minutes and the flow-through discarded. To further dry the spin-column and prevent solvent contamination of RNA, a 'dry-spin' was performed at 9,400g for 5 minutes with a new collection tube.

The collection tubes were replaced with RNase-free eppendorf tubes and 30 μ l of RNase-free water were added to each spin column. The columns were centrifuged at 9,400g for 2 minutes to elute the RNA. The same sample was passed through the column again by centrifuging at 9,400g for a further 2 minutes.
The samples were aliquoted into three tubes of 10 μ l each and frozen down at -80°C. 1.5 μ l from one aliquot was used to analyse the purity and yield of each sample with the NanoDrop.

2.2.11 RNA or DNA Precipitation

In situations where impure nucleic acid was obtained, a precipitation was carried out to acquire better purities.

To begin, a 1/10 volume of 3 M pH 5.2 sodium acetate was added to the RNA sample and this was mixed well by pipetting. Ice-cold 100% ethanol (2 to 2.5 volumes calculated after the addition of the salt) was added and this was again mixed well by pipetting.

For RNA precipitation, the resulting solution was centrifuged at 4°C for 30 minutes on maximum speed, 21,100g. The supernatant was carefully removed and 500 μ l of ice-cold 70% ethanol were added. The tube was quickly vortexed to allow thorough mixing of the pellet and then centrifuged at 4°C for 3 minutes at 21,100g. The supernatant was removed and another 500 μ l of ice-cold 70% ethanol were added, the tube vortexed and then centrifuged at 4°C for 3 minutes at 21,100g.

For DNA precipitation, once the ice-cold 100% ethanol had been added and mixed well, the resulting solution was placed on ice or in the -20°C freezer for 30 minutes. The solution was then centrifuged at 13,000g in a microfuge for 30 minutes at 4°C. Following centrifugation, the supernatant was carefully removed and 1 ml 70% ethanol was added which was followed by a quick vortex and another centrifugation at 13,000g for 5 minutes.

For both RNA and DNA, once the centrifugation had been completed, the supernatant was carefully removed and the pellet was allowed to air-dry for 15 minutes. 30 μ l of Nuclease-free water were added to re-dissolve the pellet, the tube was then briefly vortexed and then centrifuged to bring down any water droplets on the side of the tubes. The samples were then analysed on the NanoDrop (2.2.12)

2.2.12 RNA Quantification

The NanoDrop is a cuvette-free spectrophotometer that allows for the purity and quantification of DNA, RNA and protein samples to be measured.

The NanoDrop software was opened and the nucleic acids module was selected. The spectrophotometer was initialised by pipetting 1.5 μ l sterile MilliQ water onto the lower optic surface. A blank measurement was then performed by loading 1.5 μ l of RNase-free water. 1.5 μ l of the nucleic acid samples were then loaded onto the lower optical surface and the samples were measured. The optical surface was wiped clean with a kimwipe between each loading.

Purity was determined by calculating the ratios for the optical density 260/280 and 260/230. 260 nm represents the peak nucleic acid absorbance; 280 nm represents the peak protein absorbance, whilst 230 nm represents the peak carbohydrates, peptides, phenols and aromatic compounds absorbance.

For RNA, a ratio of between 1.8 - 2.07 is classified as acceptable for 260/280, signifying the RNA is relatively free from protein contamination. A ratio between 1.8 - 2.2 is the acceptable range for 260/230, meaning that there is a low level of contamination from the molecules that absorb in the 230 nm range. For DNA, ratios between 2.0 - 2.2 was deemed suitable for the 260/230 ratio and a value of ~1.8 was deemed suitable for the 260/280 ratio.

2.2.13 Reverse transcription – RNA to cDNA

Once RNA had been extracted from the cells, it was reverse transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Trasncription kit. A master mix for each RNA sample was prepared as shown in the following table:

Master Mix (Total volume = 20 μl, 100 ng/μl RNA)				
Reagent	Concentration	Volume for 1 sample	Volume for 5	
		(μl)	samples (μl)	
10x RT buffer	1x	2	10	
25x dNTP Mix (100	1x	0.8	4	
mM)				
10x RT Random	1x	2	10	
Primers				
Multiscribe™		1	5	
Reverse				
Transcriptase				
Nuclease-free water		Sample-dependent	Sample-dependent	
RNA	2 μg (10 μl at 200	Sample-dependent	Sample-dependent	
	ng/μl)			

Table 2.13: For each RNA sample, one Master Mix was made into an Eppendorf tube. The Master Mix was then aliquoted into five 0.2 ml tubes with 20 μ l in each. Depending on the yield of the RNA sample, the resulting volume of RNA and Nuclease-free water was adjusted accordingly to keep the final volume of RNA and water for 1 sample as always 14.2 μ l.

Once the Master Mix was made and aliquoted into 0.2 ml tubes with 20 μ l in each tube, the tubes were placed in the G-storm thermal cycler and an RT Total RNA protocol with the following settings was run:

- 1x 25°C 10 minutes
- 1x 37°C 120 minutes
- 1x 85°C 5 minutes
- Hold 4°C Infinite

Once the programme had finished running, the samples were removed from the G-storm thermal cycler and the resultant cDNA was diluted to 20ng/µl by adding 80 µl of nuclease-free water to each 20 µl cDNA aliquot. The 5 tubes were then pooled together and vortexed and placed on ice. Once an appropriate volume of cDNA had been taken out for standard curve measurements, the remaining cDNA was aliquoted into 50 µl aliquots and stored at -20°C.

2.2.14 Real Time Polymerase Chain Reaction

To quantify the expression level of target genes in samples, Real-Time Polymerase Chain Reaction was used. A Master Mix was prepared for each target probe as follows:

Master Mix (Total volume = 4 µl/reaction)				
Reagent	Concentration	Volume for 1 sample	Volume for 22	
		(μl)	samples (μl)	
TaqMan [®] Gene	1x	2.5	55	
Expression Master				
Mix				
Taqman [®] assay 20x	1x	0.5	11	
Nuclease-free water		1	22	
Table 2.14: For each Tagman [®] assay probe, one Master Mix was made into a 0.5 ml				

Table 2.14: For each Taqman[®] assay probe, one Master Mix was made into a 0.5 m Eppendorf tube.

Once removed from the freezer, the Taqman[®] assay probe was defrosted, centrifuged and then vortexed to ensure thorough mixing. A standard curve was constructed to test the efficiency of the Taqman[®] probe.

Standards (ng input) Volume of cDNA (µl) Volume of Nuclease-free water (µl) 40 0 15 20 30 0 10 $15 \,\mu$ l from 20 ng tube 15 5 15 µl from 10 ng tube 15 2.5 15 µl from 5 ng tube 15 1.25 15 μ l from 2.5 ng tube 15 6.25 15μ l from 1.25 ng tube 15 0 0 30 **NTC (Negative Control)** 0 30

The standard curves were constructed as follows:

Table 2.15: Standard curves were constructed by carrying out serial dilutions with the use of cDNA and Nuclease-free water

Once the Master Mixes were made with the various probes, they were added into respective standards wells of a 384 well plate. For the highest input of 40 ng, 3 μ l of Master Mix was used whilst for all the remaining concentrations; 2 μ l of Master Mix was used.

The cDNA standards were then added to the wells, adding 2 μ l to the 40 ng wells and 1 μ l to all the remaining wells. The final volume in all the wells was 5 μ l. The plate was sealed with a plastic film, shaken to ensure thorough mixing, and then briefly centrifuged.

A PCR standard curve was generated by selecting a standard curve protocol on the AB Viia 7 machine. The following settings were used:

- 1x 50°C 2 minutes
- 1x 95°C 10 minutes
- 40x 95°C 15 seconds
- 60°C 1 minute

From the standard curve, probe efficiency was calculated with the equation:

Efficiency calculation: $(10^{-1/-slope} - 1) \times 100$

A slope between -3.6 and -3.1 was deemed acceptable and the percentage efficiency of each probe was required to be within 10% of the others.

2.2.15 Fibroblast Bioassay

This assay utilises fibroblast cells to assess the effects of conditioned media generated by hCASMC that have undergone replicative senescence. This conditioned media models the SASP produced by the smooth muscle cells in an *in vivo* environment.

Senescent hCASMC were placed into 100 mm cell culture dishes. The cells were defined as senescent once they appeared to stop growing; SA β -galactosidase staining confirmed their permanent cell cycle arrest. The cells were kept alive with 5 ml fresh hCASMC media containing serum and other required growth factors. The media was changed every 3/4days, retaining the conditioned media for the fibroblast bioassay.

Young fibroblasts (Passages 2- 4) were seeded into three 100 mm cell culture dishes at a density of 4x10⁵ cells per dish in 10% FBS DMEM overnight. The following day, the media was

aspirated and the dishes rinsed with 5 ml pre-warmed DPBS. Then the appropriate media combination was added to each dish as follows:

- Condition 1 10 ml DMEM only
- Condition 2 5 ml DMEM : 5 ml fresh hCASMC media
- Condition 3 5 ml DMEM : 5 ml Conditioned media

The cells were then placed into the $37^{\circ}C/5\%$ CO₂ incubator for $\frac{3}{4}$ days.

After 3-4 days, the media was removed and discarded and the dishes were rinsed with 5 ml pre-warmed DPBS. 5 ml 0.25% trypsin-EDTA solution was added to each dish and then placed in the 37°C/5% CO₂ incubator for 5 minutes. The trypsin-EDTA solution was neutralised with 5 ml fresh DMEM media and the cells were centrifuged at 200g for 5 minutes. After centrifugation, the supernatant was discarded and the cells were resuspended in 1 ml fresh DMEM and the Trypan Blue cell count was carried out (**2.2.1.2**).

At the same time, the conditioned media produced by the senescent hCASMC was prepared to use in the bioassay. The media was aspirated and centrifuged at 200g for 3 minutes. The cells were rinsed with DPBS and fresh hCASMC media was added and the cells were put back into the 37°C/5% CO₂ incubator to continue the collection of conditioned media. After centrifugation, the supernatant was poured onto a fresh 100 mm cell culture dish and placed aside, ready for the treatment of fibroblasts subjected to 'Condition 3'.

Once the fibroblasts had been counted, they were reseeded at $4x10^5$ cells per dish again, and if the total cell number had fallen below that, then all the cells in the 1 ml suspension were seeded. As the cells were resuspended in 1 ml DMEM for the count, the appropriate volumes of DMEM were put into the dishes; for Condition 1, 9 ml DMEM was added to the cell suspension; for Condition 2, 5 ml hCASMC media and another 4 ml DMEM; and for Condition 3, 4 ml DMEM and the 5 ml of the conditioned media previously prepared. The final volume of media was 10 ml. The cells were then placed back into the $37^{\circ}C/5\%$ CO₂ incubator for another 3/4 days.

At the end of the bioassay, a SA β -galactosidase stain was carried out as directed in **2.2.8**.

Population doubling was calculated using the following equation:

 $PD = (Log_{10}Y - Log_{10}Z) / Log_{10}2$

PD – Population doubling

Y – Number of cells harvested Z – Number of cells seeded

2.2.16 **Proteomics Analysis**

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) is a very powerful technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. Being highly sensitive and selective, vast numbers of proteins can be identified from complex biological fluids. To determine the differences in the secretome of young early passage cells compared to aged senescent cells, LC-MS/MS was used.

Young (Passage 4) hCASMC were seeded into 100 mm cell culture dishes at a density of 1.5x10⁵ cells per dish and left to adhere to the dishes in complete medium overnight. As the growth was slow, the cells had not reached confluency overnight so they were left for another 72 hours to reach 80-90% confluency. The secretome was then collected and the cell counts were performed by trypan blue method **2.2.1.2**.

Senescent (Passage 20) hCASMC were unable to be seeded at accurate densities as the splitting of senescent and reseeding of the cells once senescent causes cell death and an inability for the cells to adhere to the plate. So instead the cells were continuously passaged until growth ceased and senescence estimated using SA β -galactosidase staining as described in 2.2.8. Confluent dishes were then selected for secretome collection and once the conditioned media had been removed the cell counts were performed by the trypan blue method (2.2.1.2).

To collect the secretome, once the cells were ready, the full culture medium was removed and discarded. The dishes were washed twice with pre-warmed DPBS followed by another two washes with pre-warmed Serum Free Media (SFM). 4.5 ml of fresh SFM was then added to the dishes and the cells were replaced into the incubator for 12 hours. After 12 hours, the media was collected and centrifuged at maximum speed (3,200g) for 10 minutes to remove all cell debris and the resulting supernatant was separated into 1 ml aliquots which were then stored at -80°C. The controls were prepared by separating SFM into eight 1 ml aliquots for each experiment and storing the aliquots at -80°C.

To begin the sample preparation, the samples were removed from -80°C and left to thaw. The samples were then placed into the SpeedVac[™] system for approximately 6 hours to allow

them to dry and concentrate into smaller more manageable volumes. The samples were then kept overnight in a -20°C freezer.

An Amicon Ultra 0.5 ml centrifugal filter was placed into a collection tube which was then cleaned and prepared by loading 500 μ l of 50 mM Ammonium Bicarbonate onto the filters and then centrifuging the tubes at 18,400g for 10 minutes at 4°C. The sample in the collection tube was discarded. 400-500 μ l of the conditioned media sample was loaded onto the filter depending on how much volume would fit; and subsequently centrifuged at 18,400g for 20 minutes at 4°C. The resulting filtrate was discarded. The process of loading conditioned media sample onto the filter followed by centrifugation was repeated until all the sample had been loaded and centrifuged.

The collection tubes were then washed 3 times by loading 300-400 μ l Ammonium Bicarbonate onto the filter and centrifuging at 18,400g for 20 minutes at 4°C; discarding the filtrate after each wash.

The filter was then placed upside down into a new collection tube and centrifuged at 18,400g for 10 minutes at 4° C – the resulting solution was retained. The filter was then turned the right way up in the same collection tube and 100 µl of Ammonium Bicarbonate were added. This was left to incubate at room temperature for 2 minutes and then the filter was turned upside down again and centrifuged at 18,400g for 10 minutes at 4°C. The resulting solution was again retained and then the protein assay was carried out immediately.

A Bicinchoninic Acid (BCA) Protein Micro Assay was used to determine the protein concentration of each sample.

The standards were prepared as directed by the table below:

Well	Final µg BSA per well
A	40
В	20
C	10
D	5
E	2.5
F	1.25
G	0.625
Н	0

 Table 2.16: The concentrations of BSA were prepared in a serial dilution manner in order to establish a protein standard curve for the BCA Assay

Once the standards had been added to the respective wells in a 96 well plate, 10 μ l of each sample was added to empty wells in the plate. The Working Solution was made fresh (**2.1.11**) and 100 μ l of this were added to all the wells – standards and samples. Sellotape was placed over the wells to prevent the solutions from evaporating during incubation. The plate was then heated at 60°C for 40 minutes-1 hour. The absorbance was read at 562 nm following incubation and a standard curve was constructed and the protein concentrations in the samples determined.

Once 10 µl of each sample had been taken out for use in the protein assay, the remainder of the samples were subjected to reduction and alkylation. 100 mM Dithiothreitol (DTT) was made into 50 mM Ammonium Bicarbonate, pH 7.6. This was added to the samples to give a final concentration of 15 mM DTT. The samples were vortexed and incubated at 60°C for 15 minutes. DTT was used to reduce the disulfide bonds of proteins, whilst the Indole-3-Acetic Acid (IAA) was used to prevent the reformation of disulphide bonds after the protein reduction step with DTT. 200 mM IAA was prepared in 50 mM Ammonium Bicarbonate, pH 7.6. The solution was wrapped in foil due to IAA being light sensitive. The IAA was added to the sample to give a final concentration of 20 mM and the samples were incubated in the dark at room temperature for 30 minutes.

Once the concentration of protein had been determined, trypsin was then added to the samples based on the resulting concentration of protein in the samples. The rule is to use 1 μ g of trypsin per 50 μ g of protein however as the protein levels were so low, it was difficult to determine protein content so instead 2 μ l of 1 μ g/ μ l trypsin was added to each of the samples

as the estimated total protein content would not exceed 100 μ g. The samples were then placed in a shaking incubator at 37°C overnight.

The reaction was stopped using formic acid – adding 2 µl of 2% formic acid to the digested samples. The samples were vortexed briefly then placed in a shaking incubator at 37°C for 30 minutes. Following incubation, the samples were centrifuged for 10 minutes at 13,500g then vortexed briefly and centrifuged at 13,500g for 10 minutes to prevent sample loss in the subsequent SpeedVac[™] step. The samples were then placed in the SpeedVac[™] for approximately 3 hours to complete dryness where a small translucent pellet remained. The samples were then reconstituted in a 20 µl solution containing 10 µl of 0.1% formic acid and 100 femptomoles of alcohol dehydrogenase. A 20 minute centrifuge spin at 13,500g was carried out and the supernatant was collected and transferred into glass screw neck vials for analysis on the Q Exactive[™] Hybrid Quadruple-Orbitrap Mass Spectrometer.

2.2.17 Propagating the pHyPer vectors

2.2.17.1 Bacterial Transformation

The pHyper vectors are mammalian expression vectors encoding a fluorescent H_2O_2 sensor called HyPer. Two vectors were used; one cytoplasmic to detect fast changes in H_2O_2 concentration in the cytoplasm, whilst the other contained a duplicated mitochondrial targeting sequence to enable detection of H_2O_2 in mitochondria.

The vectors were used because of their insensitivity to other oxidants such as superoxide and NO. HyPer was developed by inserting the circularly permuted Yellow Fluorescent Protein (cpYFP) into the OxyR-RD regulatory domain of the bacteria *Escherichia coli* (*E.coli*). An *E.coli* cell is able to distinguish between and separately measure superoxide anions and H₂O₂. It does so using its 2Fe-2S cluster protein to measure the superoxide anions, and its redox active OxyR-RD domain to measure H₂O₂. The OxyR-RD contains two critical cysteine residues critical for sensing H₂O₂. A hydrophobic pocket restricts the penetration of charged molecules such as superoxide, however when the amphiphillic H₂O₂ arrives, it oxidises one of the cysteine residues to a charged sulfenic acid. This charged molecule is repelled by the hydrophobic pocket, so the charged cysteine becomes closer to the other critical cysteine residue and a disulphide bond is formed. Disulphide bond formation results in a conformational change in the OxyR-RD. Thus due to the integration of the cyYFP into the conformation-changing region,

it enables the domain to be used as a fully genetically encoded fluorescent indicator of H_2O_2 (Rhee *et al.*, 2010; Lukyanov & Belousov, 2014).

The bacterial nature of HyPer means that it has virtually no interaction partners in mammalian cells. Therefore it is less toxic upon overexpression and also subjected to less post-translational modifications that may result in false-positive results (Lukyanov & Belousov, 2014).

For each of the HyPer vectors, α -Select Chemically Competent Cells were transformed with each starting plasmid. The cells were initially slowly defrosted on wet ice for 20-30 minutes; one set of cells for each vector. 50 µl of cells were added to a 1.5 ml eppendorf tube and 1 µl of each plasmid was added. The tubes were then gently swirled and finger flicked for a few seconds to allow thorough mixing. The bacterial tubes were incubated on ice for 30 minutes. Following incubation, the tubes were subjected to 'heat-shock' by being placed in a 42°C water bath for 30-40 seconds without shaking. The tubes were then placed back on ice for ~2 minutes.

1 ml of antibiotic-free LB growth media was added to the tubes and the bacteria and media solutions were transferred into 15 ml falcon tubes. The caps for the falcon tubes were screwed loosely and the tubes were shaken at 200 rpm in a 37°C shaking incubator for 60 minutes.

Whilst the bacteria were growing in the shaking incubator, LB agar plates were being prepared. The plates were labelled on the bottom side to identify which plasmid was in which. As the bacterial promoter used in the HyPer vector provided kanamycin resistance gene expression, a final concentration of 50 μ g/ml of the antibiotic kanamycin was added to autoclaved LB agar prior to plating. The agar bottle was swirled gently, making sure not to introduce any bubbles and then the agar was poured out whilst warm into 100 mm petri dishes. Roughly 30 ml of agar was gently poured into each plate – enough to cover the dish. Any air bubbles were brushed to the side of the plate using a sterile pipette tip. The agar was allowed to cool for ~10 minutes and then the lids were placed on them and left to one side until the bacteria were ready to streak out. Any plates that were not streaked were stored in a 4°C fridge.

Once the 60 minute shaking incubation was complete, the tubes were removed from the incubator. A bacterial 'hockey-puck' spreader was sterilised by placing it in absolute ethanol and placing this into a naked Bunsen flame. Once cooled, the bacteria were plated out onto the kanamycin-containing plates in different densities by adding 50 μ l or 300 μ l of the bacterial solution onto the plates and spread evenly using the sterile spreader. The bacteria were allowed to air-dry for ~10 minutes and then the plate was inverted and placed in a 37°C

incubator overnight. Following incubation, the plates where then stored in a 4°C fridge. These plates were safe for use for a couple of months before being discarded.

2.2.17.2 Inoculating a Starter Culture

To allow maximal bacterial growth, starter cultures were generated from the colonies on the agar plate. Working near a Bunsen flame to keep the area sterile, 5 ml of LB media containing kanamycin were added to a 50 ml falcon tube. Using a loop dipped in ethanol and subsequently flamed, one bacterial colony from the LB agar plate was touched and dipped into the media. The loop was swirled in the media to shake off all the bacteria and the tube was swirled. The cap was loosely secured onto the falcon tube, ensuring it was not air-tight and the bacterial culture was incubated for ~8 hours in a 37°C shaking incubator at 200 rpm.

2.2.17.3 Inoculating an Overnight Liquid Culture

Overnight cultures were made to allow optimum bacterial growth prior to creating glycerol stocks for long term bacterial storage and plasmid purification to obtain large amounts of DNA. Conical flasks of at least 4 times the volume of culture were autoclaved prior to use. Into the conical flask, starter culture was diluted into LB medium containing kanamycin. As HyPer is a high copy number plasmid, 100-200 μ l of starter culture were diluted into 100 ml medium. The flasks were loosely covered with foil and autoclave tape and then incubated for 12-16 hours with vigorous shaking (~200-300 rpm) at 37°C.

2.2.17.4 Glycerol Stocks

Following an overnight culture, glycerol stocks were made for long-term stable storage of the transformed bacteria. 500 μ l of the overnight culture were added to 500 μ l of 80% glycerol in a cryovial and gently mixed. These stocks were frozen at -80°C.

To recover bacteria from a glycerol stock, the cryovials were removed from -80°C and kept frozen by placing them on dry ice. Working near an open flame to maintain sterility, the cryovials were opened and bacteria were scraped off the top using a loop dipped in ethanol and subsequently flamed, or a sterile pipette tip. The aim of this was to prevent the stock from thawing and thus preventing a reduction in shelf life. The bacteria were then streaked onto an LB agar plate containing Kanamycin and grown overnight in a 37°C incubator to allow isolation of individual bacterial colonies.

2.2.17.5 Endotoxin-free Plasmid Purification (Isolating DNA from bacterial cultures)

Maxi Preparation of plasmid DNA is the procedure of isolating large and pure yields of plasmid DNA. The use of the Qiagen endotoxin-free maxi prep kit minimises the levels of endotoxin present in the resulting DNA, to enable the isolation of transfection-grade DNA.

To begin the maxi prep procedure, a starter culture was inoculated from a freshly streaked selective plate and bacteria were grown for ~8 hours in a shaking 37°C incubator. An overnight culture was then made by diluting the starter culture in selective media. As there was 100 ml of overnight culture, the cells from this culture were split into two 50 ml falcon tubes and subsequently harvested by centrifugation at 6,000g for 15 minutes at 4°C. The pellets in each 50 ml falcon tube were resuspended in 5 ml of Buffer P1, resuspended completely by vortexing and then combined into one 50 ml falcon tube. As LyseBlue reagent had been added to the buffer, the buffer P1 bottle was vigorously shaken before use to ensure LyseBlue reagent particles were completely resuspended.

10 ml of Buffer P2 was then added to the tube and mixed thoroughly by vigorously inverting the sealed tube 4-6 times. The tube was then incubated at room temperature for 5 minutes. The presence of LyseBlue in Buffer P1 caused the cell suspension to turn blue after the addition of Buffer P2. LyseBlue is a colour indicator that provides visual identification of optimum buffer mixing, to prevent common handlings that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA and cell debris. Mixing of buffer P1 and P2 should result in a homogenously coloured suspension. If the suspension contained localised colourless regions or brownish cell clumps were visible, then the solution was continuously mixed until a homogenously coloured suspension was achieved.

During incubation, the QIAfilter cartridge was prepared. The caps were screwed onto the outlet nozzle of the QIAfilter Maxi Cartridge and this cartridge was placed into a 50 ml falcon tube in a rack. 10 ml of chilled Buffer P3 was then added to the lysate and mixed immediately and thoroughly by vigorously inverting 4-6 times. Due to the presence of LyseBlue reagent, the suspension was mixed until all trace of blue had gone and the suspension was colourless. A homogenous colourless suspension indicates that the SDS had effectively been precipitated.

Following mixing with Buffer P3, the lysate was poured into the barrel of the QIAfilter Cartridge and incubated at room temperature for 10 minutes without inserting the plunger. The cap was then removed from the QIAfilter Cartridge outlet nozzle and the plunger was gently inserted into the QIAfilter Maxi Cartridge and the lysate was filtered into the 50 ml falcon tube. 2.5 ml of Buffer ER was added to the filtered lysate and this was mixed by inverting the tube approximately 10times followed by incubation on ice for 30 minutes.

A QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT and allowing the column to empty by gravity flow. The tip was allowed to drain completely and the filtrate discarded. The filtered lysate was then applied to the QIAGEN-tip and it was allowed to enter the resin by gravity flow. The tip was then washed twice with 30 ml of Buffer QC, allowing the buffer to move through the QIAGEN-tip by gravity flow. The buffer washes were discarded. The DNA was then eluted with 15 ml of Buffer QN into a fresh 50 ml falcon tube. Following elution, the eluate was transferred to a high resistance ultra-centrifugation tube. The DNA was then precipitated by adding 10.5 ml of room-temperature isopropanol to the eluted DNA. This was mixed and then immediately centrifuged at 5,000g for 60 minutes at 4°C. The supernatant was then carefully discarded without disturbing the pellet. The pellet was then washed with 5 ml of endotoxin-free room-temperature 70% ethanol and centrifuged at 5,000g for 60 minutes at 4°C. Following the ethanol wash, the pellet was not adherent to the bottom of the centrifuge tube so the ethanol could not simply be decanted. The Techne Sample Concentrator was instead used to dry the pellet using nitrogen gas.

Hyperdermic needles were inserted through both the needle guide holes and the sealing pad in the gas chamber in a pattern to suit the needs of the tubes to be placed in the aluminium block. The needles were moved away from the sample using the knobs of the carriage and nitrogen gas was released from the gas cylinder. The needles were then repositioned and lowered into the sample tubes – a reasonable distance into the tube to allow nitrogen release onto the pellet. The pellet was continuously checked to ensure it had not been over-dried and once dry (~5-10 minutes), the gas chamber was raised and the hyperdermic needles removed from the guide holes. The gas was turned off and the needles saved for reuse.

The pellet was then redissolved in 200-300 μ l of MilliQ water and transferred into a 1.5 ml eppendorf tube ready for NanoDrop analysis. DNA with ratios within 2.0 and 2.2 was deemed suitable for the 260/230 ratio and a value of ~1.8 was deemed suitable for the 260/280 ratio. Any DNA samples out of this range were precipitated to obtain better purities.

2.2.18 Transfection of the HyPer vector

Transfection is the process of deliberately introducing nucleic acids into cells. The NEPAgene electroporator was used to transfect the HyPer vectors into hCASMC. 12 transfection conditions were tested that varied in voltage and length of poring pulse in a bid to determine the optimum transfection condition.

hCASMC were grown to confluency in T75 flasks and then subcultured as normal (2.2.1.1). Following trypsinisation and centrifugation, the cell pellet was resuspended in 5 ml of hCASMC SFM which acted as Electroporation Buffer and the cells were centrifuged again at 220g for 3 minutes. Following centrifugation the supernatant was discarded and the pellet was again resuspended in 5 ml of SFM and centrifuged for another 3 minutes. The purpose of the two washes in SFM was to wash the serum containing medium completely off the cells.

The cells were then resuspended in 1 ml of SFM and a trypan blue cell count (**2.2.1.2**) was carried out to determine cell density. Once cell number had been determined, 6 well culture plates were prepared by filling the appropriate number of wells with 2 ml per well of complete culture medium and then placing this to warm up in the $37^{\circ}C/5\%$ CO₂ incubator ready for post-electroporation.

The hCASMC were diluted in sufficient SFM containing DNA in a manner such that for each electroporation there would be 300,000 cells in a final volume of 100 μ l SFM containing 10 μ g of DNA. 100 μ l of this cell and DNA solution was dispensed into each cuvette. The cells were mixed lightly without foaming by gently tapping the cuvette with a finger.

The electroporation parameters were set and the cuvette was placed into the CU500 Cuvette chamber. A note of the impedance value was made and the electroporation programme was executed. Once this was completed, the cuvette was taken out of the chamber and a sufficient amount (~300 μ l) of cell culture growth medium from the respective well of the 6 well culture dish was added to the cuvette using the included pipette. The cells and medium in the cuvette were mixed well using the pipette and the sample was completely removed from the cuvette and dispensed into the respective well. The process was repeated for the remaining samples and the cells were incubated in $37^{\circ}C/5\%$ CO₂ incubator for 72 hours. Once 72 hours had passed, the cells were treated with various compounds with the aim to detect H₂O₂.

The optimum electroporation condition chosen was 200V of poring pulse over 5 milliseconds.

2.2.19 Data analysis and statistical testing

All data are presented as mean and standard error of mean (SEM) or standard deviation (SD) as appropriate. Statistical analyses were performed using GraphPad Prism[®] 6 Software (GraphPad Software, Inc., CA, USA). Statistical significance of the data was determined by applying a one-way ANOVA or two-way ANOVA with an appropriate post-test using GraphPad for comparisons between three or more groups. The Dunnett multiple comparison test was used when the means of each treatment were being compared to the mean of the control; whilst the Tukey multiple comparison test was used when the mean of every other treatment, including the control. For comparison with the means of only two groups, an unpaired t test was used. A P-value of ≤0.05 was deemed significant.

2.2.19.1 Proteomics Analysis

Functional analysis was carried out using Ingenuity® Pathway Analysis (IPA, Ingenuity Systems Inc., CA, USA). IPA is a commercial, web-based interface that utilises a variety of computational algorithms to identify and establish cellular networks, pathways and biological processes that statistically fit the input experimental data. Using IPA, a 'Core Analysis' was carried out which enabled the differentially expressed proteins to be related to the information present in the Ingenuity® Pathway Knowledge Base. The quality controlled Knowledge Base is a repository of curated biological interactions and functional annotations created from millions of individually modelled relationships between proteins, genes, complexes, cells, tissues, drugs and diseases. Once the Core Analysis was completed, IPA mapped the differentially expressed proteins to canonical pathways, disease and biological functions, networks and toxicology lists. A righttailed Fisher's exact test was used to determine the level of significance for each network, pathway and toxicity list where the p-value is displayed as a score, which is negative log of the p-value, -log₁₀ (p-value). By default, IPA defines significance in canonical pathways; downstream biological and diseases functions; and toxicity lists with a $-\log_{10}$ (p-value) of ≥ 1.3 which equates to a p-value of <0.05. For network analysis, the IPA default score of \geq 3 (p<0.001) was considered significant, which provided 17 significant networks. UniProt/Swiss-Prot Accession IDs, raw fold change values and p-values for the differential proteins were used for IPA analysis.

CHAPTER THREE

Generating Reactive Oxygen Species and Inducing Senescence

Chapter 3 Generating Reactive Oxygen Species and Inducing Senescence

3.1 Background

3.1.1 Vascular Smooth Muscle Cells and Senescence

VSMC are the predominant cellular element of the vascular media, and following vascular injury, they migrate to the intima where they synthesise ECM components, enabling them to control vascular construction (Rudijanto, 2007). Whilst VSMCs are important in normal vascular repair, their migration and proliferation can be detrimental as it contributes to the growth of atherosclerotic lesions. Senescent VSMC have a significant presence in atheromas, and thus the induction of senescence is important to understand as senescent VSMC may contribute to pathology. The SASP components released may amplify the pro-atherogenic inflammatory environment, causing dysfunction in other local vascular cells in a paracrine manner (Naylor *et al.*, 2013; Munoz-Espin & Serrano, 2014).

ROS are known to be capable of inducing senescence; both replicative by inducing the formation of the mutagenic oxidised guanine base, as well as oxidative stress-induced damage to biomolecules such as DNA, lipids and proteins triggering SIPS. Atherosclerotic plaques have a highly oxidative environment due to the higher proportion of active macrophages present as well as lipid laden macrophages, or foam cells; which increase ROS levels in the lesion (Channon, 2002; Park & Oh, 2011). Elevated levels of oxidised lipoproteins also increase ROS levels, notably the hydroxyl radical, which is the most damaging ROS (Wang & Bennett, 2012). Consequently, modulating the levels of ROS may be a potential therapeutic strategy in preventing premature VSMC senescence and its contribution to atheroma pathogenicity.

Human Coronary Artery Smooth Muscle Cells (hCASMC) were used as the primary cell type because of their importance in cardiovascular diseases. Coronary arteries are prone to atherosclerosis and this contributes to the development of Coronary Artery Disease (CAD). As there are difficulties with studying the senescence-inducing effects of ROS on primary cells due to replicative senescence, there is an advantage in utilising a suitable cell model. hTERT hASMC are human aortic smooth muscle cells that have been transfected with the catalytic subunit of the human enzyme telomerase (hTERT) (McKee *et al.*, 2003; Klinger *et al.*, 2006). As they are simply immortalised as opposed to transformed, they can be used as a model for VSMCs. The incentive of using this cellular model stems from the slow growth of primary SMCs as well as their finite lifespan.

3.1.2 Characteristics of hTERT hASMC

The purpose of engineering hTERT hASMC was to develop small-calibre arteries for bypass surgery. It was thought that the limited proliferative capacity of SMCs, the main cellular component of such mechanically robust vessels, thwarted previous attempts. In 2003, hTERT was introduced into human aortic SMCs isolated from a 2 year old male (McKee *et al.*, 2003). The cells were infected at early passage with control or hTERT retroviral vectors to generate stably infected polyclonal populations. The reactivation of telomerase activity arrested telomere shortening and extended the lifespan of the hTERT-hASMCs (McKee *et al.*, 2003).

When the hTERT hASMC were engineered, they were tested against control cells for various factors such as their expression of cell cycle regulators, p53 and Rb1; and their ability to form tumours in nude mice over a course of 2 months. Taken together the findings indicated that hTERT hASMC, although immortal, have a non-transformed, non-tumourigenic phenotype (McKee *et al.*, 2003; Poh *et al.*, 2005; Rensen *et al.*, 2007). The rate of cell growth remained stable in hTERT populations implying continued intact cell cycle regulation (Klinger *et al.*, 2006). Previous studies by this group also showed that the hTERT protein was highly expressed in hTERT hASMC using Western blotting, with low expression detected in primary hVSMC (Mistry, 2009). Finally, previous studies confirmed hTERT hASMC were still phenotypically smooth muscle cells. Characteristics of growth, morphology and cytoskeletal protein expression of SM-MHC were probed. Growth rate of hTERT hASMC was faster – an approximate of 1.1 PD per day compared with typically 0.4 PD per day at the exponential stage of hVSMC cultures; a growth rate characteristic of synthetic SMC *in vitro* (Hao *et al.*, 2003; Mistry, 2009). This correlated with past published data on these cells, where the hTERT hASMC

populations continued to divide far beyond the senescent point of control cells – theoretically long enough to engineer a robust blood vessel (McKee *et al.*, 2003).

3.1.3 Suitability of hTERT hASMC

Are hTERT-infected cells able to undergo the process of senescence? The ectopic expression of the TERT subunit not only prevents telomere shortening but it also prevents replicative senescence. Despite not being able to senesce due to telomere shortening, cells expressing TERT ectopically are still able to undergo SIPS. hTERT hASMC have previously been demonstrated by this group to undergo SIPS with AngII treatment (Herbert *et al.*, 2008). hTERT hASMC were exposed to AngII for 24 hours and results demonstrated a concentration-dependent increase in the induction of premature senescence with the maximal effect being observed at 10 nM AngII; a ~1.8 fold increase over the control (Herbert *et al.*, 2008).

In conclusion, despite not being 'normal cells', the hTERT cells could be used to model ROS generation and the induction of stress-induced premature senescence.

3.2 Aims & Objectives

The aims of this chapter include investigating the induction and detection of mitochondrial ROS in hTERT hASMC following treatment with mitochondrial inhibitors Antimcyin A (AA) and rotenone (Rot). Following this, the next aim is to subsequently determine whether SIPS can be induced in hTERT hASMC with successive exposure to either mitochondrial inhibitor or exogenous H_2O_2 .

Additionally, the aim includes investigating the induction and detection of ROS in hCASMC following treatment with mitochondrial inhibitors, AA and Rot; or the targeted redox cycler, MitoParaquat (MitoPQ). And finally, to determine whether SIPS can be induced in hCASMC with successive exposure to the mitochondrial inhibitors, MitoPQ, or exogenous H_2O_2 .

3.3 Results

3.3.1 Comparison of hTERT with primary hCASMC

As the hTERT hASMC showed smooth muscle characteristics in previous work, they were utilised for the initial studies of this thesis. The photomicrographs in **Figure 3.1** show that despite the higher passage number, the appearance of the hTERT hASMC is comparable to primary hCASMC.







The cells show comparable appearance and morphology. Images taken on the EVOS Core XL light microscope (magnification: x100). **A**, Near confluent hTERT hASMC at p32. **B**, Near confluent hCASMC at p6. (Figure also available on CD – Appendix 2).

3.3.2 Primary hCASMC undergo senescence with continuous cell culture

To confirm the hCASMC were primary cells capable of undergoing replicative senescence, the cells were continuously passaged in tissue culture. Cells were stained using the SA β -galactosidase method at an early passage (passage 3) to determine the number of senescence-positive cells then again once the hCASMC had slowed in cell growth following culture over 4 weeks. The results showed a significant increase in the level of SA β -galactosidase positive cells at later passage compared to the early passage cells. 80% of the passage 6 hCASMC stained positive for SA β -galactosidase compared to ~10% of passage 3 hCASMC (**Figure 3.2A**).

The results confirmed the ability of the cells to undergo replicative senescence and verified their primary nature.

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hCASMC (passage number)





A, Cells were cultured continuously until they stopped growing. Data presented as mean+SEM; n=3 (***p<0.001). **B**, Representative photomicrographs showing morphology and SA β -galactosidase staining of continuously cultured hCASMC at early passage 3 and late passage 6. Images taken using the EVOS® XL light microscope (magnification: x100). (Figure also available on CD – Appendix 2).

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3.3.3 Investigation of mitochondrial superoxide and SIPS induction by Antimycin A and rotenone in hTERT hASMC

3.3.3.1 Effect of Antimycin A on cell viability of hTERT hASMC

In order to investigate the induction of ROS and SIPS in hTERT hASMC by mETC inhibition, firstly, non-cytotoxic concentrations of AA and Rot were sought. hTERT hASMC growth and ATP content in the presence of mitochondrial inhibitor, AA, was assessed over a range of concentrations in order to establish suitable AA concentrations that would not induce extensive cell death following treatment.

The CCK-8 assay was used to measure the number of hTERT hASMC following a single exposure to AA over a period of 4-24 hours (**Figure 3.3A** and **Figure 3.3B**). DMSO (0.1%) was used as the solvent control. Cell viability at both 4 hours and 24 hours appeared to be unaffected when compared to the DMSO control. As cell death was deemed at anything below 80% viability compared to the control, 50-100 μ M AA was defined as non-cytotoxic.

ATP levels were then examined. As expected with complex III inhibition, at concentrations as low as 0.39 μ M, AA drastically reduced ATP levels to approximately 50% of the control (**Figure 3.3C**). Further reduction in ATP levels was apparent at 50-100 μ M AA. Subsequent studies with the hTERT hASMC were conducted with both 50 μ M and 100 μ M AA.



Antimycin A (µm)

Figure 3.3: Mitochondrial ETC inhibitor Antimcyin A does not affect hTERT hASMC cell number but decreases ATP levels.

Cells were treated with two concentrations of AA for 4 hours (**A**) or 24 hours (**B**). Cell number was measured using the CCK-8 Cell Viability Assay. 1 mM H_2O_2 was used as a positive control. Data presented as mean+SEM; n=6 (****p<0.0001 compared to the 0.1% DMSO control). **C**, Cells were treated with AA for 24 hours. ATP content was measured using the luminescence assay. 1 mM H_2O_2 was used as a positive control. Data presented as mean+SEM; n=5 (**p<0.001, ***p<0.0001 compared to the 0.1% DMSO control).

3.3.3.2 Effect of rotenone on cell viability of hTERT hASMC

hTERT hASMC growth and ATP content in the presence of mitochondrial complex I inhibitor, Rot, was assessed over a range of concentrations in order to establish suitable Rot concentrations that would not induce extensive cell death following treatment.

The CCK-8 assay was used to measure the number of hTERT hASMC following a single exposure to Rot over a period of 24 hours. Rot exposure caused a significant reduction in cell number from as low as 0.78 μ M (**Figure 3.4A**). ATP levels were subsequently examined and, as expected, all concentrations of Rot impaired ATP production by over 50% (**Figure 3.4B**). Due to the cytotoxic nature of Rot, no further studies were carried out with hTERT hASMC.



Rotenone (µM)



Figure 3.4: Mitochondrial ETC inhibitor rotenone causes hTERT hASMC cytostasis and reduction in ATP levels.

Cells were treated with Rot for 24 hours with 0.1% DMSO acting as the solvent control. Cell number was measured using the CCK-8 Cell Viability Assay (**A**) and ATP content was measured using the luminescence assay (**B**). 1 mM H_2O_2 was used as a positive control. Data presented as mean+SEM; n=3 (****p<0.0001 compared to the 0.1% DMSO control).

3.3.3.3 Antimycin A induces mitochondrial superoxide production in hTERT hASMC

The ability to induce mitochondrial superoxide production was examined using the MitoSOX^m Red fluorogenic dye. Cells were treated with 50 μ M or 100 μ M AA for 24 hours and 100 μ M was found to increase superoxide production significantly compared to the control. Superoxide levels were unaltered with 0.1% DMSO treatment (**Figure 3.5**).



Antimycin A (µM)

Figure 3.5: Antimycin A induced mitochondrial superoxide production in hTERT hASMC.

Cells were treated with two concentrations of AA for 24 hours. Mitochondrial superoxide was detected by flow cytometry using the MitoSOX[™] dye. 0.1% DMSO was used as a solvent control. Data presented as a mean+SEM; n=5 (*p<0.05 compared to the 0.1% DMSO control).

3.3.3.4 Successive treatment with Antimycin A is unable to cause Stress Induced Premature Senescence in hTERT hASMC

As 100 μ M AA was able to generate mitochondrial superoxide production without causing cell death in hTERT hASMC, the question of whether SIPS could be induced with the inhibitor was raised. Cells were treated with 2 concentrations of AA (50 μ M and 100 μ M) for 4 hours over 3 successive days. 50 μ M AA was investigated despite the concentration failing to reach statistical significance when used to detect mitochondrial superoxide generation (section **3.3.3.3**). Exposure to either concentration slowed cell growth compared to the control (**Figure 3.6A**), however neither concentration was able to induce senescence as detected by the SA β galactosidase assay (**Figure 3.6B**).



Figure 3.6: Successive treatment with the mitochondrial inhibitor AA slowed cell proliferation and failed to induce senescence in hTERT hASMC.

Cells were treated in a successive manner for 4 hours over the course of 3 days with 50 μ M or 100 μ M AA. **A**, The number of live cells were counted using the trypan blue exclusion assay. Data presented as mean±SEM; n=3 (*p<0.05, **p<0.01 compared to the control). **B**, SA β-galactosidase histochemical staining was carried out once the cells had recovered 48 hours later. Images taken on the EVOS® XL Light microscope (magnification: x100). (Figure also available on CD – Appendix 2).

The same successive treatment was conducted using 2 concentrations of H_2O_2 (50 μ M and 100 μ M). Again, whilst appearing to slow hTERT hASMC growth (**Figure 3.7A**), neither concentration was able to induce senescence as detected by the SA β -galactosidase assay (**Figure 3.7B**).



Figure 3.7: Successive treatment with H₂O₂ failed to induce senescence in hTERT hASMC.

Cells were treated in a successive manner for 4 hours over the course of 3 days with 50 μ M or 100 μ M H₂O₂. **A**, The number of live cells were counted using the trypan blue exclusion assay. Data presented as mean±SEM; n=3 (**p<0.01 compared to the control). **B**, SA β-galactosidase histochemical staining was carried out once the cells had recovered 48hours later. Images taken on the EVOS[®] XL Light microscope (magnification: x100). (Figure also available on CD – Appendix 2).

3.3.4 Investigation of mitochondrial superoxide and SIPS induction by Antimycin A, rotenone and MitoPQ in hCASMC

3.3.4.1 Effect of Antimycin A on cell viability in hCASMC

Primary hCASMC growth and ATP content in the presence of mitochondrial inhibitor, AA, was assessed over a range of concentrations in order to establish suitable AA concentrations that would not induce extensive cell death following treatment.

The CCK-8 assay was used to measure the viability of hCASMC following a single exposure to AA over a period of 4-24 hours. Cell number was only significantly reduced at the highest concentration of AA tested (200 μ M) following 24 hours of exposure (**Figure 3.8A**). Following 4 hours of treatment, there was a fall in cell number at 100 and 200 μ M; however there appeared to be a significant increase in cell number at the lower concentrations of 3.13 and 6.25 μ M suggesting AA stimulated cell growth at low concentrations (**Figure 3.8B**). Corresponding to this, at 4 hours, AA exposure caused a decrease in ATP levels at concentrations of 12.5 μ M and above. The IC₅₀ value was estimated to be 103 μ M AA (**Figure 3.8C**).

hCASMC are more tolerant to AA in terms of a reduction in ATP concentration compared to hTERT hASMC (**Figure 3.3C**) but the latter were less affected in terms of cell number (**Figure 3.3A** and **Figure 3.3B**). 50 μ M AA was selected as the concentration to proceed with as it did not alter cell number with either time period.



Figure 3.8: Antimcyin A reduced cell number and ATP levels in a concentration-dependent manner in hCASMC.

Effect of AA on cell number over a 24 hour (**A**) and 4 hour (**B**) period and ATP content over a 4 hour period (**C**). Cell number was measured using the CCK-8 Cell Viability Assay and ATP content was measured using the luminescence assay. 0.1% DMSO was used as a solvent control and 1 mM H_2O_2 was used as a positive control. Data presented as a mean+SEM; n=4 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.001 compared to the 0.1% DMSO control).

3.3.4.2 Effect of rotenone on cell viability in hCASMC

Primary hCASMC growth and ATP content in the presence of mETC inhibitor Rot, was assessed over a range of concentrations in order to establish suitable concentrations that would not induce extensive cell death following treatment.

The CCK-8 assay was used to measure the number of hCASMC following a single exposure to Rot over a period of 4-24 hours. The 24 hour treatment caused a significant reduction in cell number at all the concentrations investigated (**Figure 3.9A**), however within the shorter 4 hour exposure, cell number remained unaffected across the concentrations of Rot investigated (**Figure 3.9B**). As expected, Rot exposure caused a significant decline in ATP levels within a 4 hour exposure (**Figure 3.9**C). The IC₅₀ value was estimated to be 3.7 μ M Rot.

As the 4 hour treatment caused no alterations in cell number compared to the control, 50 μ M Rot was selected as the concentration to proceed with.



Figure 3.9: Rotenone reduced cell number with longer treatments and ATP levels

Effect of Rot on cell number over a 24 hour (**A**) and 4 hour (**B**) period and ATP content over a 4 hour period (**C**). Cell number was measured using the CCK-8 Cell Viability Assay and ATP content was measured using the luminescence assay. 0.1% DMSO was used as a solvent control and 1 mM H_2O_2 was used as a positive control. Data presented as a mean+SEM; n=4 (***p<0.001, ****p<0.0001 compared to the DMSO control).

Whilst not causing cell death, Rot treatment was found to alter hCASMC morphology (compared to **Figure 3.1**) suggesting that the depletion of ATP may affect proteins that control cell morphology as well as other possible effects within the cell apart from complex I inhibition.



Figure 3.10: 4 hour treatment with 10 μ M rotenone caused a change in hCASMC morphology Control, untreated hCASMC (A) vs 10 μ M Rot treated hCASMC. Image taken on the EVOS® XL light microscope (magnification of both imsges: x100). (Figure also available on CD – Appendix

2).

3.3.4.3 Effect of MitoParaquat on cell viability in hCASMC

As an alternative to the mETC inhibitors, the mitochondrial-targeted redox cycling paraquat moiety, MitoParaquat (MitoPQ) was used. Paraquat was targeted to the mitochondria with the use of the TPP⁺ cation, allowing it to generate superoxide within the mitochondrial matrix only, with negligible production in other parts of the cell (Robb *et al.*, 2015). Whilst AA and Rot may have other effects within the cell, MitoPQ is assumed to have minimal effects outside the proximity of the mitochondria and may therefore serve as a more useful tool in investigating the role of mitochondrial ROS in pathology and signalling *in vitro*.

Primary hCASMC growth and ATP content in the presence of MitoPQ was assessed over a range of concentrations in order to establish suitable concentrations that would not cause cytotoxicity following treatment.

The CCK-8 assay was used to measure the number of hCASMC following a single exposure to MitoPQ over a period of 4 hours. Cell number was only significantly reduced at the highest concentration of MitoPQ tested (100 μ M; **Figure 3.11A**). The corresponding ATP assay showed a fall in ATP content at concentrations of 12.5 μ M and above following MitoPQ exposure (**Figure 3.11B**). The IC₅₀ value was estimated to be 42 μ M.




Effect of MitoPQ on cell number (**A**) and ATP content (**B**) over a 4 hour period. Cell number was measured using the CCK-8 Cell Viability Assay and ATP content was measured using the luminescence assay. 1 mM H_2O_2 was used as a positive control. Data presented as a mean+SEM; n=4 (**p<0.01, ****p<0.0001 compared to the control).

3.3.4.4 Induction of mitochondrial superoxide production in hCASMC

The ability of mitochondrial inhibitors, AA and Rot, and MitoPQ to induce mitochondrial superoxide and H_2O_2 production was examined using the MitoSOXTM Red fluorogenic dye and the Amplex Red assay respectively.

hCASMC were treated with either AA or Rot for 4 hours. Both ETC inhibitors significantly increased mitochondrial superoxide levels compared to the control, showing inhibition of

complexes I and III generates a rise in reduced ETC components capable of donating electrons to molecular oxygen (Figure 3.12).



Figure 3.12: Antimycin A and rotenone induced mitochondrial superoxide production in hCASMC

Cells were treated with 50 µM AA or Rot for 4hours. Mitochondrial superoxide was detected by flow cytometry using the MitoSOX[™] dye. 0.1% DMSO was used as a solvent control. Data presented as mean+SEM; n=4 (*p<0.05 compared to the 0.1% DMSO control).

The ability for MitoPQ to induce ROS production was examined using MitoSOX[™] fluorescence and the Amplex Red assay. Flow cytometry could not be utilised due to limited cell numbers. Initial investigations utilised the Amplex Red assay to detect H₂O₂ following MitoPQ exposure to hCASMC over a 4 hour period (**Figure 3.13A**). The results suggested that H₂O₂ levels increased in a concentration dependent manner however this failed to reach statistical significance.

As the highest concentration of MitoPQ failed to induce a significant increase in H_2O_2 levels, MitoSOXTM fluorescence was adopted. The cells were treated with the compounds for 4hours and MitoSOXTM dye added 30 minutes prior to the end of the treatment time. The cells were then imaged on the fluorescence microscope and images of 5 fields of view taken. Fluorescence was quantified on ImageJ software. Again, MitoPQ failed to induce significant changes in mitochondrial superoxide (**Figure 3.13B**). Using this method to examine fluorescence however, can be perceived as inaccurate as it appears to contradict the flow cytometry results observed when the hCASMC are exposed to AA and Rot for the same length of time (**Figure 3.12**). There are also some limitations to this method as it appears that MitoSOX[™] can at times be localised to the area of the dish where it is added. Additionally, cells that undergo shear stress due to pipetting display significantly higher levels of fluorescence compared to other cells within the same treatment (**Figure 3.14**).



Figure 3.13: MitoParaquat does not induce ROS generation in hCASMC

A, Cells were treated with MitoPQ for 4 hours and extracellular H₂O₂ was measured using the Amplex Red assay. 50 μM Menadione (Mena) was used as a positive control. Data presented as mean+SEM; n=5 (*p<0.05 compared to the control). B, Cells treated with MitoPQ for 4 hours and mitochondrial superoxide was examined using MitoSOX[™] fluorescence. Images taken on the EVOS fluorescence microscope and fluorescence quantified using ImageJ software. 50 μM AA and 50 μM Rot used as positive controls. Data presented as mean+SEM; n=3 (*p<0.05 compared to the control).



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Cells were treated with AA for 4 hours and mitochondrial superoxide production examined using the MitoSOX[™] fluorogenic dye. **A**, Cells subjected to shear stress from pipetting. **B**, Cells within the same treatment, not subjected to mechanical stress. Images taken from the same well on the EVOS[®] fluorescent microscope (magnification: x100). (Figure also available on CD – Appendix 2).

3.3.4.5 Induction of SIPS in hCASMC by successive treatment with H₂O₂

Exogenous H_2O_2 has been found to be able to induce senescence in vascular cells (Mistry, 2009; Liu *et al.*, 2012; Zhao *et al.*, 2015). The hCASMC were treated with increasing concentrations of H_2O_2 for 3 successive days. The H_2O_2 was added to the media and not removed as studies had found that H_2O_2 is degraded within a matter of hours by peroxidases and other enzymes present in the culture media (Guelden *et al.*, 2010).



Figure 3.15: Stress Induced Premature Senescence induced with successive treatment of $H_2 O_2$

Cells were treated with H_2O_2 for 3 successive days. Following treatment cells were left to recover for 48 hours and a SA β -galactosidase stain was carried out. Data presented as a mean+SEM; n=3 (**p<0.01 compared to the control).

100 μ M H₂O₂ was able to induce SIPS in the hCASMC as determined by SA β -galactosidase staining, as observed by a ~4 fold increase in the number of senescent cells. The results show the ability of H₂O₂ to cause SIPS in the hCASMC with an established method from the literature (Toussaint *et al.*, 2000).

3.3.4.6 Investigation of SIPS induction in hCASMC by successive treatment with mETC inhibitors

The data presented in section **3.3.4.4** suggest AA and Rot possess the ability to generate mitochondrial superoxide production in hCASMC. Therefore, the question of whether SIPS could be induced with the inhibitor was raised as hTERT hASMC were unaffected with successive treatment of these inhibitors. Cells were treated with 50 μ M AA or 50 μ M Rot for either 1 hour or 4 hours for 3 successive days. Exposure to AA did not increase the number of SA β -galactosidase positive cells significantly, however, Rot increased the levels significantly to ~80% (***p<0.001; **Figure 3.16A**). There was no difference in the level of senescence between the two time courses suggesting that 1 hour successive treatments are sufficient to induce SIPS in these cells. Representative photomicrographs show the level of β -galactosidase positivity in each treatment (**Figure 3.16B**).



Figure 3.16: Stress Induced Premature Senescence induced with successive treatment of mitochondrial electron transport chain inhibitors

Cells were treated with either 50 μ M AA or 50 μ M Rot for 1 hour or 4 hours each day over the course of 3 days. Following treatment cells were left to recover for 48 hours and a SA β -galactosidase stain was carried out. **A**, Data presented as a mean+SEM; n=5 (***p<0.001 compared to the control). **B**, Representative photomicrographs showing morphology and SA β -gal staining of the control, AA-treated and Rot-treated cells. Images taken using the EVOS® XL light microscope (magnification: x100). (Figure also available on CD – Appendix 2).

Despite the inability to detect ROS production following MitoPQ treatment (**3.3.4.4**) in hCASMC, the ability to induce senescence with successive exposure was investigated. Cells were treated with increasing concentrations of MitoPQ for 4 hours over the course of 3 days. Successive exposure to MitoPQ caused an increase in SA β -galactosidase staining (**Figure 3.17**). This reached statistical significance at 50 μ M MitoPQ.



Figure 3.17: Stress induced premature senescence induced with successive treatment of MitoParaguat

Cells were treated with MitoPQ for 4 hours each day over the course of 3 days. Following treatment cells were left to recover for 48 hours and a SA β -galactosidase stain was carried out. 50 μ M Rot was used as a positive control. Data presented as a mean+SEM; n=3 (*p<0.05; **p<0.01 compared to the control).

3.3.4.7 Single treatment with mETC inhibitors is not sufficient to cause Stress Induced Premature Senescence in hCASMC

Successive treatment of mETC inhibitors successfully induced SIPS so a single hit treatment was investigated to determine if the cells could recover from a single stress. Cells were treated with either 50 μ M AA or 50 μ M Rot for 24 hours. Exposure to either inhibitor failed to cause any changes in the number of SA β -galactosidase positive cells (**Figure 3.18**). A 24 hour single exposure to MitoPQ was also unable to induce senescence in hCASMC (data not shown). The results suggest that the repeated exposure quoted in literature (Toussaint *et al.*, 2000) is the best method to study SIPS induction by mitochondrial compounds as opposed to a single long term exposure (Mistry, 2009).



Figure 3.18: A single exposure of mitochondrial electron transport chain inhibitors is not sufficient to induce Stress Induced Premature Senescence

Cells were treated with 50 μ M AA or 50 μ M Rot for 24 hours. Following treatment, the cells were allowed to recover for 48 hours and a SA β -galactosidase stain was carried out. Data presented as mean+SEM; n=3.

3.4 Discussion

With the aid of a multitude of studies in both human and mouse models, cellular senescence has been strongly associated with ageing and cardiovascular disease (Adams, 2009; Naylor et al., 2013; Baker et al., 2011). From an evolutionary perspective, this association can be explained by the theory of antagonistic pleiotropy (Adams, 2009; Campisi, 2003). The theory proposes that in evolutionary history, aged individuals comprised a small proportion of the population, and so there was little or no selective pressure to improve phenotypes that manifested only at advanced ages. Cellular senescence may be an example of evolutionary antagonistic pleiotropy, suppressing cancer development in early life, yet driving ageing and age-related pathology in later life (Campisi et al., 2011). A 2011 study eliminated p16expressing senescent cells with the use of senolytic drugs in BubR1 progeroid mice, and found a delay in the onset of age-related deficits; a decrease in the levels of senescent markers, and improved muscle function (Baker et al., 2011; Peeper, 2011). A later study using ABT263, a specific inhibitor of the anti-apoptotic proteins BCL-2 and BCL-xL, found oral administration of the senolytic drug caused the clearance of senescent bone marrow hematopoietic and muscle stem cells in vivo; demonstrating the potential of senolytic drugs as anti-ageing agents (Chang et al., 2016). The complex phenotype of cellular senescence; its link with age-related diseases and cancer; and its clearance promoting longevity, make the process even more intriguing to investigate.

Previous studies within the group discovered that treatment with a mitochondria-targeted antioxidant prevented the onset of senescence; implicating mitochondrial ROS in the senescence signalling pathway. The aim of this chapter was to investigate whether mETC inhibitors and the targeted redox cycler MitoPQ, induced senescence and mitochondrial ROS generation; and to establish the suitability of hTERT hASMC as a suitable *in vitro* smooth muscle cell model for senescence induction.

3.4.1 Comparison of primary hCASMC with immortalised hTERT hASMC

Previous studies investigated the characteristics of hTERT hASMC and they were found to be comparable to normal SMCs. They maintained the expression of calponin and SM-MHC, both differentiation markers. They also upregulated tumour suppressor protein, p53, in response to DNA damage by UV irradiation; and the TERT enabled continuous passage till at least 100

population doublings (McKee *et al.*, 2003). Once in culture, the morphology of the cells was investigated and the hTERT hASMC were found to be comparable to primary hCASMC. However, these cells grew and reached confluency significantly faster than the hCASMC.

3.4.2 Determination of sub-cytotoxic concentrations of the mitochondrial compounds.

AA and Rot are both inhibitors of the mETC, with AA blocking the respiratory chain at complex III, ubiquinol:cytochrome c oxidoreductase; and Rot inhibiting complex I, NADH:ubiquinone oxidoreductase. Inhibition of the complexes causes an increase in the leakage of free electrons, which subsequently causes the reduction of molecular oxygen, forming the superoxide anion free radical (West *et al.*, 2011; Turrens, 2003). MitoPQ however is a mitochondria-targeted redox cycler that when reduced at the Flavin site of complex I in the ETC, reacts rapidly with molecular oxygen to generate superoxide. The localised redox cycling leads to the production of superoxide restricted to the mitochondrial matrix, with minimal ROS generation in other parts of the cell (Robb *et al.*, 2015).

The three compounds were used as a method to increase the levels of mitochondrial superoxide anions which subsequently forms H_2O_2 by spontaneous dismutation, or rapid catalysis by the mitochondrial manganese superoxide dismutase (Murphy, 2009). Combined, this means that the addition of AA, Rot and MitoPQ should not only increase superoxide levels, but also increase H_2O_2 levels. As H_2O_2 is able to freely diffuse out of the mitochondria, H_2O_2 is believed to be the signalling molecule for any downstream signalling pathways (Cordeiro & Jacinto, 2013; Sies, 2014; Taylor *et al.*, 2003). However some studies believe that superoxide can be transported into the cytoplasm via voltage-dependent anion channels (Turrens, 2003).

To determine cell viability following inhibitor treatment, both the ATP and CCK-8 assays were employed. The aim of these assays was to find suitable concentrations that were not cytotoxic to either cell type. hTERT hASMC could tolerate AA exposure at concentrations as high as 100 μ M. Although ATP levels decreased at sub-nanomolar concentrations, cell number did not decrease at 100 μ M suggesting that the cells were able to survive despite the depletion of ATP. The viable cells may be reliant on the glycolytic pathway following the inhibition of oxidative phosphorylation. 100 μ M of AA was deemed as the suitable concentration to proceed with experiments increasing mitochondrial superoxide as it did not appear to cause cell death. hCASMC, on the other hand, showed a concentration-dependent response to AA treatment with reductions in cell number occurring at the highest concentrations of 100-200 μ M.

AA toxicity is reflected in studies with primary ECs. Human umbilical vein and calf pulmonary artery ECs were used to evaluate the effects of AA. Their results showed that 50 μ M and 100 μ M AA decreased cell number and growth and increased the number of apoptotic cells at 24 hours (You & Park, 2010). Studies using the HeLa cancer cell line also found that exposure to 50 μ M AA caused a ~50% reduction in cell viability emphasising the toxicity of the compound in various cell types (Han *et al.*, 2008). However, both the hTERT hASMC and the hCASMC appear more tolerant of slightly higher AA concentrations as there was no apparent change in cell number at 50 μ M for either cell line. The maintenance of cell number observed in the CCK-8 assays may signify a balance between cell proliferation and cell death. A cell death assay with the use of the death marker, Annexin V, would be able to elaborate more to the effects of AA on both hCASMC and hTERT hASMC. At the lower concentrations, there also appeared to be a significant increase in hASMC cell number with 4 hour AA treatment suggesting the compound may have stimulated a cell proliferation response, however, there is no evidence in the literature to support this nor was the increase reflected in the 24 hour treatment.

AA depleted ATP levels in hCASMC with an IC_{50} of approximately 100 μ M. Studies using a rabbit renal proximal tubule model found exposure to AA caused a rapid depletion in ATP levels; 97% reduction within 2 minutes (Harriman *et al.*, 2002). Although not as significant a reduction compared to the control, the results found in both the hCASMC and the hTERT hASMC are in line with previous studies as complex III inhibition has a drastic effect on ATP synthesis, which may sensitise cells to death responses.

Regarding Rot, hTERT hASMC cell viability fell to 70% at concentrations as low as 0.78 μ M within 24 hours and ATP levels also fell to ~40% in that time frame. Due to this significant cell number decline, Rot was deemed toxic to the hTERT hASMC, and thus studies with the hTERT hASMC were discontinued.

hCASMC seemed to tolerate the toxic effects of Rot over a 4 hour period. Longer treatments caused a ~50% reduction in cell number, highlighting the possibility that the complex I inhibitor was more toxic to the hCASMC than AA. Complex I inhibition meant that ATP levels were also reduced with Rot exposure, with an IC₅₀ of ~3 μ M.

Both Rot and AA are well known to have other effects within a cell, which goes beyond simple ETC inhibition including for example, the ability for AA to inhibit autophagy in various cell lines in a dose and time-dependent manner, independent of ATP and ROS generation (Ma et al., 2011). Figure 3.10 shows the change in hCASMC when treated with 10 μ M Rot, a concentration found to still maintain cell viability at 4hours. The cells appear flatter and epithelioid in shape and are also more difficult to detach from culture dishes during cell culture. Other studies with Rot at concentrations as low as 1 µM have observed morphological changes following treatment with the complex I inhibitor; suggesting the effects are not unique to the hCASMC and possible other signalling pathways are being affected following exposure (Li et al., 2014; Wang et al., 2014). Most animal cells have three types of cytoskeletal filaments, responsible for spatial organisation and mechanical properties, including actin filaments. With the help of accessory proteins, actin filaments determine the shape of a cell's surface and are responsible for locomotion (Alberts *et al.*, 2008). Motor proteins are a subset of accessory proteins and they convert the energy of ATP hydrolysis into mechanical force that enables membrane-enclosed organelle movement along the filaments, or movement of the filaments themselves (Alberts et al., 2008). Depletion of ATP following Rot exposure may affect motor protein function, causing actin filament deregulation; and as a consequence, alterations in cell morphology. Subsequent studies in hCASMC were conducted with 50 μM Rot, with the knowledge that it was capable of altering other processes in mind.

Prior to MitoPQ synthesis, the redox cycling capabilities of paraquat was employed to induce oxidative stress and senescence (Joguchi *et al.*, 2004; He *et al.*, 2012; Wiemer & Osiewacz, 2014). Paraquat is used in many applications, but mainly as a nonselective herbicide where it quickly became one of the most important worldwide weed control methods. Despite its success, it also had its flaws including indirect effects on crops and humans where it was been found to selectively accumulate in human lungs and cause oxidative injury and fibrosis with high mortality rates (Sedigheh *et al.*, 2011; He *et al.*, 2012). Paraquat (10-20 μ M) has been found to increase H₂O₂ release, reduce growth rate and decrease lifespan of the fungus *Podospora anserine* (Wiemer & Osiewacz, 2014). Within embryonic human lung fibroblasts, a higher concentration (100 μ M) caused an induction of premature senescence following a 4 day exposure in culture (Joguchi *et al.*, 2004). More recently, paraquat was found to reduce cell survival and increase caspase activation in human bronchial epithelial cells in a concentrationdependent manner. The compound (10-20 μ M) also increased ROS fluorescence; induced the production of the inflammatory and profibrogenic factors, TNF- α , IL-6 and TGF- β ; as well as induced normal human lung fibroblast transformation. All of these effects were successfully inhibited by resveratrol, the naturally occurring polyphenol with antioxidant and antiinflammatory properties (He *et al.*, 2012). Combined, the studies highlight the effectiveness of paraquat to induce ROS production and premature senescence.

MitoPQ was synthesised as current redox cycling compounds caused superoxide generation in the mitochondria as well as in other cellular compartments. With MitoPQ, the superoxide generation is localised to the mitochondrial compartment, enabling the effects of mitochondrial superoxide to be studied specifically (Robb *et al.*, 2015). The developers found that the mitochondria-targeted version selectively increased superoxide production within the mitochondrial matrix in cells and *in vivo*. Within 20 minutes, 1-5 µM MitoPQ increased H₂O₂ efflux from mitochondria as well as increased MitoSOX[™] fluorescence and increased SOD2 protein levels. Cell death was also caused between 5-10 µM (24 hour exposure) whilst paraquat caused the same level of death at a 5 mM concentration (Robb *et al.*, 2015). Their results suggest that MitoPQ is several hundred-fold more potent than paraquat and so, to prevent toxicity, 1 µM was the desired concentration to increase mitochondrial superoxide without causing cell death.

MitoPQ experiments were conducted in hCASMC. Cell viability investigations suggested the cells could tolerate MitoPQ at concentrations as high as 50 μ M as shown by no changes in cell number following a 4 hour exposure. Whilst it cannot be excluded that MitoPQ may be causing some cell death, the lack of change could suggest a balance between cell death and cell proliferation. ATP levels were also affected in a concentration dependent manner with MitoPQ exposure, with an IC₅₀ of 42 μ M. Prior studies had not investigated ATP changes upon MitoPQ exposure so the ATP alterations may be as a result of damage to the ETC upon superoxide production or mitochondrial dysfunction. A range of MitoPQ concentrations were carried forward (1-50 μ M).

3.4.3 Detection of Reactive Oxygen Species generation

After establishing the appropriate concentrations of the mitochondrial compounds that maintained cell viability, the resulting increase in ROS generated from the treatments was determined by various methods.

Flow cytometry was used as the method of superoxide detection in hTERT hASMC with the use of the mitochondrial targeted detector, MitoSOX[™]. The dye has its limitations with respect to its ability to bind nuclear DNA, and the potential to be oxidised by cytosolic factors during transport from the cell membrane to the mitochondria; although it is assumed that the level of cytosolic oxidised MitoSOX[™] is negligible (Mukhopadhyay *et al.*, 2007). Two concentrations of AA were tested, 50 µM and 100 µM. Whilst appearing to increase superoxide levels, 50 µM did not reach statistical significance but 100 µM significantly increased mitochondrial superoxide levels. Subsequent experiments were then conducted using 100 µM AA as the aim of the study was to significantly induce the generation of oxidants.

As hCASMC are primary cells, there were many limitations with detecting superoxide using MitoSOX[™] flow cytometry. Such limitations included the slow growth of the cells; the limited numbers of cells available for repeat experiments, and the induction of senescence following serial passage. Whilst flow cytometry could be adopted for studies investigating AA and Rot, it could not be used for MitoPQ ROS-induced detection. Flow cytometry studies found a significant increase in mitochondrial superoxide following 4hour treatment with the 50 μ M concentration chosen for both AA and Rot; indicating successful complex inhibition and the reduction of molecular oxygen. To detect MitoPQ-induced ROS generation, the Amplex assay and MitoSOX[™] microscopy were employed however these failed to detect any significant changes in H_2O_2 and mitochondrial superoxide. The lack of detection could possibly have been due to the lack of sensitivity for the Amplex assay as it relies on superoxide to be converted into H_2O_2 , then diffuse out of the mitochondria, then out of the cell. Despite the developers of MitoPQ being able to detect MitoSOX[™] fluorescence at concentrations as low as 1 µM, this study was unable to do so and it could have been due to the limitations of MitoSOX[™] as discussed in section 3.3.4.4. If there were more cells, flow cytometry would have been better suited to accurately detect the superoxide changes following MitoPQ exposure.

Beyond microscopy, there are additional limitations with the use of fluorogenic dyes such as DHE and MitoSOX[™] as indicators of superoxide formation as they can provide unreliable results. DHE produces a number of non-specific products upon entry into cells such as ethidium (E+) and non-fluorescent dimers, as well as a single superoxide-specific product, 2-OH-Ethidium (2-OH-E+). Many studies have claimed that E+ is the major production of DHE oxidation, rather than 2-OH-E+ (Robinson *et al.*, 2008; Nazarewicz *et al.*, 2013b). MitoSOX[™] reacting with superoxide also yields the corresponding hydroxylated ethidium analogue 2-OH-Mito-E+ as well as the general oxidation product, Mito-ethidium (Mito-E+) (Zielonka & Kalyanaraman, 2010). Current fluorescence assays are unable to distinguish E+ from the superoxide-specific product 2-OH-E+, meaning that the results observed by flow cytometry may be partly independent of superoxide activity *in vitro*. To resolve this problem, High Performance Liquid Chromatography (HPLC) can been used to separate the oxidation products and therefore enable proper conclusions to be made about intracellular superoxide levels.

MitoSOX[™] also has other limitations apart from the species it generates. It is irreversible; it can bind to nuclear DNA following oxidation; it has been found to be oxidised by H₂O₂, some peroxidases and other intracellular processes especially those involving cytochromes and oxidases; and some of the MitoSOX[™] can also be oxidised during transport from the cell membrane to the mitochondria (Robinson *et al.*, 2006). Although it is assumed that the level of cytosolic oxidised MitoSOX[™] is negligible, it cannot be ruled out (Mukhopadhyay *et al.*, 2007). To overcome these problems, there are alternative fluorescent probes that can be employed in detecting mitochondrial ROS, such as the mitochondrial H₂O₂ detector, MitoPY1. Despite being also irreversible and having relatively slow reaction kinetics, it is much more organelle and ROS-specific and displayed a marked localised increase in HeLa cell fluorescence following H₂O₂ treatment. Co-staining with the mitochondrial stain MitoTracker[®] confirmed MitoPY1 retention within the mitochondrial compartment (Dickinson & Chang, 2008).

3.4.4 Induction of Stress Induced Premature Senescence in hTERT hASMC with the use of mitochondrial inhibitors and exogenous H₂O₂

Irrespective of the inducer, SIPS and replicative senescence have an overlap of molecular signals as well as resulting phenotype. Both mechanisms usually involve the stabilization of tumour suppressor, p53; increased expression of CDK inhibitor, p21 and subsequent hyperphosphorylation of pRb, causing growth arrest through its action on the cell cycle (Campisi & di Fagagna, 2007; Adams, 2009). The action of another CDK inhibitor, p16, is also able to regulate senescence through the action of pRb, independent of p53 (Adams, 2009).

VSMCs present in advanced human atherosclerotic plaques are characterized by multiple markers of senescence, including telomere loss, extensive DNA damage, expression of p21 and p16, and expression of senescence-associated β -galactosidase (Wang *et al.*, 2015). As well as

the many other cell types present in atherosclerotic plaques, VSMC are predominant. In early pathogenesis, it is believed that their proliferation and ECM component synthesis in response to vascular injury contributes to plaque development and growth (Rudijanto, 2007; Wang & Bennett, 2012; Robbins & Cotran, 2005). In advanced disease, plaque instability is a multifactorial process that includes increased macrophage infiltration and redox-dependent activation of matrix-degrading enzymes that lead to thinning of the fibrous cap (Xu *et al.*, 2014). As VSMC are the primary cell source of supportive extracellular matrix components, loss of their synthesis through apoptosis and senescence contributes to instability and subsequent rupture (Wang *et al.*, 2015; Xu *et al.*, 2014). Studies investigating VSMC within plaques have implicated oxidative stress from various cellular sources as a driver of senescence, and the phenotypes that may lead to plaque instability and rupture responsible for cardiovascular disease events including myocardial infarction and stroke (Xu *et al.*, 2014; Gorenne *et al.*, 2006; Matthews *et al.*, 2006).

In order to study oxidative stress-induced premature senescence in cultured VSMC, the mitochondrial inhibitors, AA and Rot, were employed along with exogenous H_2O_2 as a positive control. Both cell types were subjected to a single hit treatment and this was compared with successive treatments in order to determine which regimen was needed to cause senescence. Following treatment, the cells were allowed to recover for several days prior to measuring senescence markers in a bid to investigate senescence independent of immediate short-term cell cycle arrest triggered by the stressors (Dumont *et al.*, 2000). As explained earlier, SIPS and replicative senescence share the same phenotypes and as yet, there is no experimental technique that distinguishes between the two. Despite having its limitations, the marker chosen for senescence detection was the SA β -galactosidase histochemical stain as this is the most commonly used technique in experiments investigating senescence in VSMC as well as numerous other cell culture types and mammalian tissues (Debacq-Chainiaux *et al.*, 2009; Herbert *et al.*, 2008).

As both 50 μ M and 100 μ M AA increased mitochondrial superoxide levels in hTERT hASMC, these concentrations were used to investigate the induction of premature senescence. With the use of the successive treatment protocol, both concentrations failed to induce senescence however caused a fall in cumulative cell number. Addition of exogenous H₂O₂ (100 μ M), caused a ~50% decline in cumulative cell number compared to the untreated control. Again, the histochemical stain showed an absence of senescence positive cells suggesting the lack of SIPS induction. Exposure to AA and H₂O₂ appeared to slow cell proliferation, or cause cell

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stasis. AA has previously been found to suppress cell growth in a lung cancer cell line by inducing cell cycle arrest in the G1 phase after a 72 hour exposure, as well as inducing apoptosis in 15% of cells (Han & Park, 2009). The mETC inhibitor also increased the levels of cyclin-dependent kinase (CDK) inhibitor, p27, whilst decreasing CDKs 2, 4 and 6; and their respective regulatory proteins, cyclins D and E; which collectively facilitate progression through the cell cycle. The increase of p27 and the decrease of the cell cycle kinases, maintains the cells in G1 following AA treatment (Han & Park, 2009; Alberts *et al.*, 2008). The same phenomenon has been observed in mouse fibroblasts following H₂O₂ treatment, with a downregulation in cyclins D1 and D3, and upregulation of another CDK inhibitor, p21 (Barnouin *et al.*, 2002). The response observed in hTERT hASMC following successive exposure to both H₂O₂ and AA may be as a result of a halt in cell cycle progression.

The results suggest the inability of TERT-expressing cells to undergo SIPS however previous studies within the group have demonstrated the ability of hTERT hASMC to undergo AngII-induced senescence. 100 nM of AngII in a single acute 24 hour exposure caused the hTERT hASMC to cause a ~1.8 fold increase in senescence-positive cells compared to the control (Mistry, 2009). Additionally, another study investigating the overexpression of the Ha-Ras oncogene found that the expression of TERT does not prevent SIPS. They used fibroblasts that had been infected with the catalytic subunit of the human telomerase, TERT, and then subsequently infected with the Ha-Ras expressing vector. The results showed that the majority of cells infected with the Ha-Ras vector ceased to divide by day 5; with virtually all the surviving cells having acquired the enlarged morphology of senescent cells; stained positive SA β -galasctosidase; and upregulated expression of p16 and p21 (Wei *et al.*, 1999). Both studies highlight the capability of certain stressors to induce premature senescence in cells despite high levels of ectopic hTERT expression.

Thus, from the results in this study, it seems that the hTERT hASMC are incapable of undergoing senescence with the chosen stressors but the slowdown of growth suggests the cells may be entering quiescence instead. Analysis of cell cycle kinetics may be useful in confirming the transient cell cycle arrest and subsequent fall in cell number as opposed to being attributed to cell death.

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3.4.5 Replicative senescence

With each cell division, telomeres within primary cells shorten due to incomplete duplication (Mathon & Lloyd, 2001; Rodier & Campisi, 2011). With continuous passage, the loss of DNA eventually triggers the senescence response to ensure cells with dysfunctional telomeres are withdrawn from the cell cycle on a permanent basis, rendering them incapable of forming a tumour. Once differentiated, most human somatic cells do not express the enzyme telomerase which maintains telomere length (Mathon & Lloyd, 2001). Studies have found a relationship between telomere length and cardiovascular disease risk, with individuals with a leukocyte telomere length in the lowest or middle tertile being at an increased risk of developing coronary heart disease compared to individuals with the longest telomeres (Brouilette *et al.,* 2007). A portion of the senescence as opposed to SIPS; especially in individuals with shorter telomeres whom are predisposed to senescence compared with those with longer telomeres.

The purchased hCASMC had been isolated from human coronary arteries, staining positive for smooth muscle α -actin. Being primary cells, hCASMC eventually stop expressing telomerase and thus are able to undergo replicative senescence. In order to study this phenomenon, the cells were subjected to continuous culture to induce replicative senescence. The cell culture model system of replicative senescence has been criticised of recent as the procedure may not provide an accurate representation of ageing in organisms (Chen *et al.*, 2013). Cultivation requires that the cells continuously undergo proliferation; exposure to the trypsinisation procedure; and occasionally exposure to standard fluorescent light; all of which not only potentially stresses the cells but also does not occur in an organism (Cristofalo *et al.*, 2004; Chen *et al.*, 2013). Additionally, the low-serum content of the specific hCASMC medium contains a lower concentration of protein compared with extracellular tissue fluids; however Promocell developed the media with the selected growth factors and supplements specifically for the optimal growth of human smooth muscle cells (*in vitro*).

To define the hCASMC as senescent, the *in vitro* signs of ageing were investigated. The initial observation of senescence was when the cultures (passage 6) slowed in growth and failed to reach confluency within the usual timeframe. The cells were not passaged, but instead fed with the replacement of fresh complete medium for 2 consecutive weeks. Following that, other *in vitro* characteristics were examined. Cell morphology was observed, and it was found that the hCASMC had increased in cell size, acquiring the flattened morphology with an enlarged nuclear size. The SA β -galactosidase stain was then employed and it was found that

80% of the cells stained positive, compared to the same batch of cells at passage 3 where 10% stained positive. The significant increase in senescence staining indicated that the cells were truly primary cells and that they were capable of undergoing replicative senescence in an *in vitro* system.

A limitation of this model is that the cells purchased from Promocell come in batches and each batch may come from potentially different donors. Although Promocell cryopreserve the SMC at passage 2 shortly after isolation, the donors may differ in respect to genetics, age and sex. Therefore, the time taken for the batches to reach replicative senescence may differ. Initial studies with the hCASMC found that the cells reached senescence as early as passage 6, however later studies found that one particular batch reached senescence at passage 20.

3.4.6 Induction of Stress Induced Premature Senescence in hCASMC by mitochondrial inhibitors and exogenous H₂O₂

Previous studies within the group have used a mitochondrial-targeted antioxidant to prevent the onset of senescence caused by the hormone, Angll. This implicates mitochondria in the senescence signalling pathway and creates a need to investigate mitochondrial ROS-induced senescence with the use of mitochondrial inhibitors, AA and Rot; and the mitochondrialtargeted redox cycler, MitoPQ. Exogenous H_2O_2 was investigated as a positive control.

Previous studies have found that the addition of sub-lethal concentrations of H_2O_2 in foetal lung fibroblasts induces a number of biochemical changes including the rise in tumour suppressor protein, p53; a high increase of p21 levels, and a lack of Rb phosphorylation leading to subsequent G1 arrest. When G1 arrest was inactivated with the use of the human papillomavirus protein E7, the cells still maintained their inability to replicate demonstrating the induction of irreversible proliferation arrest (Chen *et al.*, 1998; Colavitti & Finkel, 2005). As such studies demonstrate the ability of exogenous H_2O_2 to induce senescence, the hCASMC were exposed to exogenous H_2O_2 . The higher concentration of 100 µM was found to induce cellular senescence with an observed ~4 fold increase in SA β-galactosidase positive cells; indicating the ability of exogenously added H_2O_2 to cause oxidative stress within the cells, in a concentration dependent manner, and subsequently activate the senescence pathway. Successive treatment with Rot caused a significant ~4 fold increase in senescence-positive cells compared to the control. For both inhibitors, there was no apparent difference between the time courses, suggesting that a 1 hour treatment using the successive protocol is sufficient as opposed to 4 hour treatments. Complex I inhibition by Rot appears to be able to induce senescence more effectively than complex III inhibition by AA, suggesting the amount of superoxide generated may be unable to be scavenged by the endogenous mitochondrial defences and may target biomolecules such as iron-sulphur proteins susceptible to oxidative damage; or mitochondrial DNA that has no histones associated (Duchen, 2004). Some reviews have discussed the efficiency of ROS production by mETC complexes I and III (Murphy, 2009; Chen et al., 2003). One review discusses how complex III can be induced to produce superoxide with AA inhibition; but mentions that complex III superoxide production in mitochondria under physiological conditions is negligible compared to the maximum rates of superoxide production from complex I (Murphy, 2009). Another contradicts this conclusion, stating that complex III is the dominant site of superoxide generation due to the ROS being directed away from the antioxidant defences present in the matrix. Complex I however produces superoxide into the proximity of antioxidant defences (Chen et al., 2003). Complex I may be the dominant site of ROS generation within hCASMC, based on the ability of Rot to induce SIPS.

A transcriptome analysis study conducted on a neuroblastoma cell line chronically exposed to marginally toxic and moderately toxic doses of Rot found a complex pleiotropic response to Rot that impacted a variety of cellular events including the cell cycle, DNA damage response, proliferation, senescence and death (Cabeza-Arvelaiz & Schiestl, 2012). The majority of the affected cell proliferation genes also regulate apoptosis, the cell cycle and the DNA Damage Response (DDR). Numerous Rot-deregulated genes encoded proteins that associate with or that are components of the cytoskeleton, particularly in the microtubule system suggesting Rot treatment influences processes involved in cell fate decisions. DDR repair pathways were also induced by Rot, indicated by the upregulation of genes *ATR*, *CHEK1*, *RAD17* and *ATM*. Additionally, the presence of 8-oxo-G adducts was increased by Rot indicating DNA damage by oxidative stress (Cabeza-Arvelaiz & Schiestl, 2012). Although this study was conducted by studying chronic Rot exposure, it cannot be ruled out that some of these responses may be observed in the hCASMC, hence the significant increase in senescence-positive cells following successive 3 day exposure.

Whilst the inventors of MitoPQ found 1-5 μ M caused increases in H₂O₂ and mitochondrial superoxide and induced MnSOD expression in a mouse myoblast cell line, the ability to induce premature senescence was not investigated (Robb *et al.*, 2015). As Paraquat has been shown to cause senescence in both human and plant cells, there is an expectation that MitoPQ may be capable of the same in hCASMC (Wiemer & Osiewacz, 2014; Joguchi *et al.*, 2004). When treated in successive days, 50 μ M MitoPQ showed an approximate 2.5 fold increase in SA β-galactosidase staining indicating the ability to cause senescence induction in a concentration-dependent manner. To my knowledge, this is the first demonstration that MitoPQ is able to induce SIPS in human primary cells.

Combined, the results suggest the ability of mitochondria-derived oxidative stress to induce senescence in hCASMC. As shown in **Figure 3.19**, there are many processes in the mitochondria whereby their dysfunction can trigger cellular senescence (**Figure 3.19**).



Figure 3.19: Perturbation of mitochondrial homeostasis promotes the establishment and maintenance of cellular senescence during ageing (Ziegler *et al.,* 2015).

Loss of proper mitochondrial homeostasis can promote cellular senescence through (1) excessive ROS production (orange), (2) impaired mitochondrial dynamics (brown), (3) electron transport chain defect (blue), (4) bioenergetics imbalance and increased AMPK activity (red), (5) decreased mitochondrial NAD⁺ and altered metabolism (green), and (6) mitochondrial calcium accumulation (purple). These mitochondrial signals trigger p53/p21 and/or p16/pRb pathways and ultimately lead to cellular senescence, which subsequently promotes age-related phenotypes, such as loss of tissue regeneration and function (Ziegler *et al.*, 2015)

ADP – Adenosine diphosphate; ATP – Adenosine triphosphate; AMP – Adenosine monophosphate; AMPK – AMP-activated protein kinase; ETC – Electron Transport Chain;
FIS1/DRP1 – Fission 1 protein/Dynamin-related protein 1 complex; ME – Malic enzyme; NAD – Nicotinamide adenine dinucleotide; NADH – Nicotinamide dinucleotide H (reduced); PARPs – Poly ADP ribose polymerase; Pi – inorganic phosphate; ROS – Reactive Oxygen Species; TCA – Tricarboxylic acid cycle.

AA and Rot may induce SIPS by acting on one or more mitochondrial processes. Firstly, inhibition of the ETC causes a decline in ATP production. Inhibition of ATP synthesis has been found to trigger senescence. The fall in ATP production can increase the AMP to ATP ratio, creating a bioenergetic imbalance within the cell which triggers both the p53/p21 and p16/pRb pathways; experimentally observed by the upregulation of p16 and p21 expression (Ziegler *et al.*, 2015; Zwerschke *et al.*, 2003).

Inhibition of the ETC causes a defect in the transfer of electrons between the complexes and also causes an increase in mitochondrial ROS (**Figure 3.19**). As H_2O_2 is able to diffuse out of the mitochondria, it can cause an increase in cellular oxidation activating the p53/p21 pathway with the p16/pRb pathway, promoting a ROS-dependent positive feedback loop, which reinforces irreversible cell cycle arrest. Some studies have found the prevention of senescence with the use of mitochondrial antioxidants such as MitoQ (Ziegler *et al.*, 2015; Macip *et al.*, 2002).

Other processes can be disturbed by ETC inhibition such as impaired mitochondrial dynamics and creating a fusion: fission imbalance (Figure 3.19). Mitochondria are known to be dynamic organelles, with the ability to adjust their size, shape and organisation; all of which are regulated during cell division, apoptosis, autophagy and mitochondrial biogenesis (Ziegler et al., 2015; Seo et al., 2010). Some studies believe senescent cells are typically associated with a shift towards fusion events with the presence of abnormally enlarged mitochondria in the cells; however it is unclear how fusion contributes to permanent growth arrest (Ziegler et al., 2015). Despite limited knowledge of their role, it is hypothesised that enlarged mitochondria form due to dysregulation in mitochondrial dynamics and impaired autophagy, as some studies conducted in models of replicative senescence and ageing animals have found a downregulation of fission proteins and reduced clearance of mitochondria by autophagy (Seo et al., 2010). In line with this, AA has been found to inhibit autophagy in mouse embryo fibroblast at concentrations as low as 9.5 nM in a manner independent of ATP and ROS reduction. Their results found that treatment with AA had no obvious effects on ROS under the same conditions that inhibited autophagy (Ma et al., 2011). The results imply that not only does AA have an alternative mechanism of action that does not rely on superoxide generation and ATP inhibition; but it also affects mitochondrial dynamics and this can be a contributing factor to how the complex III inhibitor causes premature senescence.

With regards to H₂O₂, due to the relatively long half-life, good membrane permeability, and higher intracellular concentration, it has been proposed to function as a second messenger in signalling pathways. H₂O₂ possesses the ability to modulate protein structure and function by inducing the transient oxidation of protein cysteinyl thiols, including the formation of disulphide bonds (Son *et al.*, 2011; Sobotta *et al.*, 2015). Some have investigated the signalling mechanism and speculated that many redox-regulated proteins are not actually direct targets

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of H_2O_2 , but rather their oxidation is mediated by thiol peroxidases (Sobotta *et al.*, 2015). The group found that peroxiredoxin II transfers oxidative equivalents to the redox-regulated transcription factor STAT3 by the formation of highly transient mixed disulphide intermediates and subsequent oxidation in the presence of H_2O_2 . This oxidation inhibits STAT3 transcriptional activity (Sobotta *et al.*, 2015). From these findings, it is clear that exogenous H_2O_2 possesses the ability to alter signalling pathways through the flow of oxidative equivalents and there may be components of senescence inducing pathways that are subjected to such oxidative transfer.

Beyond the direct abilities to induce senescence, H_2O_2 is able to act on the mitochondria which may in turn lead to the induction of senescence. One study monitored the effect of H_2O_2 on various aspects of the mitochondria (Gonzalez *et al.*, 2005). They found that incubation of the pancreatic acinar cells with H_2O_2 increased cytosolic and mitochondrial calcium concentration; induced a depolarisation of the mitochondria; and increased levels of oxidised flavine adenine dinucleotide. In complex II of the ETC, electrons are delivered into the quinone pool originating from succinate and transferred via the cofactor flavine adenine dinucleotide to ubiquinone. This electron transfer reduces ubiquinone and oxidises flavine adenine dinucleotide. The increase in the oxidised form of this nucleotide causes a loss in mitochondrial respiratory chain activity, suggesting H_2O_2 has the ability to induce the senescence pathway through mitochondrial dysfunction as shown in **Figure 3.19** (Gonzalez *et al.*, 2005; Heikal, 2010).

Together these processes converge on the induction of cellular senescence and the onset of age-related phenotypes (**Figure 3.19**).

Conclusions

Various conclusions can be drawn from the results provided in this chapter. Both AA and Rot are capable of inducing mitochondrial superoxide generation in hTERT hASMC, however Rot was found to possess cytotoxic effects. Despite inducing mitochondrial superoxide, the hTERT hASMC did not undergo SIPS as a response to either AA or H_2O_2 . They therefore may not be a suitable VSMC model for senescence; however they may be utilised to study the induction of ROS in SMC.

hCASMC are capable of undergoing replicative senescence with continuous cell culture. Both mitochondrial ETC inhibitors, AA and Rot, cause ROS generation in hCASMC however only Rot is able to induce SIPS with successive treatment. Whilst no ROS production was able to be detected following exposure of the hCASMC to MitoPQ, the compound induced premature senescence in a concentration-dependent manner; as did treatment with exogenous H₂O₂.

Together, the results show that VSMC (hCASMC) are able to undergo SIPS with successive treatment of compounds that target mETC and/or superoxide.

CHAPTER FOUR

Modulation of Mitochondria derived Reactive Oxygen Species by Antioxidants

Chapter 4 Modulation of Mitochondriaderived Reactive Oxygen Species by Antioxidants

4.1 Introduction

Antioxidants have had limited success in preventing the progression of diseases proposed to involve oxidative damage. One possible reason for this may be that most small molecule antioxidants distribute around the body, with only a small fraction being taken up by the mitochondria (Murphy, 2008). The lack of successful antioxidants leads to the need for stable small molecule antioxidants that could be selectively taken up by mitochondria within organs most affected by mitochondrial oxidative damage.

One approach to preventing mitochondrial oxidative damage that satisfies the selectivity requirement has been to conjugate lipophilic cations such as triphenyl phosphonium (TPP⁺) to antioxidant molecules (Murphy, 2008). Lipophilic cations are able to pass easily through the lipid bilayers of the membranes because their charge is dispersed over a large surface area and the electrochemical gradient drives their accumulation into the mitochondrial matrix (Murphy, 2008). An intriguing aspect of the use of mitochondria-targeted antioxidants is that in principle, they could be applied to a range of diseases as mitochondrial oxidative damage is hypothesised to play a role in the pathogenesis of many disorders including atherosclerosis (Murphy, 2008).

hTERT hASMC were initially used as a SMC model with a view of modulating exogenouslyinduced mitochondrial ROS and preventing the onset of senescence following oxidative stress. The studies highlighted in the previous chapter demonstrated that the hTERT hASMC were unsuitable as a model for senescence, however they could still be used to investigate ROS production within mitochondria. As premature senescence could not be induced with the hTERT hASMC, primary hCASMC were also used for these investigations. Previous studies have demonstrated the ability to reduce ROS levels with various antioxidants following exposure to a variety of stressors (Spagnuolo *et al.*, 2006; Lowes *et al.*, 2008; Han *et al.*, 2008; Dikalova *et al.*, 2010).

4.2 Aims & Objectives

Previous studies within this group have described the prevention of premature senescence by mitochondria-targeted antioxidant, MitoTEMPO, implicating mitochondrial ROS in the senescence pathway (Mistry *et al.*, 2013). However, the previous study failed to investigate if mitochondrial ROS was reduced as a result of treatment with MitoTEMPO. Thus, the aim of this chapter is to investigate the ability of two mitochondria targeted antioxidants and the non-targeted antioxidant, N-acetyl cysteine (NAC) to reduce ROS levels induced by mitochondrial inhibitors, AA and Rot. The significance of this investigation is to determine if senescence can be prevented by reducing mitochondria-derived oxidant species with the use of antioxidants.

4.3 Results

4.3.1 Effect of NAC on hTERT hASMC cell viability

GSH is a tripeptide, synthesised and maintained at high intracellular concentrations. In its reduced form, it is able to act as an antioxidant, known to minimise lipid peroxidation of cellular membranes as well as protein thiolation and drug detoxification. The tripeptide also acts as a co-factor for peroxidase enzymes (Rushworth & Megson, 2014). GSH synthesis is limited by the availability of its cysteine substrate, so NAC, an acetylated cysteine residue, is widely used to supplement the biosynthesis of reduced GSH (Kerksick & Willoughby, 2005). NAC has advantages over natural cysteine due to its relative resistance to disulphide oxidation and the ability to permeate the cell membrane. Additionally, NAC acts as an antioxidant by scavenging free radicals (Kerksick & Willoughby, 2005; Rushworth & Megson, 2014). Previous studies have successfully reduced ROS levels in various cells *in vitro* with the use of NAC (Han *et al.*, 2008; Dott, 2014; Spagnuolo *et al.*, 2006).

hTERT hASMC growth and ATP content, in the presence of NAC, was assessed over a range of concentrations in order to establish suitable non-cytotoxic concentrations.

The trypan blue assay was used to measure the viability of hTERT hASMC following a single exposure to NAC over a period of 24 hours. The CCK-8 assay was not utilised due to interference of NAC with the colorimetric assay.

At concentrations up to 1000 μ M, cell viability, measured using the Trypan blue assay (Figure 4.1**A**); was maintained when compared to the control. ATP levels were subsequently examined and higher concentrations of NAC were tested. There was no significant fall in ATP levels with NAC treatment (Figure 4.1**B**). 1 mM was chosen as the desired concentration for hTERT hASMC antioxidant treatment.







Figure 4.1: NAC does not alter cell number or ATP levels in hTERT hASMC.

A, Cells were treated with NAC 24 hours. Cell number was measured using the Trypan Blue assay, utilising 1 mM H_2O_2 as a positive control. Data presented as a mean+SEM; n=4 (****p<0.0001 compared to the control). **B**, Cells were treated with NAC for 24 hours. ATP content was measured using the luminescence assay, utilising 1 mM H_2O_2 as a positive control. Data presented as mean+SEM; n=7 (****p<0.0001 compared to the control).

4.3.2 Effect of NAC on superoxide levels in hTERT hASMC

As NAC is an untargeted antioxidant, superoxide levels were determined using the DHE dye to measure cytosolic superoxide levels. Cells were treated with either 1 mM NAC or 100 μ M AA for 24 hours or in combination with a 2 hour pre-treatment of NAC followed by a 24 hour co-treatment with AA and NAC. Compared to the control, superoxide levels were unaltered with NAC alone suggesting the antioxidant was unable to reduce baseline superoxide levels (**Figure 4.2**). There was also no change in DHE fluorescence intensity for any of the other treatments

(**Figure 4.2**). Therefore there was no evidence that NAC could reduce cellular superoxide levels for either basal or AA-stimulated conditions.



Figure 4.2: NAC failed to alter AA-induced cytosolic superoxide levels in hTERT hASMC

Cells were treated for 24 hours with either AA (100 μ M) or NAC (1 mM) alone or in combination with a 2 hour pre-treatment of NAC followed by a 24 hour co-treatment of the two compounds. Cytosolic superoxide levels were detected by flow cytometry using the DHE dye. Data presented as mean+SEM; n=3.

4.3.3 Cell Viability in hTERT hASMC following MitoQ exposure

As MitoQ has been found to possess many beneficial effects *in vivo* and *in vitro* including blocking oxidative damaged-induced cell death and reducing the rate of ROS production, (Jauslin *et al.*, 2003; Lowes *et al.*, 2008), its antioxidant ability in hTERT hASMC was investigated. The initial studies measured hTERT hASMC growth and ATP content in the presence of MitoQ over a range of concentrations in order to establish non-cytotoxic suitable concentrations for cell treatment.

The CCK-8 assay measured cell number after a 24 hour treatment and the cells were found to be tolerant of the antioxidant at concentrations as high as 1 μ M demonstrated by unaltered numbers compared to the control (**Figure 4.3A**). Over the same time course, the ATP levels

were reduced in a concentration dependent manner. A significant reduction in ATP was found at concentrations of 2 μ M of MitoQ and above (p<0.05; **Figure 4.3B**).



Figure 4.3: MitoQ maintains hTERT hASMC cell viability but reduces ATP levels in a concentration dependent manner.

A, Cells were treated MitoQ for 24 hours and cell number was measured using the CCK-8 cell viability assay, utilising 1 mM H_2O_2 as a positive control. Data presented as mean+SEM; n=4 (****p<0.0001 compared to the control). **B**, Cells were treated with MitoQ for 24 hours and ATP content was measured using the luminescence assay, using 1 mM H_2O_2 as a positive control. Data presented as mean+SEM; n=3 (*p,0.05; ****p<0.0001 compared to the control).

4.3.4 Effect of MitoQ on Antimycin A induced superoxide production in hTERT hASMC

As higher concentrations of MitoQ were reported to have detrimental effects on the mETC (Reily *et al.*, 2013) and a reduction in ATP levels were observed at μ M concentrations of MitoQ (**section 4.3.3**); 100nM and 500nM of MitoQ were used to attempt to modulate the increased superoxide levels caused by AA.

MitoSOX[™] flow cytometry was utilised and the treatments were conducted over a 24 hour period. The hTERT hASMC were treated with AA in the presence or absence of MitoQ (**Figure 4.4**). AA significantly increased the mitochondrial superoxide levels to ~3 fold compared to the control. Neither concentration of MitoQ was able to reduce the baseline superoxide levels; nor did the treatment with 100 nM MitoQ reduce the superoxide levels caused by AA. However, the higher concentration of MitoQ appeared to potentiate the effects of AA, further stimulating the production of superoxide compared to AA alone (p<0.05). As the antioxidant failed to act as an antioxidant, further studies with MitoQ were discontinued.



Figure 4.4: Mitochondria-targeted antioxidant MitoQ fails to reduce mitochondrial superoxide levels in AA-stimulated hTERT hASMC.

Cells were treated for 24 hours with AA (100 μ M) in the presence or absence of MitoQ (100 nM or 500 nM). Mitochondrial superoxide levels were detected by flow cytometry using the MitoSOXTM dye. Data presented as mean+SEM; n=4 (**p<0.01 compared to the control; #p<0.05 compared to AA alone).

4.3.5 Cell Viability in hTERT hASMC following MitoTEMPO

exposure

Once MitoQ was found to be ineffective in modulating AA-stimulated superoxide production, experiments were carried out with MitoTEMPO. Again, the cytotoxicity assays were performed, with the CCK-8 assay measuring cell number and ATP measured by luminescence following antioxidant treatment.
The CCK-8 assay was conducted following 24 hour treatment with MitoTEMPO and there was no significant change in cell number (**Figure 4.5A**). Unlike MitoQ, there was no significant change in ATP levels, even at the high concentration of 5 μ M (**Figure 4.5B**).

The cytotoxicity assays showed that any concentration of MitoTEMPO up to 5 μ M could be used to investigate modulation of AA-induced superoxide generation.





 $M \ ito TEMPO \ (\mu M)$

Figure 4.5: Mitochondria targeted antioxidant MitoTEMPO maintains cell number and ATP levels in hTERT hASMC.

Cells were treated with MitoTEMPO for 24 hours. Cell number was measured using the CCK-8 cell viability assay (**A**) and ATP content was measured with the luminescence assay (**B**). 1 mM H_2O_2 was used as a positive control. Data presented as mean+SEM; n=5 (**A**) and n=3 (**B**) (*p<0.05; ****p<0.0001 compared to the control).

4.3.6 Effect of MitoTEMPO on Antimycin A-induced mitochondrial superoxide in hTERT hASMC

The initial studies attempting to modulate AA-stimulated superoxide production utilised lower concentrations of MitoTEMPO as previous studies have found success with nanomolar concentrations (Dikalova *et al.*, 2010).

MitoSOX[™] flow cytometry was used to investigate mitochondrial superoxide levels following AA stimulation (**Figure 4.6A**). Neither 50 nM nor 100 nM MitoTEMPO altered the baseline superoxide levels. When treated in combination with AA, neither concentration of MitoTEMPO decreased the superoxide levels induced by AA.

Subsequently, co-treatments with higher concentrations of MitoTEMPO were investigated. Again, neither 100 nM nor 500 nM MitoTEMPO affected mitochondrial superoxide induced by AA (**Figure 4.6B**).



Figure 4.6: Effect of mitochondria-targeted antioxidant MitoTEMPO on mitochondrial superoxide levels in AA-stimulated hTERT hASMC.

A, Cells were treated for 4 hours with AA (100 μ M) in the presence or absence of MitoTEMPO (50 nM or 100 nM). Mitochondrial superoxide levels were detected by flow cytometry using the MitoSOXTM dye. Data presented as a mean+SEM; n=3 (ns, no significant difference). **B**, Cells were treated for 24 hours with AA (100 μ M) in the presence or absence of MitoTEMPO (100 nM or 500 nM). Mitochondrial superoxide levels were detected by flow cytometry using the MitoSOXTM dye. Data presented as mean+SEM; n=5 (ns, no significant difference; *p<0.05 compared to the control).

4.3.6.1 Effects of micromolar concentration of MitoTEMPO on Antimycin A-induced mitochondrial superoxide production

As nanomolar MitoTEMPO concentrations did not decrease superoxide levels, a higher (1 μ M) concentration was used over a time course of 4 hours and 24 hours.

MitoSOX[™] flow cytometry was utilised for both time courses and as shown (Figure 4.7**A**), 1 μM MitoTEMPO did not decrease the AA-induced superoxide levels for either time course (Figure 4.7**A** and **B**).



Figure 4.7: Micromolar MitoTEMPO failed to reduce mitochondrial superoxide levels in AAstimulated hTERT hASMC.

Cells were treated for 4 hours (**A**) or 24 hours (**B**) with AA (100 μ M) alone, MitoTEMPO (1 μ M) alone or co-treated with both AA and MitoTEMPO. Mitochondrial superoxide levels were detected by flow cytometry using the MitoSOXTM dye. Data presented as mean+SEM; n=7 (**A**) and n=4 (**B**) (ns, no significant difference; ****p<0.0001 compared to the control).

4.3.6.2 Effect of pre-treatment with MitoTEMPO on Antimycin A- induced mitochondrial superoxide production

As the 1 µM MitoTEMPO co-treatment appeared to slightly reduce AA-induced superoxide production (no statistical difference; section **4.3.6.1**), the experiment was altered so that the hTERT hASMC could first be loaded with MitoTEMPO for 2 hours as a pre-treatment. Following the pre-treatment, the cells were co-treated with 1 µM MitoTEMPO and AA, and MitoSOX[™] flow cytometry was conducted. The MitoTEMPO treatment alone did not reduce baseline superoxide levels however the co-treatment appeared to slightly reduce the superoxide levels although this did not reach statistical significance (**Figure 4.8A**).

Despite the lack of significance between the AA and 1 μ M MitoTEMPO + AA treatments (p-value - 0.8942), the confidence intervals were investigated and all the treatments were compared against each other. As the difference between the AA and the MitoTEMPO + AA overlapped, it was clear to see that the difference between the means of the groups was not statistically significant (**Figure 4.8B**).

A power calculation was then performed using the Decision Support System research website (DSS, 2016). The means and standard deviations from both the AA group and the MitoTEMPO + AA group were used to calculate the theoretical sample size required to reach statistical significance (p<0.05). The sample size needed for there to be a statistically significant difference between the means of the two groups is 19 (**Table 4.1**).



Figure 4.8: A pre-treatment with 1 μ M MitoTEMPO failed to reduce mitochondrial superoxide levels in AA-stimulated hTERT hASMC.

A, Cells were pre-treated with MitoTEMPO (1 μ M) for 2 hours and subsequently co-treated for 4 hours with both AA (100 μ M) and MitoTEMPO (1 μ M). Mitochondrial superoxide levels were detected by flow cytometry using the MitoSOXTM dye. Data presented as mean+SEM; n=7 independents determinations (**p<0.01 compared to the control). **B**, MitoSOXTM flow cytometry results presented in a confidence intervals graph.

	100 μM	100 μM Antimycin A + 1 μM	Sample Size	
	Antimycin A	MitoTEMPO	Required	
Mean	13.27	11.28		
Standard	3.88	3.67	19	
Deviation				
Table 4.1: Power Calculation				
α error level or Confidence level = 5%				
β error level or Statistical Power = 80%				
Sample Size Output = 19				

4.3.6.3 The reversible effects of Antimycin A

Possibly due to the lack of sensitivity of the MitoSOX[™], partly caused by the associated variation within the assay, a large sample size would be required to demonstrate an effect by MitoTEMPO (**Table 4.1**). Therefore, another approach was tested. AA was loaded into the hTERT hASMC for 2 hours. Following this treatment, the AA was removed, a wash with PBS was carried out then media containing 1 µM MitoTEMPO or media alone was added for another 2 hours. The results showed that the effect of AA was reversible as the replacement of fresh media following AA treatment brought the mitochondrial superoxide back down to baseline levels, identical to the replacement of media containing MitoTEMPO (**Figure 4.9**). The results suggest that the binding of AA to complex III is not permanent as when the media containing AA was removed, AA diffused out of the mitochondria possibly to maintain a diffusion gradient.



Figure 4.9: The effect of mitochondrial inhibition by AA in hTERT hASMC is reversible.

Cells were loaded with AA (100 μ M) for 2 hours. Following the loading, the treatments were replaced with media containing MitoTEMPO (1 μ M) or media alone. Mitochondrial superoxide levels were detected by flow cytometry using the MitoSOXTM dye. Data presented as mean+SEM; n=3.

4.3.6.4 Effect of pre-treatment with MitoTEMPO on Antimycin A- induced cytosolic superoxide production

The cytosolic superoxide levels were also investigated to determine if the use of the targeted antioxidant MitoTEMPO had any SOD mimetic activity outside the mitochondria.

DHE flow cytometry was utilised as DHE detects cytosolic superoxide and the treatments were conducted over a 4 hour period, with a prior 2 hour pre-treatment with MitoTEMPO. No significant difference was observed with the AA treatment (mean DHE FL intensity = 159.76) when compared to the MitoTEMPO and AA co-treatment (mean DHE FL intensity = 156.77).

Therefore, there was no evidence that MitoTEMPO had pro- or antioxidant activity in the cytoplasm.

4.3.6.5 Effect of higher concentrations of MitoTEMPO on Antimycin A-induced mitochondrial superoxide production

Despite previous successes with low concentrations of MitoTEMPO (Dikalova *et al.*, 2010), a higher concentration was used to treat the hTERT hASMC as a higher concentration of the antioxidant may be required.

Mitochondrial superoxide levels were determined using MitoSOXTM flow cytometry following a co-treatment with 2 μ M of MitoTEMPO and 100 μ M AA. The results show no statistically significant difference between the AA treatment (mean MitoSOXTM FL intensity = 8.24) and the 2 μ M of MitoTEMPO + AA co-treatment (mean MitoSOXTM FL intensity = 7.93).

4.3.7 Cell viability in hCASMC following NAC treatment

Following the studies with various antioxidants in the hTERT hASMC cell model (sections **4.3.2**, **4.3.4** and **4.3.6**); hCASMC were utilised as it was suggested that the lack of NAC, MitoQ and MitoTEMPO antioxidant efficacy could be due to the specific properties of the hTERT-immortalised cells.

Cell viability studies were again performed to determine suitable concentrations of NAC for subsequent experiments. NAC levels up to 5 mM did not affect cell number over a 4 hour time course (**Figure 4.10A**). Investigating ATP content at the corresponding timeframe indicated that NAC exposure caused no change in hCASMC ATP levels (**Figure 4.10B**).



Figure 4.10: NAC does not alter cell number or ATP levels in hCASMC.

Cells were treated with NAC for 4 hours. Cell number was measured using the CCK-8 Assay (**A**) whilst ATP content was measured using the luminescence assay (**B**). 1 mM H_2O_2 was used as a positive control. Data presented as mean+SEM; n=4 (**p<0.01, ***p<0.001 compared to the control).

4.3.8 Effect of NAC on Antimycin A and Rotenone-induced H₂O₂ in hCASMC

The studies with NAC in hTERT hASMC were carried out using DHE flow cytometry to detect cytosolic superoxide levels. However, as the hCASMC were primary cells, with limited growth potential, flow cytometry could not be conducted, as large numbers of cells were required. Instead, the Amplex Red assay was employed to detect extracellular H_2O_2 . GSH reacts with H_2O_2 in a reaction catalysed by glutathione peroxidase (Abedinzadeh *et al.*, 1989) and so if NAC was boosting GSH levels, this should cause rapid detoxification of H_2O_2 , detectable by the Amplex Red assay.

Following treatment with mETC inhibitors, AA or Rot, and varying concentrations of NAC, H_2O_2 levels were measured using the Amplex Red assay (**Figure 4.11**). Both AA and Rot appeared to reduce H_2O_2 levels when compared to the control, however statistical significance was not reached (**Figure 4.11A**). The high NAC starting concentrations of 1.25 mM and 2.5 mM were selected based on successful reduction of ROS by studies in this group albeit in a different cell type (Dott, 2014). The 4 hour 2.5 mM NAC treatment caused a significant increase in H_2O_2 levels compared to the untreated control. When used as a co-treatment with AA, both 1.25 mM and 2.5 mM appeared to slightly increase H_2O_2 levels compared to the AA treatment however no statistical significance was reached. With Rot, again the two concentrations appeared to slightly increase H_2O_2 levels compared with the Rot treatment alone, with no statistical significance (**Figure 4.11A**).

Lower NAC concentrations were adopted and the same assay was carried out. Compared to the AA alone, the co-treatment with 500 μ M NAC appeared to increase H₂O₂ levels whilst the co-treatment with 100 μ M NAC maintained the same H₂O₂ levels. With Rot, both 100 μ M and 500 μ M NAC appeared to increase H₂O₂ levels, however again no statistical significance was reached (**Figure 4.11B**).

Lower 1 μ M and 10 μ M NAC concentrations were tested, with the hope of finding a suitable concentration with antioxidant capacity. For both AA and Rot, co-treatments with NAC caused no change in the levels of H₂O₂ compared to the treatment with the respective inhibitor alone (**Figure 4.11C**).

The results from this series of experiments demonstrate the inability of NAC to act as a suitable antioxidant in modulating the H_2O_2 levels of hCASMC stimulated with either AA or Rot. This may be due to the fact that both AA and Rot already appear to cause reductions in H_2O_2 levels compared to the control, so NAC may not be able to make any further decreases. Additionally, as NAC was at times shown to cause increased H_2O_2 levels compared to the control (**Figure 4.11A**), the results suggest that NAC could be acting as a pro-oxidant; generating superoxide that could cause subsequent H_2O_2 production, which will be discussed further in the section **4.4**.



Figure 4.11: NAC does not reduce the extracellular hydrogen peroxide levels following hCASMC treatment with either AA or Rot.

hCASMC were treated with AA (50 μ M), Rot (50 μ M) or the different concentrations of NAC; **A**, (1.25 mM or 2.5 mM); **B**, (100 μ M or 500 μ M); **C**, (1 μ M and 10 μ M) for 4 hours. Extracellular H₂O₂ levels were measured using the Amplex Red assay. Data presented as mean+SEM; n=3 (*p<0.05 compared to the control).

4.3.9 Cell viability and ATP levels in hCASMC following MitoTEMPO treatment

As NAC appeared unable to reduce H_2O_2 production in hCASMC following AA and Rot stimulation, the mitochondrial targeted MitoTEMPO was utilised. Work with MitoQ was not carried out as the antioxidant had demonstrated the ability to act as a pro-oxidant and potentiate the effects of AA in superoxide generation (**Figure 4.4**).

Cell cytotoxicity was determined with the CCK-8 and the ATP assays over 4hours. hCASMC tolerated MitoTEMPO treatment at concentrations up to 1 μ M; whilst at 2 μ M, cell number was found to be at 78% of the control (**Figure 4.12A**). ATP levels were unaffected from the MitoTEMPO treatment (**Figure 4.12B**). Both the cell number and ATP results demonstrated that MitoTEMPO had no cytotoxic effects on the hCASMC so 1 μ M of MitoTEMPO was used to act as a potential antioxidant for subsequent experiments.



MitoTEMPO (µM)



Cells were treated MitoTEMPO for 4 hours. Cell number was measured using the CCK-8 cell viability assay (**A**) and ATP content was determined using the luminescence assay (**B**). 1 mM H_2O_2 was used as a positive control. Data presented as mean+SEM; n=4 (***p<0.001, ****p<0.0001 compared to the control).

4.3.10 Modulation of Antimycin A and rotenone-induced H₂O₂ by MitoTEMPO in hCASMC

Once an appropriate concentration of MitoTEMPO was chosen, it was used in treatments with AA and Rot to attempt to modulate the ROS induced by these inhibitors. Due to the slow growing nature of the hCASMC, the Amplex Red assay was utilised and extracellular H_2O_2 was measured. Quantitative superoxide detection could not be conducted due to the number of cells required for flow cytometry studies.

AA and Rot cause an increase in superoxide levels due to mETC inhibition. However, as the Amplex Red assay detects H_2O_2 , the experiment relies on superoxide molecules generated from mitochondrial inhibition to be converted into the soluble H_2O_2 . Numerous studies have employed the Amplex Red assay to measure ROS following treatment with either AA or Rot (Chen *et al.*, 2003; Sipos *et al.*, 2003; Droese & Brandt, 2008).

hCASMC were treated with AA or Rot in the presence or absence of 1 μ M MitoTEMPO. Cells were also treated with MitoTEMPO alone to determine if the antioxidant caused any changes to baseline H₂O₂ levels (**Figure 4.13**).

At the end of the 4 hour treatment, Amplex Red reagent was added and the H_2O_2 levels were monitored over a period of 30 minutes. With incubation, the fluorescence increased as the horseradish peroxidase captured H_2O_2 to form the fluorescent product, resorufin (**Figure 4.13A**). Levels of H_2O_2 in cells treated with AA remained lower than the control throughout the time course, and the presence of MitoTEMPO did not alter this (**Figure 4.13A**).

Rot alone also appeared to produce lower levels of H_2O_2 compared to the control, however the H_2O_2 levels were much higher in the presence of MitoTEMPO (**Figure 4.13B**). At the 30 minute time point, the data were gathered into a bar chart to highlight the different levels of H_2O_2 for the treatments and to facilitate statistical analysis (**Figure 4.13C**). H_2O_2 levels were significantly reduced by AA. However, MitoTEMPO did not affect H_2O_2 levels in cells treated with AA (**Figure 4.13C**).

Although Rot alone did not affect H_2O_2 levels compared to the control (**Figure 4.13C**), there was a significant difference between the Rot treatment and the Rot with MitoTEMPO treatment. This difference suggests that MitoTEMPO modifies the effects of Rot, as found with MitoQ and AA-induced superoxide production in hTERT hASMC (**Section 4.3.4**).



Figure 4.13: Effects of MitoTEMPO on extracellular H_2O_2 levels in hCASMC treated with mETC inhibitors.

Cells were treated for 4hours with inhibitors AA (50 μ M) or Rot (50 μ M) in the presence or absence of MitoTEMPO (1 μ M). Following treatment, extracellular H₂O₂ levels were measured using the Amplex Red assay. 50 μ M menadione was used as a positive control (not shown, [H₂O₂] Arb fluorescence units = 2189). Data presented as a mean+SEM; n=4 (*p<0.05 compared with the control; *p<0.05 compared with Rotenone alone).

4.3.11 Gene expression of endogenous antioxidants following hCASMC treatment with AA, Rot and MitoTEMPO

The Amplex Red assay showed that hCASMC treatment with AA and possibly Rot caused a decrease in H₂O₂ levels compared to untreated cells. This suggests that the mitochondrial inhibitors may be stimulating antioxidant expression or activity to prevent oxidative stress within the cell, thereby resulting in lower H₂O₂ levels. As the Amplex Red assay was conducted over a 4 hour treatment period, it was unclear whether the results were based on gene expression changes of endogenous antioxidants or protein modifications resulting in enzyme activity changes. Gene expression changes were examined at 4 hours.

To quantify the expression level of various endogenous antioxidant genes in the hCASMC following treatment, Real-Time qPCR was employed. The cells were treated with AA or Rot in the presence or absence of MitoTEMPO. Following treatment, RNA was extracted, and cDNA was reverse transcribed. To begin, PCR amplification efficiencies of the probes were determined by construction of standard curves using cDNA pooled from all the treatment samples. The standard curve also highlighted the linear range of the assay and the concentration of cDNA suitable for subsequent assays (standard curve in Appendix 1). Probe efficiency within 10% was deemed acceptable and the percentage efficiency of each probe was within 10% of the others; except GAPDH, which was slightly higher (**Table 4.2**). Despite the efficiency of GAPDH being out of range, the housekeeper was still included and averages between the 3 housekeepers were used for comparative CT calculation.

Gene	Efficiency			
SOD1 – Superoxide Dismutase 1	88%			
SOD2 – Superoxide Dismutase 2	89%			
GPX I – Glutathione Peroxidase	90%			
CAT – Catalase	90%			
PRDX I – Peroxiredoxin I	84%			
PRDX III – Peroxiredoxin III	87%			
GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase	96%			
TBP – TATA Binding Protein	92%			
B2M – β-2-microglobulin	83%			
Table 4.2: Taqman probe efficiency for the antioxidants and housekeeper genes				

The Taqman probes, except the housekeeper GAPDH, were within 10% of each other.

Once standard curves had been constructed and probe efficiencies determined, the comparative CT method was used for relative quantification. The target genes were normalised to the averages of the 3 housekeeper genes; β 2M, TBP and GAPDH. There were no differences in the expression of the 6 endogenous antioxidants across the treatment groups (**Figure 4.14**). This suggests the results observed in the Amplex Red assay were not dependent on gene expression changes, but may possibly be reliant on enzyme activity (**Figure 4.14**).



Figure 4.14: Mitochondrial inhibitors and MitoTEMPO do not alter gene expression profiles of endogenous antioxidants in hCASMC.

Cells were treated for 4 hours with AA (50 μ M) or Rot (50 μ M) alone or in combination with MitoTEMPO (1 μ M). Following treatment, RNA was extracted; cDNA reverse transcribed, and gene expression of 6 endogenous antioxidants was determined using Real Time qPCR. Data are presented as a mean+SEM; n=8.

4.3.12 Investigation of the potential protective effects of Antimycin A

The reduction in H_2O_2 observed following AA treatment of hCASMC appeared not to be due to changes in antioxidant gene expression. Therefore, a cell viability assay was used to determine if the decrease in H_2O_2 conferred any protective effects on the cells. The aim was to determine if the AA-treated cells were protected due to a possible increase in endogenous enzyme activity. hCASMC were treated with AA for 4 hours and various concentrations of H_2O_2 were added to both untreated cells and the AA-treated cells. The control cells were fed with fresh media for 4 hours then increasing concentrations of H_2O_2 were added, identical to the AA-treated cells.

In the untreated cells, increasing H_2O_2 caused slight reductions in cell numbers although these failed to reach statistical significance (**Figure 4.15**). However, for the AA-treated cells, the increasing concentrations of H_2O_2 caused noticeable reductions in cell number which reached statistical significance at 200 μ M H_2O_2 (p<0.05; **Figure 4.15**). This suggests that a 4 hour pretreatment offers no protective effects to the cells, rather it sensitises them to cell death. As the addition of exogenous H_2O_2 was able to cause a decrease in cell number, the results suggest there is no increased enzyme activity to combat the rise in oxidants within the cells. Nevertheless, it could be argued that the exogenous H_2O_2 acts on an alternative pathway that relies on antioxidants that were not investigated in the gene expression studies in section **4.3.11**.



Figure 4.15: Antimycin A pre-treatment offers no protective effects against H₂O₂ in hCASMC

Cells were treated with 50 μ M AA (blue bars) or with fresh media (orange bars) for 4 hours. Following the treatment, the media was removed and replaced with media containing increasing concentrations of H₂O₂ (20-200 μ M) for 1 hour. A control was left for each (green bars). Cell number was measured using the CCK-8 assay and 1 mM H₂O₂ for 1 hour was used as a positive control. Data presented as mean+SEM; n=5 (****p<0.0001 compared to the media control; #p<0.05 compared to the AA control).

4.4 Discussion

Studies performed previously in this group found MitoTEMPO treatment was able to prevent Angll-induced premature senescence implicating mitochondrial ROS in the senescence signalling pathway; however ROS levels were not investigated (Mistry *et al.*, 2013). The purpose of this chapter was to determine if it was possible to modulate mitochondrial-induced ROS with the use of targeted and untargeted antioxidants, MitoQ, MitoTEMPO and NAC in hTERT hASMC and hCASMC. All three antioxidants were reported to reduce ROS levels in other studies (Jauslin *et al.*, 2003; Han *et al.*, 2008; Nazarewicz *et al.*, 2013; Dott, 2014), however, no study has conducted the investigations in VSMC. It must be noted that many of these previous studies utilise inaccurate oxidant species detection methods such as DCFH-DA fluorescence which can be oxidised by various oxidants and redox-active metals, leading to false positive results (Kalyanaraman *et al.*, 2012).

4.4.1 N-acetyl cysteine as an antioxidant

The untargeted antioxidant used to attempt ROS modulation was NAC. As mentioned earlier, other studies in different cells (Spagnuolo *et al.*, 2006; Han *et al.*, 2008), as well as previous work within the group had reported ROS reduction with NAC administration in L6 cells, a rat skeletal muscle cell line (Dott, 2014).

In aiming to modulate ROS in hTERT hASMC, 1 mM NAC was used with a 2 hour pre-treatment prior to stimulation of superoxide production to enable the uptake of the compound into the cells, allowing GSH synthesis, as done by other studies (You & Park, 2010; Dott, 2014; Mukherjee *et al.*, 2007). Flow cytometry results found no change in cytosolic superoxide levels in cells treated with NAC alone or as a co-treatment with AA. These results indicated the inability of NAC to alter cytoplasmic superoxide levels.

In studies with hCASMC, the Amplex Red assay was used to detect extracellular H_2O_2 levels rather than using DHE flow cytometry, which detects cytosolic superoxide. It was assumed that NAC and its boosting of GSH levels should cause rapid detoxification of H_2O_2 as *in vivo*; GSH reacts with H_2O_2 to form glutathione disulphide and water in a reaction catalysed by glutathione peroxidase (Abedinzadeh *et al.*, 1989). Thus alterations in H_2O_2 are better detected by the Amplex Red assay. With hCASMC, a wider range of NAC concentrations was investigated ranging from 1 μ M to 2.5 mM; however none of these reduced H_2O_2 levels following mitochondrial complex inhibition.

4.4.1.1 The pro-oxidant nature of NAC

In hCASMC, it was found that the high NAC concentration of 2.5 mM significantly increased extracellular H_2O_2 levels compared to the control. This has been mirrored in studies carried out in HeLa cells where 500 μ M and 2 mM NAC increased ROS levels compared to the control 2 minutes after treatment, which was sustained up to 60 minutes. This increase was not observed with exposure to lower concentrations (100 μ M) (Han *et al.*, 2008). The explanation for the increase in ROS following NAC exposure will be discussed below.

In hCASMC, when treated with AA, the higher concentrations of NAC appeared to potentiate the effects of mETC inhibition. Although failing to reach statistical significance, the cotreatments with 1.25 mM and 2.5 mM NAC appeared to suggest that there were higher levels of H_2O_2 than either mitochondrial inhibitor alone. Both AA and Rot had reduced H_2O_2 levels compared to the control; however the addition of the higher NAC concentrations brought these levels nearer to the control levels. A possible explanation for the reduction in H_2O_2 following mitochondrial inhibition may be the induction of endogenous antioxidant gene expression to combat the increased oxidative burden within the cells, or alternatively, increased antioxidant enzyme activity; both of which will be discussed in further detail.

When the lower 100 μ M and 500 μ M NAC concentrations were combined with the mitochondrial inhibitors, the pro-oxidant effects were more pronounced in the Rot co-treatment, although none of these results reached statistical significance. Even lower 1 μ M and 10 μ M NAC caused no change in H₂O₂ levels for either AA or Rot.

Together, these results show that in this *in vitro* system, NAC fails to act in any antioxidant capacity over the wide range of concentrations tested. The effect of NAC is believed to be a balance between antioxidant and pro-oxidant activity, dependent on concentration; however this also appears to be dependent on cell type and the stimulator of oxidative stress. Many *in vitro* studies have found NAC (and MitoQ) to exert biphasic effects, with low concentrations offering protection against depletion of reduced GSH and oxidative stress; whilst high concentrations enhance the production of ROS, along with carbonylation and glutathionylation of cellular proteins (Mukherjee *et al.*, 2007). High concentrations of NAC also play a role in preventing metal ion toxicity as demonstrated by studies on the H460 human lung cancer cell line (Luczak & Zhitkovich, 2013). 10 mM NAC was found to rescue cell viability of the cells following lethal exposure to carcinogenic metals; chromium, cobalt and cadmium. NAC also caused a concentration-dependent inhibition of the accumulation of the metals during 24 hour

incubations with 10 mM having maximal inhibition. The study indicates that NAC possesses chemoprotective activity against metal ions (Luczak & Zhitkovich, 2013).

Whilst some studies show low NAC concentrations to be favourable, other studies have found the converse to be true. Previous work in HeLa cells (Han et al., 2008) found 100 μM NAC caused no effect on ROS levels when compared to the control as measured by DCFH-DA fluorescence. However the higher concentration of 500 μ M significantly increased the levels of ROS, which were further increased with 2 mM NAC. These data show the higher concentrations of NAC caused an elevation in ROS levels within resting, unstimulated HeLa cells. Despite the elevation in ROS, all the concentrations reduced ROS levels in cells stimulated with 50 µM AA (Han et al., 2008). The same laboratory found 2 mM NAC to reduce ROS levels, again measured by DCFH-DA in Human Umbilical Vein Endothelial Cells (HUVECs) stimulated by 50 μM AA. Conversely, when using DHE to detect superoxide specifically, 2 mM NAC caused a significant increase in superoxide levels compared to cells treated with only AA (You & Park, 2010). The discrepancies between the two ROS detection methods results are interesting. DCFH-DA detects many species under the umbrella term 'ROS', whilst DHE is supposedly specific to superoxide. However, beyond being a precursor for GSH synthesis, NAC contains a thiol group, and thus is able to scavenge various species including the myeloperoxidasederived oxidant HOCl, ${}^{\bullet}OH$, H_2O_2 and superoxide; with different rate constants (Benrahmoune et al., 2000; Aruoma et al., 1989). Studies have shown it has a similar efficacy to the endogenous antioxidant, SOD, enabling NAC to reduce superoxide levels at concentrations of 10⁻⁶molL⁻¹ or above (Luo *et al.*, 2009).

NAC may act as a pro-oxidant by accepting free electrons from the ETC. Thus if NAC acts as the electron acceptor, it may be the intermediate species that reduces molecular oxygen, forming the superoxide anion; which subsequently increases H_2O_2 levels. NAC has also been proposed to undergo auto-oxidation in the presence of metals such as iron in the following reaction:

 $NAC-SH + Me^n \rightarrow NAC-S^{\bullet} + Me^n$ (a) $NAC-S^{\bullet} + O_2 \rightarrow NAC-S-S-CAN + O_2^{\bullet-}$ (b)

NAC reacts with a transition metal (Me^n), where it becomes a thiol radical (a), which then proceeds to reduce molecular oxygen, forming the superoxide radical (b). Once the pathway is

initiated, other oxidant species can be generated including H_2O_2 and 'OH (Misra, 1974; Chan *et al.*, 2001).

In summary, the data presented highlight the inability of NAC to function as an antioxidant in both hTERT hASMC and hCASMC stimulated by mitochondrial inhibitors, AA and Rot. Other untargeted antioxidants may prove more successful including the naturally occurring polyphenol found in red wine, resveratrol, which has been found to prolong lifespan in lower organisms (Ungvari *et al.*, 2009). Resveratrol was also found to reduce MitoSOX[™] fluorescence to below baseline levels, following AA and Rot stimulation in a mouse epithelial cell line, highlighting the effectiveness of the antioxidant following mitochondrial perturbation (Babu *et al.*, 2015). The studies suggest that resveratrol may prove a better method of mitochondrial ROS modulation than NAC.

4.4.2 Mitochondria targeted ubiquinone; MitoQ

The use of TPP⁺ cations was introduced more than 3 decades ago to study the mitochondrial electropotential gradient (Murphy & Smith, 2007). Since then, many targeted antioxidants and fluorescent compounds have been synthesised targeting the mitochondrial compartment including the targeted ubiquinone, MitoQ. MitoQ accumulates several hundredfold in the mitochondria, where it becomes reduced to the antioxidant ubiquinol by complex II of the ETC. The compound prevents oxidative damage by being oxidised to the ubiquinone form, which is then recycled through reduction by complex II (Murphy & Smith, 2007). Previous studies have documented beneficial *in vitro* and *in vivo* effects in MitoQ treatment thus the effects in VSMC, specifically hTERT hASMC, were investigated (Jauslin *et al.*, 2003; Ng *et al.*, 2014).

Cell viability studies were conducted and found that exposure to MitoQ had no effect on total cell number; however ATP levels fell significantly at micromolar concentrations. MitoQ toxicity has been reported in the literature, with the authors claiming that for mammalian cells in culture, concentrations in the range 0.1-1 μ M generally avoid short-term toxicity; however this is largely dependent on cell density, cell type and incubation conditions (Murphy & Smith, 2007). A study has looked into the effects of MitoQ and other targeted antioxidants and their linker chains, on cellular bioenergetics using a series of experiments which measure oxygen

consumption rate (OCR) and extracellular acidification rate (ECAR) (Reily *et al.*, 2013). The results of this study suggest that 1 μ M MitoQ caused a steady decline in oxygen consumption over 2 hours and caused an initial transient increase in ECAR that returned back to basal levels within 2 hours. Upon injection of ATP synthase inhibitor, oligomycin, glycolysis increased minimally and was not further stimulated with AA treatment, nor treatment of the uncoupler, FCCP. These data suggest that MitoQ disrupted the ability of the respiratory chain to transfer electrons to oxygen (Reily *et al.*, 2013), and suggests a mechanism for the fall in ATP levels observed in hTERT hASMC at the higher concentrations of MitoQ.

Thus nanomolar concentrations were used for flow cytometry studies to investigate MitoQ modulation of AA-induced mitochondrial superoxide. Baseline levels of the hTERT hASMC were unaffected by MitoQ administration indicating no antioxidant activity. 100 nM MitoQ had no effect on mitochondrial ROS, whilst 500 nM increased the mitochondrial superoxide levels in AA-stimulated cells, indicating pro-oxidant activity (mechanism discussed below).

4.4.2.1 MitoQ as a pro-oxidant

Along with the reported successful studies, there are also studies reporting the lack of efficacy of MitoQ.

MitoQ has been found to possess pro-oxidant activity *in vitro*. One study assessed the protective effects of MitoQ in experimental acute pancreatitis and found that MitoQ caused a biphasic effect on ROS production. The authors used phorbol myristate acetate (PMA) to activate NAD(P)H oxidase in isolated polymorphonuclear leukocytes and the subsequent ROS generation was measured with the use of a chemiluminescent assay. At 8 minutes, MitoQ inhibited the PMA-induced ROS peak, whilst the higher 10 μ M MitoQ concentration reduced the levels down to the control level. The initial inhibition was suggested to be attributed to the ROS scavenging effects of MitoQ. However, at the later 40 minute time point, in the presence of MitoQ, there was increased PMA-induced ROS production, suggesting its effect is time-dependent. Their conclusions were that MitoQ would be deemed as inappropriate as a therapeutic strategy for acute pancreatitis (Huang *et al.*, 2014).

MitoQ contains a quinone group known to have redox cycling properties. Quinones react with flavine containing reductases to form semiquinone radicals that can reduce oxygen to the superoxide anion. A study investigating the effects of MitoQ on endothelial and mitochondrial ROS production found that commonly used concentrations of MitoQ produced large amounts

of superoxide, when exposed to isolated mitochondria and intact ECs (Doughan & Dikalov, 2007).

4.4.2.2 Mechanism of oxidant generation

MitoQ, like all ubiquinols, possesses the ability to deprotonate in water to form the ubiquinolate anion, which can facilitate the formation of superoxide from molecular oxygen (*c* & *d*), subsequently forming H_2O_2 (*e*) (James *et al.*, 2004; Murphy & Smith, 2007; Doughan & Dikalov, 2007):

 $UQH^{-} + O_{2} \rightarrow O_{2}^{-} + UQ^{-} + H^{+} (c)$ $UQ^{-} + O_{2} \rightarrow UQ + O_{2}^{-} (d)$ $2O_{2}^{-} + 2H^{+} \rightarrow H_{2}O_{2} + O_{2} (e)$

Ubiquinolate monoanion – UQH⁻; ubisemiquinone radical anion – UQ⁻⁻, and oxidised ubiquinone – UQ

However, for the equation above to occur (c), a pH level of 9 or above is required for the ubiquinolate anion to form; and as deprotonation is required for autoxidation, ubiquinol within a phospholipid bilayer is unlikely to be oxidised in this manner, except if the membrane is damaged and there has been an increased infiltration of water to the core of the membrane (James *et al.*, 2004).

Nevertheless, once oxidation of a quinol is initiated, a chain reaction of autoxidation can occur as superoxide can react with UQH2 (reduced ubiquinol) or UQ (f & g) to form further ubisemiquinone radicals, which will initiate further oxidation and through these reactions superoxide can act as chain carrier to propagate UQH2 autoxidation (James *et al.*, 2004):

 $UQH_2 + O_2^{-} \rightarrow UQ^{-} + H_2O_2 (f)$ $UQ + O_2^{-} \rightarrow UQ^{-} (g)$

Reduced ubiquinol – UQH₂

The addition of SOD however can protect against the autoxidation process by neutralising the superoxide anions, blocking the chain-propagation process (James *et al.*, 2004).

The results of MitoQ exposure potentiating the effects of AA observed in section **4.3.4** can be explained by its redox cycling capabilities (as explained above). By inhibiting complex III of the ETC, AA causes a blockade, creating large amounts of electrons between complex I and complex III. This electron accumulation provides MitoQ with electrons for reduction, forming the ubisemiquinone radical anion and subsequent superoxide anions as shown in the equations above.

Many studies have documented beneficial effects of MitoQ, however many of these studies do not directly measure oxidant species following MitoQ exposure; find no change in ROS levels, despite positive outcomes; or use heavily criticised experimental procedures of ROS detection within their studies (Jauslin *et al.*, 2003; Lowes *et al.*, 2008; Mercer *et al.*, 2012; Ng *et al.*, 2014). An example being, dichlorodihydrofluorescein diacetate (DCFH-DA), a probe that does not react directly with H_2O_2 therefore cannot be used as a direct measure of H_2O_2 ; and can be oxidised by several oxidant species including the 'OH, several species formed from ONOO⁻ decomposition, cytochrome c and redox-active metals (Kalyanaraman *et al.*, 2012). All of which lead to false positive results and improper data interpretation.

Whilst some studies use better ROS detection methods, they have found that MitoQ does not act in an antioxidant manner, but rather as a protective agent in preventing lipid peroxidation. Interaction of MitoQ with mitochondrial ROS within Rot-treated fibroblasts was investigated and it was found that whilst MitoQ did not decrease superoxide levels as measured using the DHE dye, it did prevent lipid peroxidation although the exact mechanism for this is unknown (Koopman *et al.*, 2005; Murphy & Smith, 2007).

In summary, further studies with MitoQ were discontinued due to the apparent narrow antioxidant/pro-oxidant window and the ability to induce superoxide generation as a redox cycler. Subsequent studies utilised MitoTEMPO as the targeted antioxidant for both the hTERT hASMC and the hCASMC.

4.4.3 The alternative mitochondrial targeted antioxidant; MitoTEMPO

Nitroxide compounds are low molecular weight, membrane permeable stable free radicals. They have been shown to possess antioxidant activity and protect against a variety of oxidants. Their proposed mechanism of action for antioxidant activity includes SOD or CAT mimicity, detoxification of secondary organic radicals or oxidation of reduced metals that would otherwise catalyse the formation of hydroxyl radicals (Suy *et al.*, 1998). TEMPO is an example of a nitroxide compound that has been shown to cause cell death in a breast cancer cell line making it a potential therapeutic strategy in cancer therapy (Suy *et al.*, 1998).

MitoTEMPO is a derivative of TEMPO, with a conjugated TPP⁺ molety causing accumulation within the mitochondria. It is believed to act as a mitochondria-targeted superoxide scavenger and therefore be defined as a SOD mimetic (Nazarewicz *et al.*, 2013). Previous studies within the group have used MitoTEMPO to prevent AngII-induced premature senescence (Mistry *et al.*, 2013). The results suggest that scavenging of mitochondrial superoxide plays a role in senescence inhibition, and thus the investigation of modulating mitochondrial ROS by MitoTEMPO exposure becomes an attractive therapeutic strategy for pathologies where cell senescence may be a contributing mechanism.

Cell viability studies found MitoTEMPO exposure was well tolerated at all concentrations examined for both hTERT hASMC and hCASMC. The lack of a change in ATP content indicates that the antioxidant does not affect the ETC at the concentrations investigated; suggesting MitoTEMPO has less toxic effects than MitoQ at similar concentrations in VSMC.

4.4.3.1 Antioxidant capabilities of MitoTEMPO in hTERT hASMC

Once cytotoxicity studies had been conducted, MitoTEMPO was used to attempt to modulate AA-induced mitochondrial superoxide generation within hTERT hASMC utilising MitoSOX[™] flow cytometry.

Within the hTERT hASMC, the initial treatments were carried out as co-treatments with the AA and MitoTEMPO loaded concurrently. The results of these co-incubations did not show an antioxidant effect of MitoTEMPO at all the concentrations tested. When a MitoTEMPO pre-treatment was conducted to allow loading of the antioxidant prior to AA stimulation, the results suggested possible reductions in mitochondrial superoxide, however this result was not statistically significant (**section 4.3.6**). To determine whether the results would ever truly be

statistically significant, a power calculation was performed. Power is defined as the probability of detecting an effect within a specified sample size if it is present, and is related to the Type II or β -error associated with hypothesis testing (Lovell & Omori, 2008). The results from the calculation indicated that based on the means and standard deviations between the two groups (AA versus MitoTEMPO + AA), for the data to reach statistical significance, a sample size of 19 is required. This means that the data will be statistically significant if 19 independent determinations were made; however the power calculation must be used with caution as it is merely an assumption and it depends on the standard deviations (Lovell & Omori, 2008).

As a sample size of 19 independent experiments could be deemed as large, the conclusion was drawn that MitoTEMPO was unable to modulate AA-induced mitochondrial superoxide generation in the cell model system studied. The lack of efficacy could be attributed to the lack of sensitivity of the MitoSOX[™] flow cytometry, which may be unable to detect small changes in superoxide levels. Alternatively, the hTERT hASMC may be resistant to mitochondrial ROS modulation and so the levels of ROS generated from complex III inhibition may be too great to be reduced by exogenous antioxidant activity. Additionally, the hTERT hASMC may be insensitive to mitochondrial changes as there have been reports of hTERT presence within the mitochondrial compartment as well as the nucleus (Santos et al., 2004). Studies conducted within virally-introduced telomerase-expressing fibroblasts found that hTERT potentiated the damaging effects of H_2O_2 on mtDNA; with greater levels of mtDNA damage consistently found in hTERT-overexpressing cells exposed to H_2O_2 compared with their normal counterparts (Santos et al., 2004). Telomerase-expressing fibroblasts required more of the divalent metal chelator, desferrioxamine, to prevent H₂O₂-mediated mtDNA damage. From these results, the authors believed that hTERT sensitised cells to oxidative mtDNA damage partly by modulation of metal homeostasis (Santos et al., 2004). An alternative study found hTERT to function as an endogenous inhibitor of the intrinsic apoptotic pathway (Massard et al., 2006). The results showed that hTERT suppression with the use of siRNA sensitised HeLa cells to apoptosis induction by DNA damaging agents as well as by ROS; with a requirement for the regulator of the intrinsic apoptotic pathway, Bax. The study suggests that inhibition of TERT expression by siRNA may constitute a valid strategy of chemosensitisation (Massard *et al.*, 2006). Both studies provide examples of the effects TERT can have within the mitochondria, and support the notion that the hTERT hASMC may not respond to ROS modulation in a manner that primary VSMC would.

4.4.3.2 Antioxidant capabilities of MitoTEMPO within hCASMC

Whilst flow cytometry was used for the hTERT hASMC, ROS modulation within hCASMC utilised the Amplex Red assay, as being primary cells, the hCASMC had many limitations including the limited number of cells available for repeat quantitative experiments using flow cytometry.

The Amplex Red assay revealed H_2O_2 levels were reduced following treatment with AA compared to the control. Co-incubation of AA with MitoTEMPO did not further reduce the H_2O_2 levels suggesting there was no additional antioxidant activity with the addition of the targeted antioxidant. H_2O_2 levels following Rot exposure appeared to also be reduced however this failed to reach statistical significance. However, the H_2O_2 levels observed in the Rot co-incubation with MitoTEMPO was significantly increased compared to Rot treatment alone. This suggested that MitoTEMPO potentiated the effects of Rot in the same manner that MitoQ potentiated the effects of AA in the hTERT hASMC. The possible mechanism for the observed rise in H_2O_2 by MitoTEMPO following Rot exposure could be the action of MitoTEMPO as a SOD mimetic, catalysing the conversion of superoxide to H_2O_2 .

4.4.3.3 Effect of Antimycin A on endogenous antioxidant gene expressiom

As the Amplex Red assay suggested H₂O₂ levels following AA exposure were significantly reduced compared to the control, it was proposed that complex III inhibition might either cause the upregulation of endogenous antioxidant genes or cause increased antioxidant enzyme activity. The studies began by investigating gene expression following treatment of the mitochondrial inhibitors and MitoTEMPO over the same time period in hCASMC (**section 4.3.11**). Real Time qPCR studies found no change in gene expression indicating that AA exposure did not cause gene expression changes in any of the 6 endogenous antioxidants investigated, nor did any of the other treatments. The lack of expression alterations suggests that the reduced H₂O₂ levels observed following AA treatment may be as a result of changes in enzyme activity. Thus, future studies should investigate the activity of the endogenous antioxidants investigated, in particular peroxiredoxin I and III.

The peroxiredoxin (PRX) antioxidants are a family of thiol-specific peroxidase enzymes that rely on thioredoxins as the hydrogen donor for the reduction of H_2O_2 , peroxynitrite and lipid peroxides (Sobotta *et al.*, 2015; Rhee *et al.*, 2012). There are 6 PRX isoforms, however I and III were selected for this study as III is exclusively localised in the mitochondria, playing a key role in mitochondrial defence against oxidants generated by the respiratory chain; whilst I is cytosolic. Both PRX I and PRX III are 2-cysteine (Cys) PRX isoforms, meaning that they exist as homodimers (Sobotta *et al.*, 2015). The PRX scavenge H_2O_2 as shown in **Figure 4.16** however the antioxidants also possess the ability to transfer the oxidation state of the Cys to a specific target protein through direct protein-protein interaction, as opposed to eliminating the oxidative equivalent. Due to their high rate constant, high abundance, and high affinity for H_2O_2 , PRX are thought to be prime candidates for scavenging H_2O_2 associated with redox signalling (Sobotta *et al.*, 2015; Rhee *et al.*, 2005).



Figure 4.16: H₂O₂ scavenging cycle of the 2-Cys Peroxiredoxin enzymes. *Adapted from* (Rhee *et al.*, 2005)

Upon oxidation with H₂O₂, one Cys residue of the PRX is oxidised to Cys-SOH, which then reacts with the neighbouring Cys-SH of the other subunit to form an intermolecular disulphide bond (PRX-S-S-PRX). Thioredoxin catalyses the conversion back to the reduced oxidant-scavenging state. PRX enzymes can also be inactivated during catalysis, where the Cys-SOH undergoes further oxidation to sulfinic acid Cys-SO₂H, a reaction that is reversible with the use of the sulfiredoxin enzyme (Sobotta *et al.*, 2015).

PRX enzymes can be inactivated during catalysis, forming the hyperoxidised form which increases in accordance with the accumulation of H_2O_2 (Baek *et al.*, 2012). Thus, the activity of

the PRX enzymes following AA stimulation in hCASMC could be measured with the use of nonreducing electrophoresis and Western blotting; enabling it to be possible to monitor and quantify the interconversion of individual PRXs in cultured cells (Cox *et al.*, 2010; Baek *et al.*, 2012). The working hypothesis is that mitochondrial ETC inhibition results in an upregulation of PRX activity, thus monitoring the PRX enzymes may provide answers to why AA exposure causes reduced H₂O₂ levels, and possibly why MitoTEMPO appears to potentiate the effects of Rot.

A protective assay was conducted to determine if a 4 hour exposure to AA offered any protective effects to hCASMC upon exogenous H_2O_2 treatment (**section 4.3.12**). The results showed the contrary; AA sensitised the cells to a reduction in cell number at the highest concentration of 200 μ M H_2O_2 , which may be indicative of cell death. The results suggest that the addition of exogenous H_2O_2 stimulates an alternative pathway that may not involve the activation or upregulation of the antioxidants investigated in section **4.3.11**. Intriguingly, studies have reported that endogenously produced and exogenously added H_2O_2 have differential selectivity for target signalling pathways. One study investigating the role of PRX II in platelet derived growth factor (PDGF) signalling found that exogenously added H_2O_2 did not have the identical signal messenger role that endogenous H_2O_2 had; by studying PDGF receptor phosphorylation as well as hyperoxidation of the PRX isoforms (Kang *et al.*, 2005). Thus the exogenously added H_2O_2 , in the protective assay, may not accurately reflect the reduction in H_2O_2 following AA stimulation shown by the Amplex Red assay, as H_2O_2 signalling is extremely complex and different pathways may stimulated for both.

AA was found to affect H_2O_2 levels in hCASMC in a manner not previously recorded in the literature, and the reasons for this observation are yet to be determined. Both micromolar and nanomolar (data not shown) concentrations of MitoTEMPO were investigated with the aim of reducing AA and Rot-induced ROS production, however no antioxidant activity was detected; though other studies have reported antioxidant activity (Nazarewicz *et al.*, 2012; Dikalova *et al.*, 2010).

4.4.4 Conclusion

Of the three antioxidants investigated, none were able to reduce AA and Rot-induced mitochondrial ROS in either hTERT hASMC or hCASMC. The antioxidants may have been reported to reduce ROS in some studies; however the different cell types, different stressors, and different procedures to measure oxidant species, may explain the inconsistencies between studies. A wider range of MitoTEMPO needs to be investigated, with other methods of ROS detection, including the use of the H₂O₂-specific fluorescent probe, HyPer. Alternative antioxidants may also be used including resveratrol which has been reported to reduce both AA and Rot-induced mitochondrial ROS (Babu *et al.*, 2015). Finally, alternative mitochondrial ROS inducers may be utilised such as MitoPQ, which has been found to induce superoxide generation within the mitochondrial compartment at low concentrations (Robb *et al.*, 2015).

CHAPTER FIVE

Angiotensin II derived Reactive Oxygen Species and Cellular Senescence

Chapter 5 Angiotensin II derived Reactive Oxygen Species and Cellular Senescence

5.1 Introduction

The octapeptide hormone AngII is a major effector of the renin-angiotensin- system (RAS) and affects many organs including the heart, kidney and brain. It primarily functions as a vasoconstrictor by inducing contraction in VSMC, ensuring ample blood flow; however it has other effects including cell growth, differentiation, pro-inflammatory cytokine induction and apoptosis (Zhang *et al.*, 2007). AngII exerts its effects on cells by binding to G-protein coupled plasma membrane receptors; AT1R (type 1) and AT2R (type 2).

As AngII is crucial in regulating the physiological and pathological responses in the cardiovascular system, it is not surprising to find that studies have implicated the hormone in the pathogenesis of CVD, with VSMCs as important targets for its actions (Kim & Iwao, 2000; Ruiz-Ortega *et al.*, 2001; Herbert *et al.*, 2008). Numerous studies on AngII have documented its participation across a range of renal and cardiovascular diseases including atherosclerosis, hypertension, coronary heart failure, vascular thickening and post-myocardial infarction (Kim & Iwao, 2000). Several factors such as sympathetic activity and haemodynamic factors such as shear stress likely contribute to the age associated increase in AngII within the arterial wall. The highest expression of AngII is observed in the thickened intima in ageing (Najjar *et al.*, 2005). Blocking the AT1R has been shown to positively impact the morbidity and mortality outcomes of CVD, thus highlighting the key pathological effects of AngII (Min *et al.*, 2009; Ferrario, 2006).

Recent *in vivo* and *in vitro* studies of its molecular and cellular effects have shed light onto the involvement of AngII and the RAS on CVD. These include promoting endothelial NO production and subsequent dysfunction; inducing VSMC production of cell adhesion molecules and chemokines, creating a local pro-atherogenic environment, and altering ECM protein synthesis thus enabling fibrosis and cardiac remodelling (Ferrario, 2006; Mehta & Griendling, 2007). Apart from these processes, AngII is also well known to promote the onset and progression of
premature senescence in the vasculature and the understanding of this process will be invaluable in the quest for therapeutic strategies.

5.1.1 Angiotensin II and VSMC Senescence

AngII is able to act upon various cells in the vasculature, however VSMC are its principal targets (Mehta & Griendling, 2007). Several *in vitro* studies have investigated the link between strong, sustained AngII stimulation and characteristics of senescence in human and animal vascular cells. AngII induced premature senescence in a p53/p21-dependent manner in VSMCs and induced the expression of p21, IL-1 β , IL-6 and MMP-2, in a mouse model of atherosclerosis (Kunieda *et al.*, 2006).

Studies within the Herbert group implicated the intracellular accumulation of ROS, particularly H_2O_2 , as a critical component of AngII signalling in VSMCs (Herbert *et al.*, 2008). The study found that AngII binding to AT1R resulted in SIPS in a concentration-dependent manner in VSMCs within a 24 hour exposure, accompanied by DNA damage, ROS production and increased p53 expression (Herbert *et al.*, 2008). A longer duration of AngII exposure was investigated and VSMC were treated with AngII on alternate days for 30 days. The results showed that AngII induced premature replicative senescence. Both the induction of SIPS and replicative senescence were prevented by co-incubation with CAT or E3174, an AT1R antagonist (Herbert *et al.*, 2008). The type of senescence induced appeared to be dependent on the length of exposure as well as the dose of AngII treatment. The authors proposed that low levels of DNA damage sustained over several population doublings would accelerate telomere attrition, thus promoting replicative senescence; sublethal but critical levels of DNA damage would rapidly induce p53-dependent SIPS; and severe and lethal DNA damage would result in apoptosis (Herbert *et al.*, 2008).

Other studies have shown that VSMC treated with AngII for a period of 14 days initially show a mitogenic response, followed by an enlarged and flattened morphology i.e. senescence (Min *et al.*, 2009).

Together, the results show that within VSMC, a predominant cellular component of atherosclerotic plaques, AngII promotes vascular senescence via AT1R-coupled signalling.

5.1.2 Blocking Angiotensin II induced senescence

Various methods of blocking AngII signalling in animal models *in vivo* have resulted in the improvement of cardiac mitochondria bioenergetics, mitochondria structure in cardiomyocytes and hepatocytes, endothelial function, intimal medial thickening and arterial stiffening and animal survival (Najjar *et al.*, 2005; de Cavanagh *et al.*, 2009). Studies blocking AngII signalling have included experimental approaches targeting the AngII receptor, targeting transcription factors capable of coordinating AngII signalling or alternatively targeting downstream aspects within the signalling pathway.

Starting from the plasma membrane, it has been reported that Angiotensin Converting Enzyme inhibitors (ACEIs) and Angiotensin II Receptor Blockers (ARBs) could protect against the effects of vascular ageing in animals and humans by attenuating oxidant damage to mitochondria (Min *et al.*, 2009). The Losartan metabolite E3174 is a potent specific AT1R receptor antagonist and its use on hVSMCs was found to inhibit the resulting DNA damage and the percentage of SA β -galactosidase positive cells following AngII treatment (Herbert *et al.*, 2008; Mistry *et al.*, 2013). Consistent with these findings, another ARB, Valsartan, also blocked the senescenceinducing effects of AngII (Min *et al.*, 2007; Min *et al.*, 2009; Ichiki *et al.*, 2012).

In relation to targeting downstream components, a study by Kunieda et al., (2006) showed that treatment of human VSMCs with AngII induced premature senescence and increased the production of proinflammatory cytokines through NF- κ B activation. Along with an increase in SA β -galactosidase activity, p53 transcriptional activity was increased in a concentration-dependent manner accompanied by elevated expression of p21 and p53 proteins. The results showed that p21 knockdown significantly inhibited NF- κ B activation by AngII treatment and the introduction of ROS scavenger, NAC, also effectively inhibited AngII-induced senescence. These results suggest a critical role for p21 and again ROS in the induction of AngII-induced senescence (Kunieda *et al.*, 2006).

Finally, antioxidants have also been used to prevent AngII-induced senescence. Resveratrol is known to have beneficial effects on cardiovascular diseases, diabetes and cancer (Ichiki *et al.*, 2012; Szkudelski & Szkudelska, 2015). The naturally occurring polyphenol is believed to

activate the protein coding gene *SIRT1*, but also possesses anti-oxidative, anti-inflammatory and anti-apoptotic effects (Ungvari *et al.*, 2009; Ichiki *et al.*, 2012). Their findings indicated that resveratrol treatment reduced the number of senescent-positive cells induced by AngII in a concentration dependent manner and suppressed expression of both p53 and 21. The mechanism for the action of resveratrol was unclear, however, as the authors could not detect an effect of the polyphenol on ROS production (Ichiki *et al.*, 2012).

5.1.2.1 Mitochondrial superoxide and its involvement in AngII-induced senescence

As studies with non-targeted antioxidants have proven successful in inhibiting AngII induced senescence, it is clear that ROS play a crucial role in the AngII signalling process. Previously, AngII has been found to stimulate mitochondrial ROS, so the use of a targeted antioxidant scavenging mitochondrial superoxide may be detrimental to the downstream processes of AngII signalling (de Cavanagh *et al.*, 2009).

Studies within this research group investigated the action of mitochondrial targeted nitroxide antioxidant, MitoTEMPO, on AngII stress-induced hVSMC senescence. A pre-incubation with 25 nM MitoTEMPO was conducted, followed by a 24 hour exposure to 10 nM AngII to cause SIPS. Cells exposed to AngII alone showed a ~1.5 fold to 2 fold increase in senescence-positive cells, whilst the presence of MitoTEMPO completely prevented AngII-induced senescence (Mistry *et al.*, 2013).

Following AnglI stimulation, crosstalk between the NOX enzymes and the mitochondria has been proposed (Dikalov, 2011) and this suggests that mitochondrial superoxide is a major mediator in the senescence signalling pathway upon AnglI stimulation (Mistry, 2009; Mistry *et al.*, 2013). Within the previous study however (Mistry *et al.*, 2013), superoxide levels were not investigated so it is unclear whether mitochondrial superoxide levels were reduced in the cells pre-treated with MitoTEMPO compared with those only exposed to AnglI (Mistry *et al.*, 2013). This is important as the MitoTEMPO may be scavenging superoxide elsewhere in the cell, as opposed to the mitochondrial compartment specifically.

5.2 Aims & Objectives

This chapter investigates the induction and detection of cytosolic and mitochondrial ROS in hTERT hASMC and hCASMC following AngII treatment. Subsequently, the propensity for AngII to induce premature senescence (SIPS) in hTERT hASMC and hCASMC will be investigated.

5.3 Results

5.3.1 Effect of Angiotensin II on superoxide and H₂O₂ production and induction of SIPS hTERT hASMC

Previous studies investigating the ability of AngII to induce cellular senescence have found an effect at concentrations as low as 10-100 nM (Herbert *et al.*, 2008; Kunieda *et al.*, 2006; Min *et al.*, 2009). Some studies insist on replacing the media of the cells with media containing a reduced level of serum or serum free media prior to AngII exposure to render the cells in a state of quiescence where the cells become synchronised in the G0 phase of the cell cycle (Kunieda *et al.*, 2006; Herbert *et al.*, 2008; Min *et al.*, 2007; Touyz & Schiffrin, 1997a). This reduced serum prevents downregulation of AT1R gene expression by growth factor receptor activation (Mistry, 2009; Nickenig & Murphy, 1994). The experiments conducted in both the hTERT hASMC and hCASMC were thus subjected to serum starvation prior to AngII stimulation.

To determine the concentration of AngII that hTERT hASMC could withstand AngII, a CCK-8 cell viability assay was used. hTERT hASMC were exposed to reduced serum media for 24 hours. The cells were then treated with a range of AngII concentrations for 24 hours. hTERT hASMC tolerated AngII at concentrations up to 1 μ M (**Figure 5.1**). 10 nM and 100 nM were selected as the concentrations to proceed with as they had been used to induce senescence previously (Herbert *et al.*, 2008).



Figure 5.1: Effects of Angiotensin II on hTERT hASMC viability

Cells were treated with AngII for 24 hours following serum starvation. Cell number was measured using the CCK-8 Cell Viability Assay. 1 mM H_2O_2 was used as a positive control. Data presented as a mean+SEM; n=8 (****p<0.0001 compared to the control).

5.3.1.1 Effect of AngII on superoxide production in hCASMC

AngII is known to effectively activate NAD(P)H oxidase activity by AT1R signalling, where cytosolic subunits translocate to the membrane to form an active NAD(P)H oxidase (Zhang *et al.*, 2007). Evidence has also shown AngII stimulation upregulates mRNA and protein levels of all major oxidase subunits including the catalytic and cytosolic components (Zhang *et al.*, 2007). This activation causes sustained generation of superoxide, subsequently followed by increased intracellular production of H₂O₂. Evidence of mitochondrial crosstalk has also been provided (Zhang *et al.*, 2007; Kimura *et al.*, 2005; Doughan *et al.*, 2008). Thus based on the evidence provided, the ability of AngII to induce superoxide formation in hTERT hASMC, both in the cytosol and within the mitochondrial compartment was investigated using both DHE and MitoSox[™] flow cytometry respectively.

The hTERT hASMC were subjected to 24 hours of serum starvation prior to treatment with AngII. Neither concentration of AngII induced superoxide within either compartment compared to the control during the course of the treatment (**Figure 5.2A and B**). The positive control, AA, caused a ~4 fold increase in mitochondrial superoxide levels measured by MitoSOXTM; however caused a lesser increase in cytosolic superoxide levels measured by DHE.

Extremely high concentrations of AngII were investigated for a longer time course. 1 μ M and 10 μ M again failed to induce cytosolic superoxide levels compared to the control (**Figure 5.2C**). According to previous literature, at such high concentrations, AngII should be causing NAD(P)H oxidase activation, a substantial amount of superoxide and hydrogen peroxide generation and possibly apoptotic cell death, however the results suggest otherwise.

The positive controls for these experiments were not initially serum starved prior to treatment because cells subjected to AA treatment following serum starvation were unable to cope with the toxicity and thus 100% cell death was observed.



Figure 5.2: Angiotensin II failed to increase both mitochondrial and cytosolic superoxide in hTERT hASMC

Cells were serum starved for 24 hours then treated with AngII for 4hours. Mitochondrial superoxide levels were detected by flow cytometry using MitoSox[™] (**A**) and cytosolic superoxide levels were detected by flow cytometry using the DHE dye (**B**). Higher AngII concentrations were applied for 24 hours (**C**). Cells were treated with 100 µM AA or 50 µM tBHP as positive controls and were not serum starved prior to treatment, however the control was serum starved. Data presented as mean+SEM; n=3 (**p<0.01 compared to the control).

5.3.1.2 Investigation of SIPS induction in hTERT hASMC by Angiotensin II

Despite the inability to detect superoxide, the ability to cause SIPS was investigated as there may be a possible alternative mechanism for senescence induction that does not involve oxidant generation; alternatively the method(s) for oxidant detection may lack sensitivity.

Previous studies conducted within the group investigated the effects of AngII and found a concentration-dependent increase in cell senescence with a maximal effect at 10 nM which induced a 3 fold increase in SA β -galactosidase positive hVSMC (Mistry, 2009). The experiment was replicated in hTERT hASMC where the cells were subjected to a 24 hour period of serum starvation followed by AngII treatment for 24 hours. The cells were then allowed to recover in full serum containing media to allow the cells to resume proliferation. Allowing the cells to recover following treatment is an important aspect in measuring SIPS *in vitro* as judgments can be made that are relatively independent from purely adaptive responses (Mistry, 2009; Toussaint *et al.*, 2000). Following recovery, the hTERT hASMC were stained for SA β -galactosidase and neither concentration of AngII altered the number of SA β -galactosidase positive cells compared to the control (**Figure 5.3**). tBHP, however, was able to induce a 2 fold increase in hTERT hASMC, showing that although the cells were not susceptible to SIPS by AngII, they were still susceptible to SIPS by tBHP.



Figure 5.3: Stress Induced Premature Senescence is not induced with Angiotensin II

treatment

Cells were subjected to serum starvation for 24 hours then treated with AngII for 24 hours. Following treatment, the cells were then allowed to recover for 48 hours before a SA β -galactosidase stain was carried out. 50 μ M tBHP was used as a positive control. Data presented as mean+SEM; n=3 (*p<0.05 compared to the control).

5.3.1.3 Investigating the expression of the AngII Receptors in hTERT hASMC

As superoxide levels within hTERT hASMC were not stimulated with AngII treatment, nor was premature senescence induced following a 24 hour exposure, it was proposed that the lack of response to AngII may be due to the cells not expressing the AT1R or AT2R receptors. Although the cells had always been serum starved prior to treatment to prevent downregulation of the AngII receptors (Nickenig & Murphy, 1994), the possible absence of receptors on the hTERT hASMC was considered as the cells had undergone numerous passages and freeze-thaw cycles and may possibly not contain the markers of normal smooth muscle cells. In a bid to determine if the AT1R and AT2R receptors were expressed, qPCR studies were conducted to determine gene expression.

Probe investigation

The initial steps involved investigating the efficiency of the Taqman[®] gene expression assay probes. The AT1R and AT2R Taqman[®] gene expression assay probes, AGTR1 and AGTR2 respectively, were tested for efficiency against a housekeeper, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The hTERT hASMC were serum starved for 24 hours or 48 hours, the RNA was extracted and cDNA produced through reverse transcription. The resulting cDNA was then pooled with cDNA from hTERT hASMC that had not been subjected to serum starvation. With the pooled cDNA, a standard curve was constructed as shown in **Figure 5.4**. The AT2R could not be detected which could be perceived as a lack of gene expression from the hTERT hASMC or the levels were too low to be detected. The efficiencies of the AGTR1 and GAPDH Taqman® assay probes were found to be within 10% of each other (92.0% and 90.6% respectively). Beyond being unable to detect AT2R in the pooled cDNA, the suitability of housekeeper GAPDH was questioned. Studies have found GAPDH to be an unsuitable housekeeper gene in *in vitro* experiments involving serum alterations. A relationship between serum-stimulation and expression of GAPDH was statistically significant, thus causing experimental results to appear statistically significant (Schmittgen & Zakrajsek, 2000).



Figure 5.4: Investigating Taqman[®] probe efficiency in hTERT hASMC

RNA was extracted from cells grown in complete media and cells that had been subjected to serum starvation. cDNA was reverse transcribed and aliquots of cDNA from all the samples were pooled. A standard curve was conducted from the pooled cDNA. qPCR was carried out using Taqman[®] gene expression probes. The efficiency of AGTR1 was found to be 92.0% and GAPDH as 90.6%, whilst AGTR2 was undetected.

Efficiency was calculated as: 10^(-1/gradient)-1 (Applied Biosystems, 2008).

Due to the questionable suitability of GAPDH as a housekeeping gene for studies involving serum manipulation, B2M and TBP were investigated. The expression of B2M has been found to be unaffected by serum and TBP was also used so that an average of the two values could be taken when comparative CT analyses were conducted (Schmittgen & Zakrajsek, 2000).

Again, pooled cDNA from different time points of serum starved hTERT hASMC and control hTERT hASMC were used to construct a standard curve for the efficiency of all the probes to be calculated (Figure 5.5). AT2R was again undetected.

Efficiencies for the probes were calculated to be 97.0% for AGTR1, 87.1% for B2M and 88.2% for TBP. As the efficiencies were all within 10%, the comparative quantitation analysis was undertaken with both B2M and TBP as the housekeeping genes. The standard curve also highlighted the dynamic range of the assay and as a result, 10ng of cDNA was used in the comparative quantitation assay.



Figure 5.5: Investigating Taqman[®] probe efficiency in hTERT hASMC

A, The Amplification Plot of the Standard curve. cDNA from all the hTERT hASMC samples was pooled and a standard curve was constructed. AGTR1 (blue), TBP (yellow) and B2M (pink). AGTR2 was below the limit of detection. **B**, Standard Curve. RNA was extracted from control, untreated hTERT hASMC and cells that had been subjected to serum starvation. cDNA was reverse transcribed and the cDNA from all the samples pooled. A standard curve was conducted from the pooled cDNA. qPCR was carried out using Taqman[®] gene expression probes. The efficiency of AGTR1 was found to be 97.0%, B2M as 87.1% and TBP as 88.2%, whilst AGTR2 was undetected.

Efficiency was calculated as: 10^(-1/gradient)-1 (Applied Biosystems, 2008).

Investigating receptor gene expression

Once the housekeepers had been selected and the efficiencies calculated, the comparative CT method of relative quantification assay was adopted (Applied Biosystems, 2008). Real Time PCR was carried out on the hTERT hASMC samples to show the differences in AT1R gene expression following 24 hours or 48 hours of serum starvation; expressed in fold change when compared to the unstarved control (**Figure 5.6**). The results show there is no difference in expression between cells subjected to serum starvation and cells that had not been. The detection of *AT1R* gene expression in the hTERT hASMC suggests that the lack of superoxide production and the lack of SIPS induction following AngII treatment may be due to the batch of AngII used as opposed to the cells being unable to respond to treatment. Therefore, a new solution of the hormone was purchased and its effect on another cell type was investigated.



Serum Starvation



Cells were serum starved for 24 or 48 hours, RNA was extracted and cDNA was subsequently transcribed. Real Time qPCR was used to determine AGTR1 expression. Data presented as mean+SEM; n=3

5.3.2 Effects of Angiotensin II on H₂O₂ production and induction of SIPS in hCASMC

As studies with AngII had been unsuccessful in the hTERT hASMC, a new vial of AngII was obtained and prepared as advised (Personal communication: Dr Salil Srivastava, Kings College London).

Once prepared, the AngII was used to treat hCASMC that had been serum starved for 24 hours in a concentration-dependent manner to determine if the AngII had any cytotoxic effects. A CCK-8 cell viability assay was used to determine cell number following treatment with AngII for 6 hours. AngII caused no significant changes in cell number (**Figure 5.7A**). Previous studies have found AngII concentrations as low as 200 nM cause cell death within hCASMC (Srivastava *et al.*, 2015); however none of the concentrations within this study reached statistical significance. ATP levels were also unchanged by AngII treatment (**Figure 5.7B**). The results suggest that any concentration of AngII could be used for subsequent experiments or alternatively, the cells simply did not respond to AngII.



Angiotensin II (μM)



Figure 5.7: Angiotensin II does not affect hCASMC viability

Cells were subjected to serum starvation then treated with AngII for 6 hours. Cell number was measured using the CCK-8 cell viability assay (**A**) and ATP content was measured using the luminescence assay (**B**). 1 mM H_2O_2 was used as a positive control. Data presented as a mean+SEM; n=4 (**A**) and n=3 (**B**) (**p<0.01, ****p<0.0001 compared to the control).

5.3.2.1 Induction of H₂O₂ by Angiotensin II in hCASMC

Once it was established that the AngII caused no adverse cytotoxic effects on the hCASMC, the ability to induce ROS generation was tested. Superoxide detection using flow cytometry could not be utilised due to the limited availability of hCASMC so the Amplex Red assay was used to detect extracellular H_2O_2 .

Following 24 hours of serum starvation, hCASMC were treated with AngII for 6 hours. Results from the Amplex Red assay revealed that within 6 hours, the lower concentrations of AngII failed to generate any detectable H_2O_2 , however 100 μ M AngII caused a significant increase in H_2O_2 compared to the control (**Figure 5.8A**). A longer 72 hour treatment time was investigated with the thought that long term administration of AngII in hCASMC may cause oxidative stress, which may be a better measure. The results show that at 72 hours, the lower concentrations failed to cause any changes in H_2O_2 levels compared to the control however interestingly, the 10 μ M caused a significant reduction in H_2O_2 similar to the mitochondrial inhibitors AA and Rot (**Figure 5.8B**). These data may not be representative of the effect of AngII as it may not be suitable to detect the smaller more subtle changes in H_2O_2 or alternatively any changes in H_2O_2 may have been missed by the end of the 72 hour treatment time.

The fluorescence levels in the control cells following serum starvation varied with the length of exposure to reduced serum media. The control cells subjected to a total of 96 hours of serum starvation (**Figure 5.8B**) exhibited ~3 fold greater fluorescence than the control cells subjected to a total of 30 hours of serum starvation prior to H_2O_2 measurement (**Figure 5.8A**). The results indicate the increased generation of H_2O_2 following serum starvation.







Figure 5.8: Detection of Angiotensin II induced H₂O₂ in hCASMC

Cells were subjected to serum starvation for 24 hours by replacing fresh media with reduced serum media. Following starvation, cells were treated with AngII for 6 hours (**A**) or 72 hours (**B**). Extracellular H_2O_2 levels were measured using the Amplex Red assay. 50 μ M AA and 50 μ M Rot were used as controls in the 72 hour experiments, with the cells being maintained in complete media containing serum; and 50 μ M menadione was used as a positive control for the 6 hour experiments. Data are presented as mean+SEM; n=3 (*p<0.05, **p<0.01, ****p<0.0001 compared to the control).

5.3.2.2 Detection of AngII-induced H₂O₂ using pHyPer vectors

As the Amplex Red assay was unable to detect any changes in H_2O_2 following stimulation with the lower concentrations of AngII, an alternative method was utilised. The HyPer fluorescent vectors, pHyPer-dMito and pHyPer-cyto, were used to detect alterations in mitochondrial and cytoplasmic H_2O_2 respectively. As the vectors are insensitive to other oxidants, it provides assurance that any alterations in fluorescence can be attributed to H_2O_2 only (Rhee *et al.*, 2010; Lukyanov & Belousov, 2014).

After transfection with the HyPer probes, the cells were allowed to recover for 48 hours, and then the cells with the AngII treatments were serum starved for 24 hours. The H_2O_2 positive control was not subjected to serum starvation. The cells were then treated with AngII for 6 hours, and then visualised on the EVOS fluorescence microscope.

In the transfections using the pHyPer-dMito vector, the cells treated with AngII demonstrated increased mitochondrial H_2O_2 levels, as the fluorescence within these cells was increased compared to the untreated control (**Figure 5.9**). The results suggest that the nanomolar concentrations of AngII increase H_2O_2 levels more than the higher micromolar concentrations, however the data is not quantitative so no statistical significance can be determined. In the transfections using the pHyPer-cyto vector, AngII treatment appeared to have no effect on cytosolic H_2O_2 levels as there was no noticeable difference in the fluorescence of the cells treated with AngII and the untreated control (**Figure 5.10**).





Following transfection with the pHyPer-dMito vector, cells were subjected to serum starvation then treated with AngII for 6 hours. $100 \ \mu M \ H_2O_2$ was used as the positive control. Images taken using the EVOS[®] fluorescent microscope (magnification: x200). (Figure also available on CD – Appendix 2).



Figure 5.10: Effect of Angiotensin II on cytosolic H₂O₂

Following transfection with the pHyPer-cyto vector, cells were subjected to serum starvation then treated with AngII for 6 hours. $100 \ \mu M \ H_2O_2$ was used as the positive control. Images taken using the EVOS[®] fluorescent microscope (magnification: x200). (Figure also available on CD – Appendix 2).

5.3.2.3 Effect of Angiotensin II on senescence induction in hCASMC

Given that there was some evidence that mitochondrial H_2O_2 was increased by nanomolar concentrations of AngII, the ability to induce SIPS was investigated. hCASMC were subjected to serum starvation for 24 hours then a 24 hour or 72 hour AngII treatment with a 48 hour period of recovery as directed by previous studies in the group (Mistry, 2009). The cells were also subjected to successive 6 hour AngII exposures over 3days, followed by a 48 hour period of recovery. The SA β -galactosidase assay was used to measure senescence by counting cells positive for SA β -galactosidase. The results found that AnglI exposure in all 3 strategies failed to induce premature in hCASMC (Table 5.1).

	24 hour SIPS (Mean fold change compared to control)	72 hour SIPS (Mean fold change compared to control)	6 hour 3 day successive SIPS (Mean fold change compared to control)
Control	1.00	1.00	1.00
10 nM Angli	0.5 ± 0.17	0.8 ± 0.12	0.73 ± 0.11
100 nM Angli	0.70 ± 0.15	1.77 ± 0.58	1.30 ± 0.36
500 nM Angli	0.80 ± 0.12	2.07 ± 0.66	1.65 ± 0.53
1 μM Angli	0.97 ± 0.12	1.40 ± 0.76	1.78 ± 0.48
Rotenone positive control	1.40 ± 0.32	2.16 ± 0.20	3.62 ± 0.47 **

Table 5.1: Angiotensin II does not induce premature senescence

Cells were subjected to serum starvation for 24 hours then treated with AngII for 24 hours, 72 hours or a successive 6 hour treatment over 3 days. Following treatment the cells were allowed to recover for 48 hours prior to SA β -galactosidase staining. Cells exposed to 50 μ M Rot were used as a positive control. Data presented as mean±SEM; n=3 (**p<0.01 compared to the control).

5.4 Discussion

The octapeptide hormone AngII, known to function primarily as a vasoconstrictor, appears to also play a critical role in regulating many of the signals that govern vascular ageing and atherogenesis (Kunieda *et al.*, 2006; Zhang *et al.*, 2007). Thus, AngII has been proposed to contribute to the pathogenicity of atherosclerosis and inhibition of AngII activity has been shown to markedly delay age-associated increases in collagen content and intimal medial thickening in rodents (Kunieda *et al.*, 2006; Najjar *et al.*, 2005). Whilst the mechanisms of the detrimental effects of AngII on the cardiovascular system are not fully understood, AngII-induced vascular senescence is believed to contribute to the process.

Studies have found AngII exposure of VSMCs induced premature senescence, with increased ROS generation, DNA damage, and increased expression of p53 and p21 (Kunieda *et al.*, 2006; Herbert *et al.*, 2008; Min *et al.*, 2009). AngII signalling has been targeted as a therapeutic strategy in hypertension. Moreover, ACEIs and ARBs have been used to block the senescence inducing effects of AngII, as have antioxidants such as resveratrol through unknown mechanisms (Ichiki *et al.*, 2012; Min *et al.*, 2009; Mistry *et al.*, 2013).

Previous studies within the group induced senescence in primary VSMCs and immortalised hTERT hASMCs upon AngII exposure at nanomolar concentration (Herbert *et al.*, 2008). Treatment with the mitochondria-targeted antioxidant, MitoTEMPO, was able to reduce this effect implicating mitochondrial ROS in the signalling pathway (Mistry *et al.*, 2013). The aim of this chapter was to investigate the ability of AngII to induce oxidant species generation within hTERT hASMC and hCASMC; and subsequently, to induce premature senescence upon AngII exposure.

5.4.1 Effect of Angiotensin II on hTERT hASMC

The AngII hormone has been found to be cytotoxic in a concentration and time-dependent manner in various cell types (Bascands *et al.*, 2001; Ruiz *et al.*, 2007; Ravassa *et al.*, 2000). Low AngII concentrations (10 nM) caused an increase in expression of the pro-apoptotic protein, Bax- α within cardiomyocytes derived from both hypertensive and normotensive rat models (Ravassa *et al.*, 2000). Exposure to higher concentrations (1 μ M) within rat aortic VSMCs cultures caused a significant increase in apoptosis at 24 hours and 48 hours; increased expression of the pro-apoptotic protein, Bcl- x_s ; and increased DNA fragmentation (Ruiz *et al.*, 2007). The results signify the ability of a range of AngII concentrations to cause apoptotic cell death; thus the initial studies investigated the cell viability of the hTERT hASMC following AngII exposure to determine concentrations that were not cytotoxic. The study found that the hTERT hASMC were resistant to AngII at concentrations as high as 1 μM.

The activation of redox-dependent signalling cascades is critical to underlying processes involved in various CVD. AngII has been observed to exert its effect by inducing the generation of ROS, mainly via the activation of NAD(P)H oxidase enzymes (Zhang *et al.*, 2007). AngII treatment has been shown to increase NAD(P)H oxidase activity in a time-dependent manner; induce NAD(P)H-mediated superoxide production as detected by chemiluminescence; and increase cytosolic superoxide levels as detected by DHE fluorescence (Kimura *et al.*, 2005; Herbert *et al.*, 2008; Min *et al.*, 2009; Kim *et al.*, 2011).

Whilst most investigations have focused on NAD(P)H oxidase as key mediators, increasing evidence suggests that the mitochondrial ROS play a role in AngII signalling. KATP channels have been identified in the inner membrane of the mitochondria, and a study revealed that Angll stimulated the generation of mitochondrial ROS through the opening of K_{ATP} channels in VSMC (Kimura et al., 2005; Zhang et al., 2007). Another study showed that treatment with a mitochondrial KATP blocker prevented the increase in mitochondrial H₂O₂ induced by AngII. The authors proposed a positive feedback loop between NAD(P)H oxidase and the mitochondria (Doughan et al., 2008). Studies have also identified AnglI in the mitochondria of mouse hepatocytes, heart myocytes and brain neurons (Abadir et al., 2011). The study found that Angll was co-localised with mitochondrial AT2R in a loose interaction and Angll was lost upon washing of isolated mitochondria (Abadir et al., 2011). Finally, as previously mentioned, studies performed by this group found AngII exposure increased MitoSOX™ fluorescence and Angll-induced senescence was blocked by the SOD2 mimetic, MitoTEMPO, suggesting the scavenging of mitochondrial superoxide plays a critical role in the signalling pathway (Herbert et al., 2008; Mistry et al., 2013). Collectively, these studies provide evidence of crosstalk between AnglI cell membrane signalling and the mitochondria.

Flow cytometry studies were used here to investigate changes in both mitochondrial and cytosolic superoxide, as AngII has been shown to increase both. However, AngII failed to induce superoxide levels in both cellular compartments; even when higher concentrations and longer time courses were utilised. It is worth noting that the AngII-induced ROS levels produced may be too low to be detected by the flow cytometry method utilised, so a more

sensitive method may have detected smaller changes. Additionally, measurement of H_2O_2 would shed some light on the ROS levels following AngII exposure as any superoxide anions generated by the NAD(P)H oxidases may have undergone spontaneous dismutation, or via SOD, to produce H_2O_2 .

The ability to cause premature senescence was investigated and AngII exposure caused no increase in senescence-positive cells; despite previous studies showing ROS generation and SIPS induction with the same concentrations (Herbert *et al.*, 2008; Kim *et al.*, 2011; Min *et al.*, 2009). The ability to induce senescence within the hTERT hASMC may be dependent on the length of exposure as a 34 day exposure to AngII was unable to induce replicative senescence however a single 24 hour treatment was able to cause SIPS in the immortalised cells (Mistry, 2009).

As AnglI treatment in the hTERT hASMC caused no apparent increase in oxidant species, nor did it induce premature senescence, the expression of AnglI receptors on the cell membrane was questioned. Previous work within the group has shown that hTERT hASMC undergo premature senescence when exposed to AnglI, indicating the presence of the receptor (Herbert *et al.*, 2008), however the freeze-thaw cycles and continuous passage of the cells may have had a detrimental effect on receptor expression.

To determine whether the observed effects were due to the absence of the AngII receptors, gene expression studies were performed. Standard curve analysis found that the gene for AT2R, *AGTR2*, was not detected as it may possibly have been below the limit of detection or it may simply not be expressed. Studies have found that whilst AT2R is highly expressed in foetal tissue (mostly in the foetal heart and aorta), its expression rapidly declines after birth, suggesting its importance may be in foetal development (Durante *et al.*, 2012; Mehta & Griendling, 2007). However, AT2R expression has been found to be upregulated in several pathological conditions including heart failure and vascular injury, and some studies have shown that AT2R antagonises AT1R by inhibiting its signalling pathway (Durante *et al.*, 2012; Mehta & Griendling, 2007). The lack of *AGTR2* detection may be simply due to a lack of gene expression or the levels may have been below the limit of detection.

In contrast to AT2R, AT1R mRNA was detected in hTERT cells. Subsequently, the relative quantification assay investigated the effects of serum starvation on AGTR1 expression. Previous investigations have discovered that starving the cells of serum prior to AngII

treatment prevents the downregulation of both the AT1R and AT2R, and studies investigating the effects of AngII exposure have adopted this in the methodology (Nickenig & Murphy, 1994; Kunieda *et al.*, 2006; Herbert *et al.*, 2008; Min *et al.*, 2009). The results showed that serum starvation for up to 48hours had no effect on gene expression of *AGTR1*. This is in contrast with an earlier study that found growth factor receptor activation markedly attenuated AT1R gene expression in cultured VSMC, as measured by the disappearance of mRNA and cell membrane-associated receptors, as well as the attenuation of the rate of AT1R gene transcription (Nickenig & Murphy, 1994).

As the gene expression results in the hTERT hASMC suggest serum starvation caused no alterations in receptor expression, the conclusion was drawn that the cells had either become unresponsive to Angll exposure; or that there were problems with the batch of Angll. Expression of the receptor protein on the cell surface may be useful in determining if the receptors are actually present on the cell membrane, as they may be continuously internalised. The presence of the receptor could be detected by investigating downstream signalling such as activation of the NAD(P)H oxidase enzymes by measuring luminescence using cell homogenates (Min *et al.*, 2007), or detecting calcium influx by measure of intracellular calcium levels using the fluorescent Fura-2 calcium indicator (Touyz & Schiffrin, 1997b; Ohtsu *et al.*, 2008). Alternatively, Western blots could be utilised to detect the receptor in the cytosol and the membrane using subcellular fractionation (Gao *et al.*, 2012).

5.4.2 Effect of Angiotensin II on hCASMC

As the studies with the hTERT hASMC proved inconclusive, the investigations were continued in primary hCASMC. A new stock of AngII was purchased and prepared as advised (Personal communication: Dr Salil Srivastava, Kings College London).

Cell viability studies were performed and the hCASMC appeared to be resistant to the toxic effects of AngII at concentrations as high as 100 μ M. This was at odds with studies that found 200nM caused apoptosis in the same cell type (Srivastava *et al.*, 2015). As AngII was well tolerated by hCASMC, the ability to generate ROS following exposure was investigated using the Amplex Red assay of extracellular H₂O₂. The shorter time course indicated a significant increase in H₂O₂ at 100 μ M, however the longer treatment of 72 hours showed a decrease in H₂O₂ at 10 μ M. The levels of AngII-induced H₂O₂ appear to differ based on the length of exposure.

It is worth noting that the H_2O_2 level in control cells after a total of 96 hours of serum starvation was ~3 fold higher than the fluorescence levels in the control cells that had been serum starved for a total of 32 hours. This baseline increase in H_2O_2 has been observed in other studies (Pandey *et al.*, 2003; Basu *et al.*, 2012; White *et al.*, 2014a). Experiments performed in the RGC-5 retinal neuronal cell line, found ~2 fold increases in ROS generation within 24 hours; which increased to ~5 fold by 72 hours of starvation (Basu *et al.*, 2012). In primary human monocytes, serum starvation caused a 2 fold increase in oxidative stress, as measured by the oxidative stress indicator, CellROX green; and the superoxide indicator DHE (White *et al.*, 2014a). Such studies demonstrate the effect serum starvation has on resting cells and support the increase observed in the hCASMC upon exposure to low serum.

As the Amplex Red assay results showed no increases in H₂O₂ levels following treatment with the lower concentrations of AngII, the fully genetically encoded fluorescent indicators, HyPer, were established and utilised. The application of HyPer probes in the detection of oxidants caused by AngII has not been described previously. The HyPer vectors are mammalian expression vectors encoding a fluorescent H₂O₂ sensor. Two vectors were utilised for the study; cytoplasmic pHyPer-cyto, to detect fast changes in H_2O_2 concentration in the cytoplasm, whilst the pHyPer-dMito contained a duplicated mitochondrial targeting sequence to enable detection of H_2O_2 in mitochondria. The vectors were used due to their insensitivity to other oxidants such as superoxide and nitric oxide and the bacterial nature ensures that it is less toxic upon overexpression and also subjected to less post-translational modifications that may lead to false-positive results (Rhee et al., 2010; Lukyanov & Belousov, 2014). Using HyPer, no alterations in cytosolic H_2O_2 with a 6 hour exposure to Angll were observed; however, Angll appeared to increase mitochondrial H_2O_2 levels. As the results are preliminary, the data is expressed in a qualitative manner and thus no statistical significance can be obtained. Subsequent studies would follow the fluorescent changes of selected cells in real-time, using an imaging method such as confocal microscopy with a 5% CO₂/37°C chamber that maintains the cells in optimal growth conditions. Angll may cause transient increases in H₂O₂ initially which may have been missed with the 6hour course, and thus was undetected by the Amplex Red assay. This would be amenable to further investigation with the HyPer vectors.

AngII is known to induce premature senescence in VSMC; however, no published study has investigated the senescence inducing effects of the hormone in SMC derived from the coronary artery (Mistry *et al.*, 2013; Min *et al.*, 2009; Kim *et al.*, 2011). hCASMC were treated

with AngII in single 24 hour and 72 hour exposures as well as with 6 hour successive treatments over a 3 day period. None of the strategies caused premature senescence in the hCASMC, which is interesting because AngII has been found to induce senescence in VSMC following a single 24 hour exposure (Herbert *et al.*, 2008; Mistry, 2009; Kim *et al.*, 2011) as well as 2 hour repeat exposures over 3 successive days (Mistry *et al.*, 2013). One study found that stimulation with 100nM AngII caused an increase in SA β -galactosidase positive VSMC derived from rat thoracic aorta, between 5-14 days after treatment, suggesting that the effect of AngII is not immediate and the hCASMC may need to be left for longer before staining for SA β galactosidase activity (Min *et al.*, 2007; Min *et al.*, 2009). This suggests an avenue for further investigation.

As Angll exposure appeared unable to induce premature senescence in the hCASMC, the expression of the receptors was investigated. Like the studies in the hTERT hASMC, AT2R was undetected indicating a lack of expression, or expression levels may be below the detection threshold. AT1R was detected, however, this was at levels too low for a standard curve to be successfully constructed, and therefore no quantitative measurements could subsequently be performed. As gene expression of AT1R was detected and the studies with the HyPer probes indicated that AnglI does stimulate mitochondrial H₂O₂ production, it can be speculated that the levels of the receptor present on the cell surface may be low. This provides the opportunity for the investigation of AT1R protein expression with the use of techniques such as Western blotting as mentioned earlier.

5.4.3 Conclusion

Although previous studies within this group have been successful in demonstrating AngIIinduced premature senescence in human saphenous vein VSMC, the current study failed to do the same using either hTERT hASMC or hCASMC. Although senescence was not induced by AngII, there was evidence in hCASMC of mitochondrial H_2O_2 production and little, if any, cytosolic H_2O_2 was detected.

CHAPTER SIX

The Senescence Associated Secretory Phenotype of VSMC

Chapter 6 The Senescence Associated Secretory Phenotype of VSMC

6.1 Introduction

In the recent years the secretome of senescent cells has generated great interest. Various investigations have been conducted with various cell types, with fibroblast cells being the most widely studied (Freund *et al.*, 2010; Lee & Lee, 2014; Hassona *et al.*, 2014). Factors that have been reported to be SASP components include proinflammatory cytokines, chemokines, growth factors, secreted proteases, ROS and various ECM components (Coppe *et al.*, 2010; Lee & Lee, 2014). The SASP creates a positive feedback loop, reinforcing the proliferation arrest of senescent cells; however it can cause detrimental effects to cells in the nearby microenvironment (Coppe *et al.*, 2010). The low grade inflammation caused by the release of SASP factors causes phenotypic alterations and hinders the tissue renewal capabilities of surrounding cells and can drive pathology and contribute to age-related diseases (Sikora *et al.*, 2011; Munoz-Espin & Serrano, 2014). Currently, it is unknown whether the SASP that is observed *in vitro* mirrors the SASP that exists *in vivo*. It is also unknown whether the SASP profile released by early senescent cells differs from that derived from cells that have been senescent for a longer period of time (Naylor *et al.*, 2013; Tchkonia *et al.*, 2013).

Despite the advances in identifying SASP factors in recent years, there is a lack of investigation of the secretome of smooth muscle cells, which is fascinating as there is an abundance of these cells in atherosclerotic lesions (Naylor *et al.*, 2013; Munoz-Espin & Serrano, 2014). Not only are VSMCs present in high numbers within atheromas, but they also display distinct morphological and biochemical characteristics of senescence and thus the SASP they produce may play a role in plaque instability and rupture (Vasile *et al.*, 2001; Minamino & Komuro, 2007; Herbert *et al.*, 2008; Munoz-Espin & Serrano, 2014).

VSMCs produce ECM components enabling the control of vascular construction. As ECM components such as collagen and fibronectin are SASP factors, their synthesis by VSMCs may

continue even when the VSMCs become senescent (Coppe *et al.*, 2010). These ECM components are able to stabilise atherosclerotic plaques, with fibronectin possessing the ability to affect cell morphology, migration and adhesion by binding to cell surface integrins and triggering downstream signalling cascades (Egeblad & Werb, 2002; Coppe *et al.*, 2010). Other factors are released such as IL-1 β which can cause NF- κ B activation by binding to the IL-1 receptor; VEGF which can cause endothelial cell proliferation and blood vessel formation; the decoy death receptor, osteoprotegerin which competes with death ligands for receptor binding to prevent the induction of the apoptotic pathway; and MMPs which are able to dissolve ECM molecules and mobilise and activate growth factors tethered to the cell surface, facilitating cell proliferation and migration. Combined, whilst affecting nearby cells directly, these factors could also alter the microenvironment within an atherosclerotic plaque and subsequently promote endothelial dysfunction and immune cell infiltration. This may continue until the plaque eventually ruptures and the blood vessel becomes occluded or thrombosis occurs (Hassona *et al.*, 2014; Braunwald, 2012).

Studies have investigated various therapeutic strategies with the aim of preventing the detrimental effects that accompany the senescence process. The drug-inducible selective killing of p16-positive cells in BubR1 mice has been found to be a highly effective approach. Age-related deficits dependent on p16 induction, such as hunchback and cataracts were delayed, and the mice showed greater exercise fitness and thicker muscle fibres (Baker *et al.*, 2011; Peeper, 2011). Alternative approaches have investigated the use of viral-based nucleotide delivery or vaccines (Tchkonia *et al.*, 2013). Some current thoughts are targeted towards the use of antibodies; using epitopes that are highly expressed in senescent cells, coupled to a potentially cytolytic agent. Such methods would not require complete elimination of senescent cells as complete removal is not required for clinical benefits to be observed (Tchkonia *et al.*, 2013). Finally, some anti-inflammatory strategies have targeted SASP components as opposed to senescent cells. Such strategies use corticosterone and cortisol which bind to the glucocorticoid receptor and cause decreases in the production and secretion of IL-1 α . This reduced IL-1 α signalling subsequently reduces NF- κ B activity and prevents the production of other SASP components (Laberge *et al.*, 2012; Tchkonia *et al.*, 2013).

Whilst therapeutic strategies are being developed, the understanding of senescence and SASP is ongoing. Numerous studies have investigated the secretory profile of mouse and human fibroblasts, ECs as well as epithelial cells, yet no group has profiled the SASP of smooth muscle

cells. Investigating the profile of these proteins may not only enable our understanding of what contributes to atherosclerotic plaque formation, growth, and instability; but it may also prove to be of therapeutic use – perhaps regulating the effects of senescent cells, rather than their abundance may be a more manageable approach.

The components of SASP can be investigated by running a proteomic analysis on the conditioned media of cells *in vitro* using commercial antibody arrays (i.e. SomaLogic[®]) or, as in this study, application of mass spectrometry.

6.2 Aims & Objectives

The initial aim of this study is to establish a hCASMC model of replicative senescence. Subsequently, to investigate the effects of conditioned media from senescent hCASMC on HDFs; specifically to study the effects on cell proliferation and senescence. The subsequent aim involves using mass spectrometry to profile the proteins present in the secretome of late passage senescent hCASMC in comparison to early passage young hCASMC. Bioinformatics analysis software will then be used to identify altered processes and pathways in hCASMC senescence with the hope of signposting potential new biomarkers to facilitate our understanding of the senescence mechanism.

6.3 Results

6.3.1 Effect of conditioned media from senescent hCASMC on HDF growth

Studies have established that the SASP composition varies dependent on cell type and the inducing factor. As the SASP of SMC has never been described, it was decided that the best method to carry out the study would be with the use of SMC that had undergone replicative senescence as opposed to SIPS. In CVD, there are a variety of factors in a plaque that could cause SIPS in the SMC; however it is difficult to recreate this environment in an *in vitro* setting. Replicative senescence is a natural phenomenon that all cells could be subjected to during the ageing process, thus SASP collected from replicative senescent cells would be the most representative.

In order to induce senescence, hCASMC were cultured continuously until they reached replicative senescence, passaging the cells once they reached confluency. Once the cells stopped growing and acquired the senescent morphology, the SA β -galactosidase assay was utilised to confirm permanent cell cycle arrest. Section **3.3.2** shows the staining of both young control hCASMC and replicative senescent hCASMC. Once senescence had been confirmed using the SA β -galactosidase assay, conditioned media was collected for bioassay analysis.

For the preliminary bioassays, the senescent hCASMC were kept alive by replacing the medium with fresh complete media every 3-4 days. Conditioned media was collected from senescent cells over a period of 72 hours. It was important that the cells were retained in complete media containing serum and growth factors because the same senescent hCASMC were used to collect the conditioned media over the course of the 2 week experiment. If they were subjected to 72 hours of serum starvation, stress responses could be triggered that could potentially alter their secretory phenotype and the results would be less representative of SASP.

Young fibroblasts usually cultured in DMEM were treated with a combination of conditioned media and DMEM; fresh hCASMC media and DMEM; or only DMEM. The purpose of the cells cultured in DMEM mixed with fresh hCASMC media was to determine if the difference in media had an effect on the cells as hCASMC media contained additional growth factors that the fibroblast media did not.

The results of the preliminary bioassay are shown in **Figure 6.1**. It was clear that the fibroblasts preferred the combination of hCASMC media with DMEM as those cells grew significantly over the course of the bioassay. The apparent increase in SA β -galactosidase positive cells (**Figure 6.1B**) may likely be due to the fact that the cells in that treatment had undergone more cell cycle divisions than the control DMEM-only cells and thus have subsequently reached replicative senescence at an accelerated pace.

The cells subjected to the conditioned media and DMEM combination were interesting as they did not grow at an accelerated pace like the hCASMC:DMEM cells but they had an increased amount of SA β -galactosidase positive cells compared to the control cells. They may not have grown as rapidly as the cells treated with hCASMC media:DMEM because of the presence of inhibitory factors in the conditioned media (SASP). Nevertheless, it cannot be discounted that the hCASMC media was possibly depleted of the nutrients and growth factors. Eventually, the cells started to die, and this could partly be attributed to simple nutrient depletion and the presence of possible toxic factors in the conditioned portion of media.



Figure 6.1: Conditioned media accelerates senescence in HDF.

Senescent hCASMC were incubated in complete hCASMC media for 72 hours to collect conditioned media. The conditioned media was then added to young HDF over a two week period with the aim of determining the effects of hCASMC SASP on fibroblasts. **A**, Treatment of HDF with both the DMEM:hCASMC media combination and the DMEM:conditioned media combination appeared to cause an increase in senescent cells compared to the control cells treated DMEM only. Data represented as mean±SEM; n=3. **B**, SA β -galactosidase histochemical staining was carried out. Data presented as mean+SEM; n=3 (**p<0.01, ***p<0.001, ****p<0.001, ****p<0.001 compared to the DMEM control; ##p<0.01, ###p<0.001, ####p<0.001 compared to the DMEM:Conditioned media).

6.3.2 Determining the composition of SMC SASP

The proteomic analysis of the hCASMC secretome is represented in **Figure 6.2A**. Serum Free Media (SFM) was used as a control for the conditioned media from young and old hCASMC. A new batch of cells was obtained and cultured continuously until they reached replicative senescence. At passage 4, some cells were retained, stained for SA β -galactosidase (**Figure 6.2B**) and subjected to 12 hours of serum starvation to enable the collection of conditioned media. 12 hours provided enough time for sufficient protein secretion, and the cells were also tolerant of serum starvation for 12 hours as they remained viable with little or no cell death. The remaining cells were continuously cultured and eventually cell growth began to slow. Once at passage 20 the cells appeared to stop dividing and were maintained in culture, with media changes every few days to make sure division had been halted. A portion of cells were then stained for SA β -galactosidase and as represented in **Figure 6.2B**, 75.6% of the cells were positive. The remaining passage 20 cells were then subjected to serum starvation for 12 hours. Cell counts were performed for both the samples in case the protein content needed to be normalised to cell number.

Following collection, the conditioned media was prepared for liquid chromatography tandem mass spectrometry (LC MS/MS) analysis as fully described in section **2.2.16**. The numbers of proteins identified within each conditioned media are shown in **Figure 6.2C** (Full protein list in Appendix 1). The Venn diagram shows the number of proteins present in each sample. The old secretome contained the most proteins, with the SFM control containing the least. There were 13 proteins that overlapped across all three samples and another 273 that were common between the young and the old conditioned media. Interestingly, 128 proteins appeared unique to the aged secretome and 77 unique to the young (**Figure 6.2C**).


Figure 6.2: Proteomic Analysis investigating the Senescence Associated Secretory Phenotype of hCASMC.

A, Visual representation of the experimental design. **B**, Young cells (passage 4) displayed 2.5% SA β -galactosidase positive cells whilst old cells (passage 20) displayed 75.6% positivity. Images taken using the EVOS XL Core light microscope (magnification: x100). **C**, LC MS/MS analysis revealed the numbers of proteins present in the secretomes of both young and old hCASMC. SFM was used as a control. Data presented as numbers of protein; n=3. (Figure also available on CD – Appendix 2).

6.3.3 Bioinformatics Analysis of the VSMC Secretome

Once the conditioned media had been subjected to mass spectrometry analysis and the protein lists generated, Ingenuity[®] Pathway Analysis (IPA) was used to analyse and map the proteins identified to molecular interactions, biological processes, networks and disease response. A flow chart of the IPA bioinformatics approach is shown in **Figure 6.3**. The data derived from the mass spectrometry analysis were organised into 2 groups; those expressed both in the young and old conditioned media, and those present in only one of the two groups. The latter are important as they show proteins that are uniquely secreted in old versus young hCASMC and some of these could potentially be new biomarkers of senescence. However, it was the proteins present in both young and old that were analysed using IPA with the aim to investigate the differentially expressed proteins.



Figure 6.3: Analysis of the detected proteins

The proteins were separated based on those expressed uniquely and those expressed in both the young and old secretome. IPA analysis highlighted the affected processes and pathways and thus enabled the generation of hypotheses.

Using IPA, a 'Core Analysis' was carried out which enabled the differentially expressed proteins to be related to the information present in the Ingenuity® Pathway Knowledge Base. The quality controlled Knowledge Base is a repository of curated biological interactions and functional annotations created from millions of individually modelled relationships between proteins, genes, complexes, cells, tissues, drugs and diseases. Once the Core Analysis was completed, IPA mapped the differentially expressed proteins to canonical pathways, disease and biological functions, networks and toxicology lists. Canonical pathways display the proteins within well-established signalling and metabolic pathways; functions relate the proteins to known biological functions and disease states; whilst networks 'grows' a network of interconnected molecules based on the number of known relations in the Knowledge Base to the proteins in the data. Also included in the Core Analysis are the downstream effects, upstream regulators and the top differentially expressed proteins present in the sample.

A right-tailed Fisher's exact test was used to determine the level of significance for each network, pathway and toxicity list where the p-value is displayed as a score, which is the negative log of the p-value, $-\log_{10}$ (p-value). By default, IPA defines significance in canonical pathways; downstream biological and diseases functions; and toxicity lists with a $-\log_{10}$ (pvalue) of ≥ 1.3 which equates to a p-value of <0.05. For network analysis, the IPA default score of ≥ 3 (p<0.001) was considered significant, which provided 17 significant networks. UniProt/Swiss-Prot Accession IDs, raw fold change values and p-values for the differential proteins were used for IPA analysis.

6.3.3.1 Cellular Location

The majority of the differentially expressed proteins were designated as cytoplasmic (54%), followed by the extracellular space (29%) and plasma membrane (11%) (**Figure 6.4**). Subcellular locations classified as 'Other' could include the endoplasmic reticulum, the lysosomes as well as the Golgi apparatus.



Figure 6.4: Cellular locations of the differentially expressed proteins derived using IPA.

The majority of the proteins present in both the young and old secretome were designated as cytoplasmic. Locations expressed as a percentage of the differentially expressed proteins only.

6.3.3.2 Canonical Pathways

IPA analysis enabled the mapping of proteins in the dataset to established metabolic and signalling pathways. Analysis revealed 88 significant pathways with the top 10 displayed in **Table 6.1** (Raw data of all pathways on CD – Appendix 2).

Top Canonical Pathways			
Name	-log ₁₀ (p-value)	Molecules	
Glycolysis I	1.24E+01	TPI1,PGAM1,ENO2,PGK1,ALDOC ,PKM,GAPDH,ALDOA,ENO1,GPI	
Gluconeogenesis I	1.24E+01	PGAM1,ENO2,PGK1,ALDOC,GAP DH,ALDOA,MDH2,ENO1,MDH1, GPI	
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1.13+EO1	IGFBP4,MMP2,COL6A3,LAMA1,C OL6A1,COL3A1,CTGF,IGFBP5,CO L1A2,COL1A1,COL6A2,COL5A1,T IMP2,A2M,FN1,CSF1,TIMP1,SER PINE1,MMP1	
Inhibition of Matrix Metalloproteases	8.75E+00	A2M,MMP2,THBS2,HSPG2,LRP1, TIMP1,MMP1,TIMP2,MMP14	
Leukocyte Extravasation Signalling	7.13E+00	CXCL12,MMP2,VCL,CTTN,EZR,AC TC1,MSN,ACTN1,CD44,RDX,TIM P2,ACTB,TIMP1,MMP1,MMP14	
NRF2-mediated Oxidative Stress Response	6.83E+00	TXN,SOD1,ERP29,ACTC1,TXNRD 1,PPIB,PRDX1,SOD3,GSTO1,ACT B,GSR,CAT,AKR1A1,GSTP1	
RhoGDI Signalling	6.21E+00	GDI1,CDH13,EZR,ACTC1,MSN,G DI2,ACTR3,CD44,RDX,CFL1,ACTB ,ARHGDIA,CDH11	
Aspartate Degradation II	5.98E+00	GOT2,MDH2,GOT1,MDH1	
Chondroitin Sulphate Degradation (Metazoa)	5.96E+00	HEXB,GALNS,HEXA,CD44,ARSB	
Actin Cytoskeleton Signalling	5.85E+00	VCL,FLNA,EZR,ACTC1,MSN,ACTN 1,ACTR3,RDX,FN1,CFL1,PFN1,AC TB,GSN,TMSB10/TMSB4X	

Table 6.1: Top 10 Canonical Pathways.

Data are presented as $-\log_{10}$ (p-value) which is also known as a P-score. A P-score of \geq 1.3 was considered as statistically significant. This value also equates to a p value of <0.05.

IPA utilised a colour coded z-score algorithm to predict overall activation or inhibition of canonical pathways as shown in **Figure 6.5**. Of these top pathways, the majority were ineligible for activation/inhibition prediction due to the lack of a clear activated state or if the pattern generated for them was too sparse. These pathways vary in processes ranging from energy metabolism in the form of glycolysis and gluconeogenesis to cell morphology and movement in the Rho GDI, actin cytoskeleton and matrix metalloprotease signalling pathways.

However, 2 of these 10 pathways were assigned an activity prediction; Leukocyte Extravasation Signalling was predicted to decrease in activity, whilst the NRF2-mediated Oxidative Stress Response was predicted to increase in activity. The increase in the NRF2mediated oxidative stress response pathway is particularly interesting as oxidative stress is an established feature of the senescent phenotype. Thus the activation of the pathway in aged hCASMC indicates the presence of oxidative stress within the cells upon senescence. However, as the literature suggests SASP to be pro-inflammatory, one might expect that there would also be an increase in leukocyte extravasation signalling by senescent cells.

Ratio is an indicator of which pathway was most affected based on the bulk overlap of proteins uploaded into IPA. In other words, it highlights cases where only a small fraction of the possible proteins on the pathway are among the proteins being analysed from the dataset, versus, cases where the large majority of proteins on the pathway overlap the proteins being analysed from the dataset. Ratio provides an amount of overlap whilst significance provides confidence of association. Ratio is calculated as the number of proteins in a given pathway that meet the cut-off criteria (cut-off is 1.3) divided by the total number of genes that make up that pathway that are in the reference protein set.



Figure 6.5: Bar chart of the Top 10 Canonical Pathways.

Data are presented as $-\log_{10}$ (p-value) which is also known as a P-score. The bar chart is colour coded to indicate activation z-scores. Orange bars with positive z-scores predict an overall increase in pathway activity while blue bars with negative z-scores predict an overall decrease in activity. White bars indicate a z-score of 0 or close to 0. Grey bars indicate pathways where prediction is unavailable.

The ratio is represented by the orange points connected by the orange line.

6.3.3.3 Top Diseases and Biological Functions

IPA analysis identified biological processes and functions that are most relevant to the proteins identified in the dataset, with the Top 5 of these shown in **Table 6.2** (Raw data of all functions on CD – Appendix 2). The p-values are expressed as a range as the functions and diseases stated in the table are high level categories and contain many lower level categories within each. Activation states were predicted for individual lower level functions based on the z-score algorithm with a z-score \geq 2 indicating an increase in the process or disease whilst \leq -2 indicates a decrease in process or disease state. However these are not shown in the table as many of the activation scores were unable to be predicted. The most affected functions correlated to cell movement, growth and proliferation; cardiovascular system development and function; organismal development and survival; and the most affected diseases include cancer, and organismal injury and abnormalities.

Diseases and Disorders		Molecular and Cellular		Physiological System	
		Functions		Development and Functions	
Name	p-value	Name	p-value	Name	p-value
Cancer	2.61E-26 – 6.57E-06	Cellular Movement	1.68E-35 – 7.04E-06	Cardiovascular System Development and Function	3.10E-27 – 7.66E-06
Organismal Injury and Abnormalities	2.61E-26 – 6.57E-06	Cellular Growth and Proliferation	5.94E-30 – 7E-06	Organismal Development	3.10E-27 – 7.48E-06
Dermatological Diseases and Conditions	7.59E-22 – 3.72E-06	Cell Death and Survival	4.46E-27 – 7.82E-06	Immune Cell Trafficking	3.98E-19 – 7.04E-06
Immunological Disease	1.21E-20 – 3.72E-06	Cell Morphology	5.59E-19 – 7.12E-06	Heamatological System Development and Function	1.53E-18 – 7.66E-06
Reproductive System Disease	1.92E-20 – 6.57E-06	Protein Degradation	2.42E – 16-3.3E-15	Organismal Survival	4.50E-18 – 1.35E-10

Table 6.2: Top 5 Diseases and Biological Functions.

These functions are high level functional categories containing several lower categories which have individual p-values, thus p-values in the table are expressed as a range. A p-value <0.05 was considered significant.

The toxicology list shows particular types of toxicity that the proteins within the dataset are involved in (**Table 6.3**, raw data on CD – Appendix 2). These include hepatic fibrosis, oxidative stress and the NRF2-mediated stress response, and cardiac necrosis, cell death and hypertrophy.

Top Toxicology Lists			
Ingenuity Toxicity Lists	-log(p-value)		
Hepatic Fibrosis	2.59E01		
Acute Renal Failure Panel (Rat)	9.26E00		
Genes associated with Chronic Allograft Nephropathy (Human)	6.61E00		
Oxidative Stress	6.07E00		
Persistent Renal Ischemia-Reperfusion Injury (Mouse)	5.61E00		
NRF2-mediated Oxidative Stress Response	5.46E00		
Cardiac Necrosis/Cell Death	5.46E00		
Recovery from Ischaemic Acute Renal Failure (Rat)	4.55E00		
Cardiac Hypertrophy	4.54E00		
Renal Safety Biomarker Panel (PSTC)	4.34E00		

Table 6.3: Top 10 Toxicology Lists.

Data are presented as $-\log_{10}$ (p-value) which is also known as a P-score. A P-score of ≥ 1.3 was considered as significant. This value also equates to a p value of <0.05.

6.3.3.4 Top Network Functions

The top network functions are listed in **Table 6.4** (Raw data on CD – Appendix 2). The networks displayed are similar to the canonical pathways and biological functions discussed previously as free radical scavenging; cell movement; cellular assembly and organisation, and immunological disease are amongst the top 5 network functions.

Network Functions	Score			
Dermatological Diseases and Conditions, Developmental Disorder, Organismal Injury and Abnormalities	43			
Free Radical Scavenging, Small Molecule Biochemistry, Cellular Movement	43			
Dermatological Diseases and Conditions, Inflammatory Disease, Skeletal and Muscular Disorders	33			
Cellular Movement, Hematological Disease, Immunological Disease	33			
Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function	29			
Table 6.4: Top 5 Network Functions.				
Data are presented as a score – an approximate 'fit' between each network and the proteins in the dataset. A score of ≥ 3 (p<0.001) was considered as significant.				

6.3.3.5 Upregulated and Downregulated Proteins

Many proteins were found to have altered expression levels between the young secretome and the old. **Table 6.5** and **Table 6.6** show the Top 10 upregulated and downregulated proteins respectively. Amongst the top differentially expressed proteins, there are some that can be recognised as altered in ageing/senescence for example, IGFBP5 (Kim *et al.*, 2007).

Top Upregulated Proteins				
Gene Name	Protein Name	Expression Value		
MT2A	Metallothionein 2	↑ 101.040		
NME1	NME/NM23 nucleoside diphosphate kinase 1	↑ 14.286		
AKAP12	A Kinase (PRKA) anchor protein	↑ 11.626		
PLBD2	Phospholipase B domain containing 2	↑ 8.923		
TAGLN	Transgelin	↑ 8.787		
ENO2	Enolase 2 (gamma, neuronal)	↑ 8.097		
CALD1	Caldesmon 1	↑ 7.913		
IGFBP5	Insulin-like growth factor binding protein 5	↑ 6.597		
GNS	Glucosamine (N-acetyl)-6- sulphatase	↑ 6.493		
PRSS3P2	Protease, serine, 3 pseudogene 2	↑ 6.031		
Table 6.5: Top 10 most upregulated proteins. Data expressed as fold change in comparison between senescent and young control cells.				

Top Down-regulated Proteins				
Gene Name	Protein Name	Expression Value		
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment derived)	↓ -14.312		
MMP1	Matrix metallopeptidase 1	↓ -12.815		
LUM	Lumican	↓ -11.885		
DCN	Decorin	↓ -10.964		
VCAN	Versican	↓ -9.823		
CFB	Complement Factor B	↓ -6.454		
PZP	Pregnancy-zone protein	↓ -5.089		
B2M	β-2-microglobulin	↓ -4.944		
MMP2	Matrix Metallopeptidase 2	↓ -4.913		
CFH	Complement Factor H	↓ -4.737		
Table 6.6: Top 10 most downregulated proteins.				

Data expressed as fold change in comparison between senescent and young control cells.

There are many proteins that are of interest in CVD pathogenesis that are differentially expressed between the secretomes. **Table 6.7** and **Table 6.8** show such proteins.

Fold change >5
A-kinase anchor protein 12
Cytosol aminopeptidase
Dipeptidyl peptidase 1
Gamma-enolase
Insulin-like growth factor-binding protein 5
Isoform 2 of Nucleoside diphosphate kinase A
Isoform 4 of Caldesmon
Metallothionein-2
N-acetylglucosamine-6-sulfatase
Putative phospholipase B-like 2
Putative trypsin-6
Transgelin
Fold change between 3 and 5
1,4-alpha-glucan-branching enzyme
Cathepsin D
Glutathione S-transferase omega-1
Isoform 2 of Dermcidin
Isoform 2 of Ectonucleotide pyrophosphatase/phosphodiesterase family member 2
Isoform 2 of Glucose-6-phosphate isomerase
Isoform 5 of Acyl-CoA-binding protein
Microtubule-associated protein 4
Myristoylated alanine-rich C-kinase substrate
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1
Phosphatidylethanolamine-binding protein 1
Phosphoglycerate kinase 1

Phosphoglycerate mutase 1
Phosphoserine aminotransferase
Plectin
Protein S100-A11
Protein-lysine 6-oxidase
Reticulocalbin-1
Seprase
Septin-9
Serum deprivation-response protein
Target of Nesh-SH3
Tetranectin
Table 6.7: Proteins in the old secretome with a significant fold change compared to the
young.

Fold change between 1.5 and 3			
Alpha-N-acetylgalactosaminidase	Isoform 4 of Calumenin		
Aminopeptidase B	Isoform 6 of Calpastatin		
Arylsulfatase B	Isoform M1 of Pyruvate kinase PKM		
Aspartate aminotransferase, cytoplasmic	Keratin, type I cytoskeletal 9		
Astrocytic phosphoprotein PEA-15	Lysosomal protective protein		
Beta-hexosaminidase subunit alpha	Microtubule-associated protein 1B		
Beta-hexosaminidase subunit beta	Microtubule-associated proteins 1A/1B light chain 3 beta 2		
Catalase	N-acetylgalactosamine-6-sulfatase		
Cathepsin Z	NAD(P)H-hydrate epimerase		
CD109 antigen	Peptidyl-prolyl cis-trans isomerase B		
Coactosin-like protein	Peroxiredoxin-1		
Collagen alpha-3(VI) chain	Plasminogen activator inhibitor 1		
Cystatin-B	Procollagen-lysine,2-oxoglutarate 5- dioxygenase 1		
Cytochrome c	Protein disulfide-isomerase		
D-dopachrome decarboxylase	Pyruvate kinase PKM		
Farnesyl pyrophosphate synthase	Rab GDP dissociation inhibitor alpha		
Fructose-bisphosphate aldolase C	Serpin B6		
Gelsolin	SH3 domain-binding glutamic acid-rich-like protein		
Glutathione synthetase	Superoxide dismutase [Cu-Zn]		
Glyceraldehyde-3-phosphate dehydrogenase	Tissue alpha-L-fucosidase		
Heterogeneous nuclear ribonucleoprotein A1	Translationally-controlled tumor protein		
Isoform 2 of Fructose-bisphosphate aldolase A	Triosephosphate isomerase		
Isoform 2 of Lysosomal Pro-X carboxypeptidase	Tubulin-specific chaperone A		
Isoform 2 of Transketolase	Ubiquitin carboxyl-terminal hydrolase isozyme L1		
Isoform 3 of L-lactate dehydrogenase A chain	Ubiquitin-40S ribosomal protein S27a		
Isoform 3 of Nucleoside diphosphate kinase B	Vimentin		
Isoform 4 of Alpha-actinin-1	Vinculin		

Table 6.8: Proteins in the old secretome with a fold change between 1.5 and 3 compared to the young.

6.3.4 Proteins Uniquely Expressed

Whilst IPA analysis was conducted on proteins present in both the young and old conditioned media to enable the identification of important proteins differentially expressed, mass spectrometry also derived a list of proteins that were exclusive to either the young or old secretomes (Raw data on CD – Appendix 2). Some of these were selected and displayed in **Table 6.9**.

The types of proteins expressed exclusively in either sample have varied functions; at a first glance it appears that many of those expressed only in the aged secretome are involved in redox biology (including metallothionein-1E, superoxide dismutase 2 and 7,8-dihydro-8-oxoguanine triphosphatase), whilst many of the proteins expressed exclusively in the young cell secretome seem to be adhesion molecules and regulators.

Exclusive to Young Secretome	Exclusive to Old Secretome
Cadherin-6	7,8-dihydro-8-oxoguanine triphosphatase
Collagen alpha-1(IV) chain	Alpha-actinin-4
Collagen alpha-1(XII) chain	Caspase-3
Collagen alpha-2(V) chain	Collagen triple helix repeat-containing protein 1
Dystroglycan	Filamin-C
EMILIN-2	Glutaredoxin-1
Fibromodulin	Glycogenin-1
Glypican-1	Gremlin-1
Low molecular weight phosphotyrosine protein phosphatase	Heat shock protein beta-6
Low-density lipoprotein receptor	Integrin alpha-7
Nidogen-1	Isoform 7 of Tumor protein D54
Periostin	Metallothionein-1E
Platelet-derived growth factor D	Metallothionein-1X
Protein CYR61	Phosphoglucomutase-1
Secreted frizzled-related protein 4	Prelamin-A/C
Testican-1	Prolargin
Thrombospondin-3	Ras suppressor protein 1
Tripeptidyl-peptidase 1	Superoxide dismutase [Mn], mitochondrial
Tyrosine-protein kinase receptor UFO	Thioredoxin domain-containing protein 12
Urokinase plasminogen activator surface receptor	Transgelin-2

Table 6.9: A selection of proteins found to be present in either the young or old secretomes only.

6.4 Discussion

VSMCs adopt one of two phenotypes; contractile or synthetic. The cells in normal mature blood vessels predominantly have the quiescent contractile phenotype as their major function is the regulation of vasoconstriction and vasodilation. The alternative is the synthetic migratory phenotype, which the cells adopt upon response to vascular injury. These cells have reduced expression of proteins required for normal contractile function, but instead have an increased capacity to synthesise ECM components, enabling the control of vascular construction (Rudijanto, 2007). This study utilises VSMC that are in a synthetic state and possess no contractile function; providing a good *in vitro* model of the senescence associated secretory phenotype.

The aim of this chapter was to investigate one of the characteristics of cellular senescence in hCASMC. The SASP has been profiled in many other cell types, especially in relation to various cancers (Coppe *et al.*, 2010; Freund *et al.*, 2010). Yet the SASP of smooth muscle cells has not previously been examined despite the role it may play in atherosclerotic plaque instability and CVD pathogenesis.

6.4.1 hCASMC 'SASP' affects fibroblast cell growth

The initial investigations into hCASMC SASP were conducted using fibroblast bioassays. Bioassays have been found to be an excellent *in vitro* experimental method to test the effects of a substance on living cells. hCASMC were cultured continuously until they reached replicative senescence, which was confirmed using the SA β -galactosidase assay. Once senescent, the cells were maintained in complete growth media to preserve cell viability. This particular batch of cells reached senescence by passage 6; the length of time taken to reach senescence varies dependent upon the donor and donor age, and it is also unknown how many passages the cells undergo prior to shipment.

For the bioassay, young fibroblasts were subjected to treatment with either normal fibroblast growth media; fibroblast growth media combined with hCASMC growth media in a 1:1 ratio; or fibroblast growth media and conditioned media containing factors secreted by hCASMC over a period of 72 hours, in a 1:1 ratio. The results showed an apparent increase in SA β -galactosidase positive cells in both the DMEM:hCASMC media and DMEM:Conditioned media treatments, although these do not reach statistical significance. An increase in senescent cells in the DMEM:hCASMC media treatment could be explained by the growth factors in the

hCASMC media including epidermal growth factor, basic fibroblast growth factor, and insulin. These could stimulate cell proliferation, causing the cells to reach replicative senescence at an accelerated rate compared to the control cells maintained in basic DMEM with only foetal bovine serum. A study on MSC expansion investigated the effects of ascorbic acid, EGF, FGF-2, IL-6, PDGF-BB, transferrin and Wnt3a on MSC expansion *in vitro* (Gharibi & Hughes, 2012). Focusing on the EGF and FGF-2 results, it was shown that there was an increase in proliferation rate in cells expanded with these growth factors compared to the cells expanded in normal growth medium. p16 expression was increased in the cells cultured with FGF-2 but not those with EGF; p21 and Rb were unaffected (Gharibi & Hughes, 2012). The data provides evidence of culture medium components affecting both cell growth and growth arrest.

Like those cultured in DMEM:hCASMC media, the cells cultured DMEM:Conditioned media also appear to increase the number of SA β -galactosidase positive cells. Unlike those cultured in DMEM:hCASMC media however, the cells subjected to conditioned media do not show the significant increase in cell number indicating reduced proliferation. The increase in senescence and the lack of cell growth and inhibited proliferation may be caused by 2 possible reasons; a lack of nutrients and growth factors within the conditioned media, or the presence of factors in the conditioned media. It could be argued that 72 hours is potentially too long to collect conditioned media from senescent cells as there could be toxic components including factors released by apoptotic cells that may affect the results. However, observing the senescent cells under light microscopy revealed minimal apoptosis – there were only a small number of apoptotic floating cells and these did not increase by 72 hours.

6.4.2 Profiling the hCASMC SASP

Once it was established that conditioned media derived from hCASMC was able to affect the growth of human fibroblasts *in vitro*, the next step was to determine the exact components of the conditioned media and whether these differed quantitatively or qualitatively between the senescent and young cells.

The assay was designed in a manner where conditioned media of young early passage hCASMC could be compared to late passage senescent hCASMC. The same batch of cells was used as cells were cultured from young to old, to prevent any differences being due to different

biological donors. As the conditioned media was obtained using SFM, the SFM was also analysed by LC MS/MS proteomics as a control.

Initial LC MS/MS analyses investigated the proteomic profile of conditioned media that was obtained over a 72 hour timescale to obtain a profile of the SASP in the bioassay. After the initial results were obtained and following extensive discussion, the investigation was amended to analysis of conditioned media obtained over a 12 hour timescale. This was to ensure that any form of a stress response was not triggered and secreted components downstream of this stress response would not alter the SASP profile. In line with this, previous groups have found 24 hours serum deprivation to be a good time point at which to obtain biologically informative secretomes with negligible cell death (Mendez & Villanueva, 2015).

Once senescence had been confirmed using the SA β -galactosidase assay, the samples were prepared for mass spectrometry analysis. To facilitate analysis of the data, the proteins were separated into 2 groups – those that were present in both but differentially expressed and those that were unique to either the young or old. The proteins in the control media were not investigated as the media had not been exposed to any SMC and the majority of these proteins were keratin subtypes. The fibrous structural protein, keratin is abundant on the outer layer of skin, in hair and nails, and is often seen as a source of contamination in mass spectrometry (Hodge *et al.*, 2013). Despite wearing protective laboratory clothing, it is still difficult to limit the exposure of keratin without using a laminar flow hood for sample preparation or a keratinfree room (Hodge *et al.*, 2013). The differentially expressed proteins were analysed using Ingenuity® Pathway Analysis and a Core Analysis was carried out whilst the uniquely expressed proteins were subjected to manual analysis where interesting proteins were selected and researched manually.

6.4.3 Cellular Location

IPA analysis revealed the cellular location of the differentially expressed proteins in the young and old secretomes. The cytoplasm appeared to be where the majority of proteins were localised, followed by the extracellular space.

Interestingly, proteins that are known to be designated nuclear and cytoplasmic were also found in the secretome. One could argue that the presence of such proteins could be attributed to cell death causing the release of these proteins into the media, yet only 6 known death markers were found in the complete dataset. These include the calcium dependent phospholipid-binding protein annexin A1 present on the plasma membrane, which becomes externalised during apoptosis; the multifunctional protein calreticulin that has been found to be expressed on the surface of apoptotic cells; the executioner of apoptosis, caspase 3; cytochrome c found in the mitochondrial intermembrane space, known to be released following the activation of the intrinsic apoptotic pathway; multifunctional enzyme glyceraldehyde-3-phosphate dehydrogenase known to accumulate prior to apoptosis; and plasma membrane-bound endocytic receptor prolow-density lipoprotein receptor-related protein 1 precursor LRP1, which is involved in apoptotic cell phagocytosis. These proteins were identified following comparison of the data with the manually curated Deathbase; a database of proteins involved in different cell death processes (Diez-Perez *et al.*, 2009).

Many proteins were found in the hCASMC secretome that are classified as non-secreted proteins including fibronectin, laminin and many collagen isoforms. However these proteins have been documented in other studies investigating the SASP phenotype, and it is currently unclear whether these proteins actively contribute to the ECM, or whether they are present in the data as a result of cellular lysis due to incubation of the cells in SFM prior to the collection of conditioned media (Hassona *et al.*, 2014).

By combining the data obtained by the IPA Core Analysis with the proteins that were found to be unique to each secretome, it became apparent that there were themes within the dataset; oxidative stress, senescence and cardiovascular disease, which will be discussed in more detail.

6.4.4 Oxidative Stress

Within the mitochondria, one electron reduction of molecular oxygen results in the generation of the superoxide anion and subsequent formation of H_2O_2 . In the presence of reduced metal ions, the highly reactive *OH may be formed. Other sources of ROS within a cell include the membrane-bound NADPH oxidases (Gough & Cotter, 2011; Liu *et al.*, 2010). Proteins, lipids and nucleic acid are vulnerable to damage and when the burden of ROS in a cell exceeds its antioxidant capability, a state of 'oxidative stress' is considered to exist (Tebay *et al.*, 2015). Many studies have shown an elevation of oxidant concentrations in cells and tissues obtained from aged organisms (reviewed in Zhang *et al.*, 2015). Oxidative stress is believed to contribute to a range of diseases including cancer, diabetes and cardiovascular diseases; thus cells employ intricate defence mechanisms to reduce the risk of cellular damage (Tebay *et al.*, 2015).

Studies have found that aged VSMC contain higher levels of oxidative stress markers such as 8oxo-deoxyguanosine indicating ROS induced DNA damage (Wang & Bennett, 2012; Malik & Herbert, 2012). Within atherosclerotic plaques there is also an increased oxidative environment leading to lipid peroxidation and the oxidative modification of proteins (Najjar *et al.*, 2005). Increased ROS production has been proposed to be a marker of unstable plaques due to the higher proportion of active macrophages present as well as lipid laden macrophages, or foam cells; which increase ROS levels in the lesion (Channon, 2002; Park & Oh, 2011).

The NF-E2-related factor (NRF2) transcription factor regulates the expression of numerous networks of genes encoding proteins that possess diverse cytoprotective activities. NRF2 itself is controlled at the level of protein stability, being sequestered by Keap1 in the cytoplasm where it undergoes continuous ubiquitination and degradation; until it becomes activated by oxidative stress or electrophilic compounds (Dinkova-Kostova & Abramov, 2015; Mimura & Itoh, 2015). Following activation, NRF2 translocates to the nucleus where it binds to antioxidant response elements (AREs) to activate the expression of a wide array of antioxidative metabolising/detoxifying genes (Guo *et al.*, 2015).

The hypothesised activation of the NRF2 mediated oxidative stress response pathway by IPA came about by the increased protein expression of antioxidant proteins, CAT, PRX I, cytosolic SOD, thioredoxin and glutathione reductase; increased levels of phase II metabolising enzymes, glutathione-s-transferase and aldo-keto reductase; and chaperone and stress response protein, protein phosphatase 1; all of which are under NRF2 regulation. Other antioxidants were exclusively detected in the secretome of the aged cells and these included

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SOD2, glutaredoxin, and various metallothionein isoforms. The toxicology list also included 'oxidative stress', whilst the top network functions included free radical scavenging; all highlighting the significance of oxidative stress following senescence induction. Whilst studies have indicated that NRF2 exhibits both anti- and pro-atherogenic effects in mice in a manner dependent on genetic background (Mimura & Itoh, 2015), some studies have reported a protective role against atherosclerosis *in vivo* through suppression of VSMC migration, proliferation and inflammation (Duckers *et al.*, 2001; Cheng *et al.*, 2012; Mimura & Itoh, 2015). There are many reports of senescent cells containing high levels of intracellular ROS (Hagen *et al.*, 1997; Macip *et al.*, 2002; Cui *et al.*, 2012); so activation of the NFR2 pathway here suggests the aged hCASMC may contain response factors that serve to preserve cell integrity and function by countering oxidative stress.

6.4.4.1 Identified proteins that combat oxidative stress

The age-dependent changes in antioxidant enzymes have been extensively studied, with great controversy as to which enzymes are increased, decreased or remain unchanged with age (Zhang *et al.*, 2015). Here, various antioxidants were found to be increased in the hCASMC aged secretome, or expressed exclusively within the aged hCASMC.

SOD isoforms catalyse the dismutation of two superoxide anion radicals into oxygen and H_2O_2 (Gough & Cotter, 2011). SOD1 expression was found to be doubled in the aged hCASMC secretome, whilst SOD2 was only found in the aged secretome. Decreases in SOD1 have been observed in various tissues and cells from young and aged human samples including skin fibroblasts (Lu *et al.*, 1999) and skeletal muscle (Pansarasa *et al.*, 1999), whilst other studies have reported the opposite; finding that SOD1 levels in human plasma increased with age with the highest levels observed in subjects aged 81-99 years (Mecocci *et al.*, 2000). SOD2 activity has been reported to be increased in skin fibroblasts (Lu *et al.*, 1999) and skeletal muscle (Pansarasa *et al.*, 1999), whilst studies in leukocytes have found no change in the activity of the antioxidant when young adults were compared to elderly adults (Niwa *et al.*, 1993). Whether SOD1 or SOD2 expression increases or decreases with age appears to be dependent on the cell type and the controversy over the age-related changes in the two antioxidant enzymes remains (Zhang *et al.*, 2015). CAT dismutases H_2O_2 into H_2O and oxygen and like SOD, its alteration upon ageing appears to be dependent on cell type. In humans, previous studies suggest the age-related changes in activity appear to be tissue specific. Some studies found decreased CAT activity in leukocytes with ageing (Niwa *et al.*, 1993; Gautam *et al.*, 2010); others found increased CAT activity in plasma samples with ageing (Kasapoglu & Ozben, 2001); whilst no alterations were observed in other studies. Whilst the current reports on age-related changes in CAT are conflicting, the senescent hCASMC secretome display elevated CAT protein levels.

The GPX antioxidant family is also known to reduce H₂O₂; however these enzymes rely on GSH to do so. GPX3 is the only family member identified in the hCASMC secretome, being detected in the young, but not the old. GPX3 is the only GPX that is secreted into the plasma so its presence as a secretory protein is not surprising (Zhang *et al.*, 2015). Whilst some studies show GPX3 increases with age in humans, others show the opposite (Niwa *et al.*, 1993; Kasapoglu & Ozben, 2001; Gautam *et al.*, 2010); but the differences are thought to be influenced by age and sex (Espinoza *et al.*, 2008; Manuel Mendoza-Nunez *et al.*, 2010). In the hCASMC, the aged cells did not secrete any GPX isoform, suggesting the antioxidant may not play a role in modulating the increased levels of oxidants within the cell upon senescence.

The PRX antioxidant family is also under NRF2 control. PRX enzymes reduce H_2O_2 and lipid hydroperoxides to H_2O and alcohols, with some utilising thioredioxin in the process. Hyperoxidised forms of PRX enzymes are known to accumulate in cells and tissues with ageing (Musicco *et al.*, 2009; Zhang *et al.*, 2015). Both PRX 1 and thioredoxin were elevated in the aged hCASMC secretome, however it is unclear if the PRX 1 was in the hyperoxidised state. Researchers have suggested that the age-related accumulation of hyperoxidised PRX appears to be linked to the increase in oxidants upon ageing; however it may also be indicative of a less efficient PRX salvage pathway within senescent cells (Zhang *et al.*, 2015).

The glutathione S-transferase (GST) superfamily plays a role in oxidative defence and detoxification by transferring GSH to electrophiles (Zhang *et al.*, 2015). Many inconsistent results have been shown, with GST isoforms showing varying expression profiles upon ageing (Ceballospicot *et al.*, 1992; van Lieshout & Peters, 1998; Gautam *et al.*, 2010). Studies within lymphocytes have found the GST π isoform did not change with age or sex, which correlated with the hCASMC secretome profile where the levels were maintained upon hCASMC ageing

(van Lieshout & Peters, 1998). However, the GST Ω 1 isoform was found to be 4fold higher in the aged hCASMC secretome than the young.

The metallothionein (MT) proteins are small molecular weight and cysteine-rich proteins that are induced by various stimuli including metals, cytokines, and ROS-producers. Although not under NRF2 regulation, the MT proteins play a role in zinc homeostasis and have protective effects against toxic metals and free radicals by scavenging several ROS such as the superoxide anion, 'OH and H₂O₂; thus enabling their classification as antioxidants (Kadota et al., 2015; Yang et al., 2014). Various MT isoforms were detected in the hCASMC secretome. These include MT1E and MT1X which were both found exclusively in the aged secretome; and MT2 which was found 100 fold greater in the aged than the young secretome. One study found a high concentration of MT in lipid-rich plaques containing SMC, suggesting an oxidantdependent mechanism for the increase in MT concentration (Gobel et al., 2000; Yang et al., 2014). The authors proposed that MT may have a protective role in SMCs and cardiovascular protection through radical scavenging activities and suppression of lipid peroxidation (Gobel et al., 2000). In vivo studies utilising MT1 and MT2 knockout mice found a decrease in mean lifespan compared to wild-type mice, which may be as a result of accelerated senescence (Kadota et al., 2015). Other phenotypes observed in the knockout male mice were hunchbacked spines, weight loss and lacklustre fur. The study suggests that the absence of MT, in particular isoforms 1 and 2, may result in accelerated senescence. At younger age, the absence of MT may be compensated for with the presence of other antioxidant proteins; however in the senescent stages MT deficiency may increase the oxidative stress burden, which becomes evident with age (Kadota et al., 2015).

Although the reason for the 100 fold increase in MT2 levels is unclear, it is an interesting find as MT2 is a major subtype of the MT family (Yang *et al.*, 2014). Its *MT2A* gene polymorphisms are associated with atherosclerosis, with a correlation between the 838C/G polymorphism and carotid artery stenosis as well as diabetic atherosclerosis. The G/C exchange within the *MT2A* locus may interfere with the normal functions of MT2 and intracellular zinc concentrations, which may contribute to atherosclerosis (Yang *et al.*, 2014).

Whilst IPA predicted an increase in NRF2 activity, LC-MS/MS did not detect any NRF2 protein in either hCASMC secretome. Despite its absence in the hCASMC secretome, studies using

carotid arteries and VSMCs derived from rhesus monkeys (aged 10 years versus 20 years) found that NRF2 protein levels and binding to the ARE decreased with age (Ungvari *et al.*, 2011). However other studies have found an increase in the gene expression level of NRF2 along with its target genes, including GPX, in spleen lymphocytes from old mice suggesting a protective mechanism to ensure the survival of long-lived memory lymphocyte cells against oxidative stress, mitochondrial dysfunction and cell death (Kim & Nel, 2005). The current data collectively suggest that basal levels of NRF2 may alter with age, but the direction may differ dependent on the cell, tissue and species (Zhang *et al.*, 2015).

The 'Regulator Effects' feature in IPA uses algorithms to connect upstream regulators and downstream functions and diseases affected within the hCASMC secretome. This enables the generation of hypotheses that can explain how the activation of an upstream regulator affects the expression of downstream target molecules, and the impact of this molecular expression on functions and diseases. The regulator effects network with the highest consistency score is shown in **Figure 6.6**. The consistency score shows how consistent and densely connected a network is. This highlights the significant link between oxidative stress and hCASMC ageing within the observed dataset. NRF2 downstream targets such as SOD2 are not shown in **Figure 6.6** as SOD2 was found exclusively in the aged secretome thus was not included in the IPA analysis but would be an additional factor to take into account in support of this proposed mechanism.



Figure 6.6: Regulator Effects network

The network generates the hypothesis that the activation of 3 upstream regulators, GH1, XBP1 and NFE2L2, drive the expression changes in the 5 antioxidants (shown in the middle tier) to cause increased degradation and reduction of H_2O_2 and modification of ROS.

CAT – Catalase; GH1 – Growth hormone 1; NFE2I2 – NF-E2-related factor (NRF2); PRDX1 – Peroxiredoxin 1; TXN – Thioredoxin; TXNRD1 – Thioredoxin reductase 1; SOD1 – Superoxide Dismuatse 1; XBP1 – X-box binding protein 1

Lines connecting molecules indicate molecular relationships. Solid lines indicate direct interactions; dashed lines indicate indirect interactions. The circular arrow originating from a molecule pointing back to itself, is termed 'self-referential' and indicates the ability of the molecule to act upon itself.

6.4.5 Senescence

Senescence is defined as a terminal differentiation state in which cells have undergone irreversible proliferation arrest where they display altered gene and protein expression profiles (Min *et al.*, 2009). Senescent cells are not only resistant to growth factors and other signals that induce cell proliferation, but they are also characterised by distinctive morphological and other phenotypic changes. The postulated evolutionary benefit of senescence was that the halt in cell division blocked immortalisation and provided a simple mechanism to stop the transmission of accumulated mutations from mother to daughter cells (Colavitti & Finkel, 2005). From the proteins differentially expressed between the young and aged secretome, IPA analysis found cancer was the top 'disease and disorder', whilst the tumour suppressor gene, TP53, was one of the top upstream regulators. This is expected as cellular senescence depends critically on two powerful tumour suppressor pathways; TP53 regulation which was reflected in the differential secretomes, and the p16INK4A regulator (Rodier & Campisi, 2011).

Many studies in various cell types have investigated the characteristics of the SASP, and have found many senescence markers including proinflammatory cytokines, IL-6 and IL-8; chemokines such as monocyte chemotactic protein; growth factors including TGF- β ; granulocyte-macrophage colony-stimulating factor; TNF- α ; IGFBPs; and MMPs (reviewed in Freund *et al.*, 2010; Rodier & Campisi, 2011; Wang & Bennett, 2012; Munoz-Espin & Serrano, 2014). Despite being recorded in many SASP reports, these markers were not present in the hCASMC secretome, even though increased secretion of IL-1 β , monocyte chemotactic protein and TNF- α has been found in aged VSMC (Csiszar *et al.*, 2012). Despite the absence of the main literature SASP markers, many other proteins that have previously been documented as being part of the SASP were found in the senescent secretome and will be discussed in detail below.

6.4.5.1 Identified proteins that are regarded as SASP factors

Insulin-like growth factor-binding protein 5 (IGFBP5) was found to be ~7 times greater in the secretome of the aged hCASMC. An earlier study found that IGFBP5 was upregulated in human dermal fibroblasts and ECs that had undergone senescence. Knockdown of the protein reduced various senescent phenotypes whilst treatment or upregulation of IGFBP5 in young HUVECs accelerated cellular senescence in a p53-dependent manner (Kim *et al.*, 2007). Induction of premature senescence in human fibroblasts was found to depend on IGFBP5; and knockdown of the binding protein inhibited the IL-6 induced ROS increase and premature

senescence (Kojima *et al.*, 2013). These investigations were also in agreement with a study that discovered IGFBP5 in conditioned media derived from senescent mesenchymal stem cells. Within the same study, IGFBP4 and IGFBP7 were also strongly upregulated and appeared to play a critical role in triggering senescence in young mesenchymal stem cells. Immunodepletion of either protein from the conditioned media reversed the pro-senescent effects of IGFBP4 and IGFBP7 (Severino *et al.*, 2013). Various signalling molecules *in vitro* regulate IGFBP5 expression, including dexamethasone, which was found to be amongst the top upstream regulators in this dataset (Kim *et al.*, 2007). Combined, the studies highlight the possibility that IGFBP5 could be a cardiovascular risk factor as its role in the induction of senescence may contribute to vascular ageing, organismal ageing and other age-associated diseases.

Matrix metalloproteinases (MMP) are proteinases that participate in ECM degradation (Visse & Nagase, 2003; Rodier & Campisi, 2011). Under normal physiological conditions, the activities of MMPs are tightly regulated at the level of transcription, precursor activation, ECM interaction and endogenous inhibition (Visse & Nagase, 2003). MMP proteolysis has been linked to numerous processes and their deregulation has been associated with various pathologies (Rodriguez et al., 2010). Senescent fibroblasts and keratinocytes have been documented to secrete various MMPs (Rodier & Campisi, 2011). MMP-2 belongs to a group of MMPs that digest denatured collagens and gelatins, and was found differentially expressed in conditioned media from senescent fibroblasts (Hassona et al., 2014). The presence of the MMPs may be to facilitate tumour cell invasiveness, which highlights the ability of senescent cells to stimulate tumourigenesis. However, the expression of MMPs in the SASP is controversial (Hassona et al., 2014). Within the hCASMC secretome, metalloproteinase inhibitor 1 & 2 (TIMP-1 & -2) were found to be expressed in higher levels in the young secretome compared to the aged. Additionally, the only MMP detected was the transmembrane protein, MMP-14, which was present at equal amounts in both the young and the aged conditioned media. TIMP-1 & -2 do not only possess metalloproteinase-inhibiting activities, but they also possess cell growthpromoting and anti-apoptotic activities (Visse & Nagase, 2003). The reduced levels of both inhibitors in the aged secretome suggest deregulation of many processes as well as uncontrolled ECM degradation following the induction of senescence.

One of the proteins identified uniquely in the aged secretome was transgelin-2, whilst its homologue and smooth muscle cell marker, transgelin, was found to be ~8 fold greater in the aged compared to the young secretome. Whilst their functional roles are unclear, both have been found to be upregulated in human cancer and thus proposed to act as potential biomarkers of certain cancers (Rho *et al.*, 2009; Dvorakova *et al.*, 2016). Studies have shown that tumour fibroblasts over-expressing transgelin supported the migration and invasion of gastric tumour cells, promoting metastasis (Dvorakova *et al.*, 2016). As the literature on transgelin and transgelin-2 is limited, it may be proposed that alterations in the expressions of these proteins play a role in ageing and tumourigenesis.

Interestingly, protein levels of the putative senescence marker β -2-microglobulin (Althubiti *et al.*, 2014), was ~5 times greater in the young secretome suggesting downregulation upon senescence. It must be noted however that as β -2-microglobulin constitutes the light chain of the class I major histocompatibility complex antigens (Bethea & Forman, 1990), it may not be present in the secretome because it may still be bound to the cell surface of the senescent hCASMC.

6.4.6 Cardiovascular Disease and Smooth Muscle Cell Senescence

Senescent VSMCs have been found in atherosclerotic plaques, displaying distinct morphological and biochemical characteristics of senescence (Vasile *et al.*, 2001; Gorenne *et al.*, 2006; Minamino & Komuro, 2007). In advanced plaques, senescence-positive VSMCs exhibit many functional abnormalities including increased expression of proinflammatory molecules possibly causing dysfunction to other cells in the vicinity in a paracrine manner, as previously observed (Nelson *et al.*, 2012; Gardner *et al.*, 2015). The detrimental effects caused by the release of VSMC derived SASP components highlight the role that senescence may play in the pathogenesis of vascular ageing (Minamino & Komuro, 2007; Naylor *et al.*, 2013; Munoz-Espin & Serrano, 2014). The hCASMC used for the study possess a synthetic migratory phenotype, and therefore secrete various proteins required for vascular construction (Rudijanto, 2007). The cells are therefore a suitable model for studying SASP *in vitro* as they have an increased secretory capacity.

The IPA Core Analysis identified the cardiovascular system as the top 'physiological system development and functions' whilst the top toxicology lists included cardiac necrosis/cell death and cardiac hypertrophy. One of the top canonical pathways the Core Analysis also identified was leukocyte extravasation signalling (6.3.3.2). IPA predicted leukocyte extravasation signalling to be decreased based on the proteins present in the hCASMC secretome. Leukocyte extravasation plays a key role in atherogenesis. Following damage and activation of the vascular endothelium, blood-borne leukocyctes including monocytes and T lymphocytes are recruited to the artery wall through cytokine and chemokine signalling as well as increased expression of adhesion molecules on EC cell surface (Rudijanto, 2007; Wang & Bennett, 2012). Studies have shown that atherosclerotic plaques contain high levels of aged senescent smooth muscle cells and continuous immune cell infiltration contributes to plaque instability (Munoz-Espin & Serrano, 2014). Thus the proposed decrease in leukocyte extravasation signalling suggests the cells may be releasing a potential feedback signal to prevent further immune infiltration within a plaque; or this decrease could be attributed to natural vascular ageing, meaning that VSMCs simply reduce extravasation signalling upon senescence induction, regardless of disease.

In cardiovascular disease, traditional proteomic studies focused on plasma biomarker discovery, however this has its limitations including the complexity and dynamic range of plasma proteins as well as the low abundance of disease-specific biomarkers. Due to the 10 orders of magnitude in protein concentration between the most abundant proteins in blood and cell-derived proteins such as interleukins; sifting the plasma for specific biomarkers can be a challenging task (de la Cuesta *et al.*, 2009; Mendez & Villanueva, 2015). In the recent years cellular and tissue proteomics has been poised as a better alternative to studying disease as secretomes are enriched with secreted proteins relevant to the particular disease (Mendez & Villanueva, 2015). These secreted proteins may then be shed into the blood and can subsequently act as disease biomarkers.

Recent studies have investigated atheroma plaque proteomics and related pathologies using both animal and human samples (de la Cuesta *et al.*, 2009). Another study used direct tissue proteomics to identify 806 proteins in 35 plaques providing the first large scale proteomics map of human coronary atherosclerotic plaques, many of these proteins being found specifically in the vascular media (Bagnato *et al.*, 2007). The proteins identified within these studies were compared to those identified exclusively or significantly upregulated within the aged secretome in this study and are shown in **Table 6.10**. Overall, there is good concordance between the hCASMC secretome data and the data currently present in the literature, indicating the validity of the data discovered in the context of cardiovascular disease, and the discovery of potential biomarkers and mechanisms.

Protein	hCASMC Secretome	Model	Methodology	Reference
α -actinin 1 α -actinin 4	α-actinin 1 – $↑$ ~2fold in aged α-actinin 4 – only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Annexin A5	Present only in aged	↑ in biopsies from affected human aortas	2DE	(de la Cuesta <i>et al.,</i> 2009)
ATP-citrate synthase	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Catalase	↑ ~2fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Cathepsin D precursor	↑ precursor is ~3fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Desmoplakin	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Endoplasmin precursor	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Fumarylacetoacetase	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Filamin C	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
γ-enolase	↑ ~8fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Glyceraldehyde-3-phosphate dehydrogenase	↑ ~2fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Gelsolin precursor	↑ ~2fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Glutathione transferase omega 1	↑ ~4fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Heat shock protein β-6	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Leukotriene A-4 hydrolase	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)

Neuroblast differentiation associated protein AHNAK (desmoyokin)	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Protein disulphide-isomerase precursor	↑ ~2fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Peroxiredoxin 1	↑ ~2fold in aged	↑ in unstable plaques from ApoE mice arota Present in 35 human coronary atherosclerotic plaques	VSMCs explant culture + DIGE Direct Tissue Proteomics	(de la Cuesta <i>et al.</i> , 2009; Bagnato <i>et al.</i> , 2007)
Plectin 1	↑ ~4fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Rho GDP-dissociation inhibitor	Equal in both young and aged	\downarrow in unstable plaques from human carotid	2DE	(de la Cuesta <i>et al.,</i> 2009)
Superoxide dismutase 2	Present only in aged	↑ in unstable plaques from human carotid Present in 35 human coronary atherosclerotic plaques	2DE Direct Tissue Proteomics	(de la Cuesta <i>et al.,</i> 2009; Bagnato <i>et al.,</i> 2007)
Superoxide dismutase 3	Equal in both young and aged	↑ in unstable plaques from human carotid Present in 35 human coronary atherosclerotic plaques	2DE Direct Tissue Proteomics	(de la Cuesta <i>et al.,</i> 2009; Bagnato <i>et al.,</i> 2007)
Thrombospondin-2	Equal in both young and aged	\uparrow in unstable plaques from human carotid	Western Arrays	(de la Cuesta <i>et al.,</i> 2009)
Transgelin-2	Present only in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)
Vinculin	↑ ~2fold in aged	Present in 35 human coronary atherosclerotic plaques	Direct Tissue Proteomics	(Bagnato <i>et al.,</i> 2007)

Table 6.10: Proteomic identification of proteins present in animal and human atherosclerotic plaques and related pathologies

Proteins from published studies were related to proteins present in the aged hCASMC secretome.

6.4.6.1 Identified proteins related to cardiovascular disease

Whilst the literature is limited on the functional roles of some of the proteins within **Table 6.10**, some proteins found in the hCASMC secretomes have been identified to play roles in cardiovascular disease and ageing and are discussed in detail below.

Plasminogen activator inhibitor-1 (PAI1) is the primary inhibitor of plasminogen activation in plasma; therefore restricting the generation of plasmin, which degrades various ECM components, controlling matrix turnover and cell invasion. Inhibition of the proteolytic cascade disrupts many processes including tissue regeneration, wound healing, immune response, angiogenesis and cancer invasion (Stoppelli, 2000). ECs undergo structural and functional changes with advancing age including expression of senescence markers, increased occurrence of polyploid nuclei, as well as increased endothelial permeability (Najjar et al., 2005). VSMC and dysfunctional ECs in aged arteries are known to secrete more PAI1, as well as senescent fibroblasts, favouring thrombus formation (Najjar et al., 2005; Yamamoto et al., 2005; Rodier & Campisi, 2011). Within the hCASMC secretome, PAI1 was found to be ~2 fold greater in the aged secretome. In accordance with this, PAI1 has also been found to be higher in p16-positive senescent cells isolated directly from fat tissue of older progeroid mice compared with nonsenescent cells from the same tissue (Tchkonia et al., 2013). These data indicate that secretion of PAI1 by senescent VSMC may contribute to the progression of the atherosclerotic process by promoting fibrin deposition and extracellular matrix accumulation in the lesions (Yamamoto et al., 2005).

Desmosomes are cell-cell adhesive junctions that confer structural integrity to various tissues, in particular those that frequently undergo mechanical stress, such as the heart and blood vessels. Desmosomes use the desmoplakin protein which belongs to a family of cytolinkers to anchor keratin and the desmin intermediate filament cytoskeleton (Albrecht *et al.*, 2015). A study reported a family with autosomal-dominant, left-sided arrhythmogenic right ventricular cardiomyopathy; which is a familial heart muscle disease characterised by structural and pathological abnormalities of the right ventricle. The study described this was caused by a frameshift mutation in desmoplakin, causing disruption of intermediate filament binding (Norman *et al.*, 2005). Desmoplakin was found exclusively within the aged hCASMC secretome; however it is unclear what role elevated levels of normal, unaltered desmoplakin plays in VSMC senescence.

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was found in human coronary atherosclerotic plaques and was found ~2 fold greater in the aged hCASMC secretome. In the previous chapter GAPDH was excluded from being used as a housekeeper gene for qPCR studies investigating AT1R expression in hCASMC due to alterations in expression upon cell manipulation. This study confirms such alterations.

Although many proteins were identified, the hCASMC secretome did not contain cytokines and growth factors that were hypothesised to be present as they are known to be at sites of atheroma. The study investigating human atherosclerotic plaques using direct tissue proteomics also failed to identify such proteins and they hypothesised the absence to be due to a number of reasons including the limitations of the method in its ability to detect low abundance proteins (Bagnato *et al.*, 2007). Such a limitation is not so applicable in this study as the secretome was collected in SFM. The presence of serum is detrimental as the protein concentrations range within serum spans at least 9 orders of magnitude, which makes detection of the smaller, less abundant proteins difficult (Merrell *et al.*, 2004). The use of SFM combined with the powerful and highly sensitive LC MS/MS technique restores confidence in the fact that the low abundance proteins were detected and any proteins that are absent may simply not be secreted by hCASMC that have undergone replicative senescence.

6.4.7 Future Work

For the first time, the hCASMC senescence secretome has been profiled; revealing proteins involved in many cellular functions including oxidative stress modulation, senescence, cell proliferation and cell survival. The hCASMC secretome displays the possible autocrine and paracrine effect of SASP. The data shows the potential effect of senescence within the senescent cell, with the activation of the NRF2 pathway and the scavenging of oxidant species; as well as the potential effect of senescence and the senescent cell on other cells, demonstrated by the decreased leukocyte extravasation signalling.
Further validation of the study needs to be performed with techniques such as Western blots and the enzyme-linked immunosorbent assay to confirm the expression of important proteins, as well as the use of hCASMC from different donors. Additionally, it is unclear whether the composition of the SASP varies with time (Tchkonia *et al.*, 2013), so it is worth profiling the SASP of cells that have just undergone senescence and comparing it to SASP derived from cells that have been senescent for much longer.

It is known that a set of core SASP factors feature in all senescent cells, with the exception of p16-induced senescence; however there are variations in the SASP quantity and quality, which is dependent on the cell type and the senescence inducer (Coppe et al., 2010). Whilst the proteomic analysis was performed on hCASMC that had undergone replicative senescence, it is worth investigating the characteristics of a secretome derived from SIPS as the proteins identified may differ. Additionally, it is worth investigating the SASP of hCASMC that had undergone senescence with the use of mETC inhibitors, AA and Rot; as well as redox cycler, MitoPQ. These compounds are proposed to induce SIPS through the generation of ROS, and as the environment within an atherosclerotic plaque is highly oxidative, the study may be extremely useful in understanding the dynamics of a plaque. A consequence of studying SASP derived from cells that have undergone SIPS is that the secretome may consist of proteins involved in the cellular response to oxidative stress and damage; not simply proteins related to senescence. Thus this composition of conditioned media may not contain proteins exclusive to the SASP. The study of replicative senescence is a 'cleaner' method as these cells are not subjected to any manipulations other than standard tissue culture growth techniques. It is however, worth noting that profiling the SASP from hCASMC that have undergone SIPS may be different from the SASP characterised in this study simply due to the different hCASMC donor, and so the study must be carried out with the inclusion of conditioned media derived from the secretome of hCASMC that have undergone replicative senescence in parallel.

ECs are known to generate microparticles (MPs), with one study finding increased MP formation in senescent cells (Burger *et al.*, 2012). Not only were no obvious phenotypic differences between the MPs derived from young and senescent ECs found, but both sets of MPs were able to significantly increase superoxide anion and H_2O_2 formation; and also induced premature senescence in a ROS-dependent manner in low-passage ECs (Burger *et al.*, 2012).

VSMC may also be capable of MP formation and the results observed in the fibroblast bioassay may not result from possible SASP factors, but may be as a result of MP activity. Future studies could investigate the possibility of MP formation by hCASMC and if treatment with the isolated MPs causes any effect on fibroblasts or young, early-passage hCASMC.

6.4.8 Conclusion

The study reveals a list of proteins that are found to be secreted by young and senescent hCASMC and subsequently provides an insight into the proteins that may be present in an atherosclerotic plaque or even exported into blood plasma. The release of these proteins could possibly promote plaque instability by facilitating cell growth and cellular dysfunction; however they can also confer some cytoprotective effects as demonstrated by the presence of various antioxidant proteins and the inhibition of leukocyte extravasation.

CHAPTER SEVEN

Perspectives and Future Work

Chapter 7 Perspectives and Future Work

7.1 Perspectives

7.1.1 Synopsis of thesis

The aim of this thesis was to investigate the role of mitochondrial oxidants in the induction of premature senescence in human VSMC. Following senescence induction by mitochondrial oxidant species, the aim was to modulate mitochondrial oxidants with mitochondria-targeted and non-targeted antioxidants and thereby regulate senescence. In general, it was found that the mitochondria-active compounds were able to induce oxidant generation and senescence; however the antioxidants used were not able to sequester the increased oxidant levels. Although previous findings had described the induction of SIPS by AngII, this proved impossible to reproduce in both of the cell types studied. The study also found that whilst not being ideal as a model of senescence, hTERT hASMC was a useful tool in investigating oxidant generation and modulation.

The second aspect of the thesis involved investigating the SASP of VSMC by first obtaining conditioned media from senescent VSMC and exposing this to fibroblasts to determine its effects on cell proliferation and senescence induction. The aim was then to profile the proteins present in the VSMC secretome, for the first time, with the hope of signposting potential new biomarkers to facilitate our understanding of the senescence mechanism. The SASP of replicative senescent hCASMC showed an array of proteins involved in numerous processes and highlighted the possibility of SASP factors to affect both the secretory senescent cell, as well as cells in the nearby microenvironment in a paracrine manner.

Future work from this study extends towards further investigation into the ability to induce and modulate ROS using MitoPQ; and also investigate the SASP of cells that have been induced into premature senescence with the use of oxidant generation.

7.1.2 Modulation of mitochondrial and cytosolic Reactive Oxygen Species and its detection

Mitochondrial ETC inhibitors, AA and Rot, were both able to induce mitochondrial superoxide generation in hCASMC and hTERT hASMC, however AngII-stimulated increases in H_2O_2 were only observed in the hCASMC, and this was at high micromolar concentrations. Although not conclusive, HyPer fluorescence indicated AngII exposure caused an increase mitochondrial H_2O_2 levels. MitoPQ was used in the hCASMC and no oxidant production was detected following exposure.

7.1.2.1 Can we detect mitochondrial oxidant species using the current tools?

The data suggest that mitochondrial oxidants can be detected as both AA and Rot were able to cause increases in MitoSOX[™] fluorescence using flow cytometry in both cell types. Using AA and Rot to induce oxidant generation is complex as it was found that within hCASMC, whilst mitochondrial superoxide was increased, H₂O₂ levels were reduced. It was postulated that PRX/CAT enzyme gene expression was altered; however this was not reflected in qPCR studies. MitoSOX[™] fluorescence was not increased with MitoPQ exposure, suggesting the redox cycler did not increase superoxide levels; however the experiment had limitations as the MitoSOX[™] appeared to preferentially remain localised to the area it was added to within the dish.

Various methods of ROS detection were used, however they each had limitations; the sensitivity of the Amplex Red assay was questioned and flow cytometry studies relied on large numbers of cells. The HyPer vectors were utilised and preliminary results showed that AngII caused detectable increases in mitochondrial H_2O_2 at nanomolar concentrations; however there was no apparent increases in cytosolic H_2O_2 . Confocal microscopy, rather than a relatively simple fluorescence microscope, may be a better method of detecting alterations in H_2O_2 as the transfected cells appeared to fluoresce weakly and the signal could not be accurately quantified. As oxidation of HyPer results in a decrease in the 420 nm excitation peak and a proportional increase in the 500 nm excitation peak, ratiometric data can be reported as well as single-wavelength imaging. Ratiometric readout prevents many imaging artefacts caused by object movement and different expression levels between cells or compartments of selected specific cells would enable observations about the length of exposure required for the ROS inducers to cause oxidant species generation.

7.1.2.2 Do mitochondrial antioxidants work as 'antioxidants'?

The 'antioxidants' used were not found to possess any demonstrable antioxidant capacity within this study. In the hTERT hASMC, MitoQ appeared to potentiate the effects of AA by further stimulating the levels of superoxide when used as a co-treatment. Work with MitoQ was subsequently discontinued as the compound appeared to have a narrow prooxidant/antioxidant window. Whilst MitoTEMPO did not stimulate further oxidant production, it also apparently did not reduce the levels.

MitoTEMPO co-treatment with Rot increased H₂O₂ levels compared to Rot alone, possibly as a consequence of MitoTEMPO catalysing the dismutation of superoxide generated by complex I inhibition. As AA reduced H₂O₂ levels compared to the control, gene expression of 6 endogenous antioxidants was investigated to determine if AA exposure caused antioxidant upregulation. None of the antioxidants showed any changes in gene expression following treatment with the mETC inhibitors and MitoTEMPO; suggesting that enzyme activity alterations may explain the reduction in H₂O₂ following AA treatment. Some studies have investigated gene expression changes of endogenous antioxidants following exposure to paraquat and found mRNA levels of SOD1, SOD2, GPX and CAT were all increased (Franco *et al.*, 1999). However this increase was after a 72 hour exposure to paraquat suggesting that gene expression may not occur so rapidly. This indicates that the short 4hour Amplex Red results may be related to more rapid changes in enzyme activity rather than altered gene expression. Pre-treatment with AA also did not appear to protect the cells from oxidative stress as might be the case if antioxidant genes were upregulated; treatment with exogenous H₂O₂ after AA exposure caused a fall in cell number, suggesting cell death or growth inhibition.

Based on the data presented in this thesis, the conclusion that none of these compounds possess any antioxidant capability can be drawn. Within both cell types, none of the antioxidants used appeared to lower the levels of oxidant species. In some instances, MitoQ appeared to act as a redox cycler, further stimulating mitochondrial superoxide; a phenomenon which has been observed in ECs and PMA-treated polymorphonuclear leukocytes (Doughan & Dikalov, 2007; Murphy & Smith, 2007; Huang *et al.*, 2014). Whilst MitoTEMPO was not found to act in a pro-oxidant manner, no evidence was found that the compound acted as an antioxidant in cells. This suggests that the previous observations that found the prevention of SIPS by MitoTEMPO following AngII exposure (Mistry *et al.*, 2013), may not be dependent on the ability of MitoTEMPO to act as a SOD mimetic. The mitochondria-targeted compound may prevent SIPS by acting on the signalling pathway through a ROS-independent mechanism.

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Mitochondria can be overloaded with the targeted compounds (MitoQ, MitoTEMPO, and MitoPQ) and this causes membrane depolarisation and affects mitochondrial bioenergetics. Murphy et al., (2007) found that non-specific mitochondrial disruption by MitoQ and decyITPP⁺ occurs at similar concentrations, indicating that the compounds are only effective antioxidants at concentrations below those that disrupt function. Therefore, it will be useful to investigate mitochondrial bioenergetics following treatment with the targeted compounds and additionally, the use of the respective TPP⁺ molecules as controls would determine if any of the results observed following treatment arise as a result of the accumulation of the TPP⁺ cation disrupting mitochondrial function (Murphy & Smith, 2007; Murphy, 2008; Reily *et al.*, 2013).

7.1.3 Induction of premature senescence by inducing mitochondrial ROS production

The hTERT hASMC model of senescence was questioned in this study as exposure to both mETC inhibitors, as well as H_2O_2 failed to induce premature senescence.

Rot and MitoPQ successfully induced premature senescence in the hCASMC. This study also found that whilst successive exposure over 3 days induced senescence, a 24 hour exposure to both Rot and MitoPQ was unable to induce senescence, suggesting the repeated exposure quoted in literature is the best method to study SIPS (Toussaint *et al.*, 2000).

AngII failed to induce premature senescence in either cell model, which may have been as a consequence of insensitivity to the hormone in both cell types; a lack of receptor expression by the hCASMC; or alternatively, an ineffective batch of AngII. Gene expression studies found that *AGTR1* was expressed by the hTERT hASMC. Subjecting the hTERT hASMC to serum starvation caused no alterations in the expression levels of *AGTR1*, suggesting there may be no need to serum starve the cells prior to AngII exposure as performed in previous studies as the cells do not appear to downregulate the receptor in the presence of growth factors (Nickenig & Murphy, 1994; Touyz & Schiffrin, 1997a; Kunieda *et al.*, 2006; Min *et al.*, 2007; Herbert *et al.*, 2008).

7.1.3.1 Does mitochondrial oxidant generation cause senescence?

The data from this study suggests that the ability of mitochondrial oxidants to induce premature senescence is questionable. Both Rot and MitoPQ were able to induce senescence however Rot exposure is able to have other effects within the cell including the alteration of cell morphology and such alterations may not be dependent on superoxide generation (Li et al., 2014; Wang et al., 2014). Thus it could be argued that senescence induction may be as a result of the deregulation of other processes within the cell, not simple oxidant generation and subsequent oxidative stress. Additionally, Rot-induced premature senescence may be as a result of a loss of mitochondrial membrane potential, which has been found to be the case in a colon cancer cell line (Behrend et al., 2005). With MitoPQ, the concentration required to induce premature senescence was quite high, and at such concentrations, the extensive accumulation of the lipophilic cation within the mitochondria may disrupt membrane integrity and respiration; meaning the senescence induction may be as a result of mitochondrial dysfunction (Murphy, 2008). Thus, before it can be confirmed whether the induction of senescence is caused by the increased mitochondrial oxidants, the effect of these compounds on the mitochondria, especially on the membrane potential, must be investigated. Initially, induction of senescence by the decyITPP⁺ moiety alone should be investigated.

Within the study, senescence was detected by phenotypic alterations including the flattened morphology, slowed cell growth and using the SA β -galactosidase histochemical stain. Whilst this stain is the commonly used method in literature (Macip *et al.*, 2002; Kunieda *et al.*, 2006; Herbert *et al.*, 2008; Min *et al.*, 2009; Burger *et al.*, 2012; Mistry *et al.*, 2013), it comes with its limitations. Such limitations include false-positive results caused by stresses induced during cell culture such as prolonged confluence in culture, contact inhibition and serum starvation and cells such as adult melanocytes and cells with a high lysosomal content such as macrophage foam cells also express the enzyme at high levels (Debacq-Chainiaux *et al.*, 2009; Campisi & di Fagagna, 2007; Sikora *et al.*, 2011; Wang & Bennett, 2012).

Some studies use multiple methods of detection such as SA β -galactosidase activity in combination with Western blots to identify senescence-associated proteins (Kunieda *et al.*, 2006; Min *et al.*, 2009). Expression of tumour suppressor proteins, p53 and p21, would have been useful to further confirm the onset of senescence. Alternative senescence detection methods could also be investigated such as identifying SAHFs, using DNA-binding dyes; detecting stable nuclear foci containing DNA damage response proteins; detecting DNA DSBs using γ -H2AX; use of the proliferation marker, Ki-67 antigen using immunostaining; or

incorporation of the radioactive thymidine analogue BrdU (Campisi & di Fagagna, 2007; Debacq-Chainiaux *et al.*, 2009; Kuilman *et al.*, 2010).

The proposed signalling mechanism for the generation of mitochondrial and cytosolic oxidants and the subsequent induction of senescence is presented in **Figure 7.1**.



Figure 7.1: Investigating the induction of premature senescence in VSMC using mitochondrial inhibitors, a mitochondrial redox cycler and Angiotensin II

AngII failed to increase both mitochondrial and cytosolic levels of superoxide and H_2O_2 , nor did the hormone induce premature senescence; so no evidence of a crosstalk between the NAD(P)H oxidase and the mitochondria could be obtained.

Mitochondrial inhibitors induced the generation of mitochondrial oxidants and Rot subsequently induced senescence; however no apparent oxidant generation was observed with MitoPQ treatment, despite the redox cycler inducing premature senescence. All the antioxidants used failed to reduce the oxidant levels within the VSMC questioning their efficacy as antioxidants.

Solid lines indicate where data support the mechanism, whilst the dashed lines represent suggested pathways but without experimental evidence in this thesis.

7.1.4 Characterising the VSMC Senescence Associated Secretory Phenotype

As mentioned earlier, the aim of the study was to profile the proteins present in the human VSMC secretome with the hope of identifying processes and pathways that are altered upon ageing, which may facilitate our understanding of the senescence mechanism. The VSMC secretome also has the potential to help drive new biomarker discoveries at the basic and clinical research levels as these proteins can be detected in biological fluids and used to compare what is present in young versus old adults or healthy individuals versus patients with CVD.

Investigations into hCASMC SASP showed that conditioned media appeared to induce premature senescence in fibroblasts and slow down cell growth and cause possible cell death.

Proteomic analysis of the conditioned media detected a total of 414 proteins in the aged secretome, compared to a total of 363 proteins in the young secretome; with 286 proteins common to both. The differentially expressed proteins were analysed with IPA whilst the proteins unique to each secretome were manually selected through a literature survey. IPA Core Analysis revealed the majority of the differentially expressed proteins were designated as cytoplasmic or in the extracellular space, as expected; however comparison with proteins in the Deathbase identified only 6 proteins as being involved with cell death. Therefore, this supports the contention that we are dealing with the secretome of senescent cells rather than a dying population.

IPA predicted the directionality of two canonical pathways identified from the proteins present in the dataset. The NRF2-mediated oxidative stress response was predicted to be activated, suggesting that the SASP may reflect the oxidative stress response of the cells. Leukocyte extravasation signalling was predicted to be decreased, indicating the effect SASP components may have on cells in the nearby microenvironment. This anti-inflammatory property is interesting as most studies in the literature investigating SASP highlight the release of proinflammatory components creating an inflammatory environment (Coppe *et al.*, 2010; Sikora *et al.*, 2011; Munoz-Espin & Serrano, 2014). However, stress-induced senescent ECs have been shown to display an anti-inflammatory phenotype with an inability to support neutrophil adhesion and transmigration even after inflammatory cytokine stimulation (Coleman *et al.*, 2013). As this EC anti-inflammatory phenotype correlates with the hCASMC data, it may be suggested that senescent vascular cells may have a unique protective role against chronic inflammation by limiting the local inflammatory response within the vasculature. This may be highly beneficial in a plaque environment as it may prevent further immune infiltration and subsequent plaque growth and instability.

The results suggest that the aged hCASMC may contain elements that preserve cell integrity and function by countering oxidative stress as many antioxidants (CAT, SOD2, PRX and various MT isoforms) were found to be upregulated or exclusive to the aged secretome. The hypothesis generated was that the activation of three upstream regulators drives the expression changes in downstream antioxidants to cause increased modification and degradation of ROS within the senescent cell. This is in line with previous studies that have found elevated levels of oxidant species in cells and tissues obtained from aged organisms (Zhang *et al.*, 2015) and higher levels of oxidative stress markers such as 8-oxodeoxyguanosine in aged VSMC specifically (Martinet *et al.*, 2001; Malik & Herbert, 2012).

Many proteins known to be related to senescence were detected, including the tumour suppressor gene, TP53, which was identified as one of the top upstream regulators. Although many of the markers present in previous SASP reports were not present in the dataset, proteins that have previously been documented as relating to senescence were detected including IGFBP5 (Kim *et al.*, 2007; Kojima *et al.*, 2013). Interestingly, a newly identified senescence marker in a bladder cancer cell line, β 2M (Althubiti *et al.*, 2014), was found to be present at higher levels in the young secretome of hCASMC.

Proteins detected in the hCASMC secretome were correlated with proteins that have been found in atherosclerotic plaques and there was good concordance between the hCASMC data and the literature (Bagnato *et al.*, 2007; de la Cuesta *et al.*, 2009). Proteins that were found in the dataset that have also been identified as present in plaques include SOD and SOD2; transgelin-2; GAPDH; and γ -enolase. Functional roles for some of the proteins identified are not well understood, and validation of these proteins by Western blots is required.

7.2 Reactive Oxygen Species, cellular senescence and cardiovascular disease

Senescent VSMC, ECs and inflammatory cells have been found in atherosclerotic plaques but their contribution to plaque stability and possible subsequent erosion or rupture is not fully understood (Vasile *et al.*, 2001; Gorenne *et al.*, 2006; Minamino & Komuro, 2007; Ovadya & Krizhanovsky, 2014). Whilst cells in the plaque can undergo senescence once their proliferative lifespan has been reached, they can also undergo premature senescence through ROS-mediated oxidative damage. This has importance given that the atherosclerotic plaque is reportedly a highly oxidative environment (Channon, 2002; Park & Oh, 2011). The signalling mechanism involved in senescence induction is complex, and understanding the process may facilitate our understanding of senescence and its contribution in CVD pathogenicity. The secretion of the proteins by senescent cells, identified as SASP components, provides an insight into the proteins present in plaques, or possibly in the bloodstream. These proteins may facilitate cell growth and dysfunction and promote plaque instability; yet they may also confer some cytoprotective effects with the release of antioxidant proteins, for example. Understanding the effects of the senescent cells within the atherosclerotic lesion will help pave the way for new therapeutic strategies.

Many early translational therapeutic interventions currently exist in the literature, with a strong focus on senescent cell clearance (Chang *et al.*, 2016; Baker *et al.*, 2011) and an increasing interest in senescence immunotherapy (Ovadya & Krizhanovsky, 2014). Exercise and other lifestyle changes have been found to be potentially therapeutic to many diseases associated with increased rates of cellular senescence; for example, p16 expression was shown to be associated with cigarette smoking and physical inactivity (Liu *et al.*, 2009; Naylor *et al.*, 2013; Ovadya & Krizhanovsky, 2014; White *et al.*, 2014b).

7.3 Future Work

7.3.1 Redox modulation

7.3.1.1 Endogenous enzyme activity

As no alterations in gene expression were observed following treatment with the mETC inhibitors, the activity of the endogenous enzymes should be investigated as this may account for the reduction in H_2O_2 levels.

7.3.1.2 Real-time ROS measurement

It may be possible that the 4-6 hour treatments of the VSMC with the ROS inducers may not enable the proper detection of ROS. There may be immediate and transient alterations in cellular ROS levels following the addition of the compounds, which would have been missed when the ROS levels were measured after the end of the desired treatment courses. A more suitable method of detecting the induction of cellular oxidative stress may be with the use of confocal microscopy with a 37°C/5% CO₂ chamber, which would facilitate the detection of realtime changes following the addition of inducers such as AA or AngII.

7.3.1.3 ROS generation with the targeted redox cycler

The studies with MitoPQ showed the redox cycler was able to induce SIPS in the hCASMC, further investigations should be continued with the use of the decylTPP⁺ cation as a control. The SIPS experiments should be repeated to determine if lower concentrations of MitoPQ can induce SIPS, as the high 50 µM concentration may be causing mitochondrial membrane depolarisation. Alterations on the cellular bioenergetics should also be investigated including the loss of membrane potential or changes in oxygen consumption or extracellular acidification rate as TPP⁺ compounds can disrupt mitochondrial function (Murphy & Smith, 2007; Reily *et al.*, 2013). Additionally, studies using ROS detection methods such as flow cytometry, and HyPer fluorescence will help to detect the smaller changes in MitoPQ-induced ROS; which can then be modulated with use of targeted antioxidants. These experiments will help determine with some certainty whether mitochondrial oxidants are able to mediate hCASMC senescence.

7.3.1.4 Use of alternative antioxidants

Many other mitochondrial targeted antioxidants have been synthesised, including MitoSOD, to degrade superoxide; the peroxidase, MitoPeroxidase; MitoTEMPOL, another SOD mimetic; and the targeted vitamin E, MitoE (Murphy & Smith, 2007). They could prove to be better at modulating ROS in VSMC however appropriate controls must be used to ensure the results can be attributed to the antioxidant, and not due to alterations to mitochondrial membrane potential, for example.

Antioxidants such as resveratrol have been promising and have been found to reduce mitochondrial superoxide levels following AA and Rot stimulation, and thus may be a better method of mitochondrial ROS modulation than the targeted antioxidants (Babu *et al.*, 2015).

7.3.2 New biomarkers of VSMC senescence by profiling the Senescence Associated Secretory Phenotype

7.3.2.1 The SASP of SIPS cells

The study profiled the SASP of hCASMC that had undergone replicative senescence. It is worth investigating the SASP released from cells that have been subjected to SIPS, especially by oxidant generation, as the plaque is a highly oxidative environment so the increase in oxidant levels may cause premature senescence in the cells nearby (Wang & Bennett, 2012). However, the proteins that may be detected within this SASP may be proteins generated as a response to oxidative stress; not simply senescence, so care in interpretation will be required.

7.3.2.2 Potential miroparticle generation

To further characterise the VSMC SASP, hCASMC derived from different donors should be investigated as well as using cells that have been senescent for longer as it is currently unknown whether the SASP composition alters with time.

Finally, the release of extracellular vesicles such as MPs or exosomes by senescent VSMC should be investigated as these vesicles may convey a wide array of molecules, including proteins and microRNAs; and have emerged as regulators of cell–cell communication and paracrine signalling mediators (Raposo & Stoorvogel, 2013; Deng *et al.*, 2015). EC-derived MPs have been found to induce senescence in young ECs, a phenomenon which has not yet been investigated in VSMCs according to current literature (Burger *et al.*, 2012). Additionally,

pulmonary artery SMC-derived exosomes were found to contain microRNA that possessed promigratory and pro-angiogenic effects on pulmonary artery ECs (Deng *et al.*, 2015). Both studies highlight the ability of these vesicles to induce alterations in a paracrine manner and as they are known to be mediators of extracellular communication in cardiovascular diseases (Deng *et al.*, 2015), the results observed in the bioassay may include effects of extracellular vesicle action and the biological mediators within, rather than the exclusive action of soluble SASP components.

Appendix

Real Time qPCR Standard Curve



Liquid Chromatography Tandem Mass Spectrometry Protein List

1,4-alpha-glucan-branching enzyme 10 kDa heat shock protein, mitochondrial 14-3-3 protein beta/alpha 14-3-3 protein epsilon 14-3-3 protein gamma 14-3-3 protein zeta/delta 45 kDa calcium-binding protein 6-phosphogluconate dehydrogenase, decarboxylating 6-phosphogluconolactonase 7,8-dihydro-8-oxoguanine triphosphatase 72 kDa type IV collagenase Actin, alpha cardiac muscle 1 Actin, cytoplasmic 1 Actin-related protein 2/3 complex subunit 1B Actin-related protein 2/3 complex subunit 2 Actin-related protein 2/3 complex subunit 3 Actin-related protein 3 Acylamino-acid-releasing enzyme Adenosine deaminase Adenosylhomocysteinase Adenylyl cyclase-associated protein 1 ADM ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 Aggrecan core protein A-kinase anchor protein 12 Alcohol dehydrogenase [NADP(+)] Alcohol dehydrogenase class-3

Aldo-keto reductase family 1 member C1 Aldo-keto reductase family 1 member C2 Aldose 1-epimerase Aldose reductase Alpha/beta hydrolase domain-containing protein 14B Alpha-2-macroglobulin Alpha-actinin-4 Alpha-amylase 1 Alpha-enolase Alpha-L-iduronidase Alpha-N-acetylgalactosaminidase Alpha-N-acetylglucosaminidase Aminoacylase-1 Aminopeptidase B Aminopeptidase N Annexin A1 Annexin A5 Argininosuccinate lyase Arylsulfatase A Arylsulfatase B Aspartate aminotransferase, cytoplasmic Aspartate aminotransferase, mitochondrial Astrocytic phosphoprotein PEA-15 ATP-citrate synthase Attractin Band 4.1-like protein 2 Basement membrane-specific heparan sulfate proteoglycan core protein Beta-2-microglobulin Beta-galactosidase Beta-hexosaminidase subunit alpha Beta-hexosaminidase subunit beta Beta-lactamase-like protein 2 Beta-mannosidase **Biotinidase** Brain acid soluble protein 1 Cadherin-11 Cadherin-6 Calmodulin Calmodulin-like protein 3 Calreticulin Calsyntenin-1 Carbonyl reductase [NADPH] 1 Carboxypeptidase A4 Carboxypeptidase Q Caspase-3 Catalase Cathepsin B Cathepsin D Cathepsin L1 Cathepsin Z CD109 antigen CD166 antigen CD44 antigen CD59 glycoprotein Chitinase-3-like protein 1 Chitinase-3-like protein 2 Citrate synthase, mitochondrial

Coactosin-like protein Cofilin-1 Collagen alpha-1(I) chain Collagen alpha-1(III) chain Collagen alpha-1(IV) chain Collagen alpha-1(V) chain Collagen alpha-1(VI) chain Collagen alpha-1(XII) chain Collagen alpha-2(I) chain Collagen alpha-2(V) chain Collagen alpha-2(VI) chain Collagen alpha-3(VI) chain Collagen triple helix repeat-containing protein 1 Collectin-12 Complement C1g tumor necrosis factor-related protein 1 Complement C1r subcomponent Complement C1r subcomponent-like protein Complement C1s subcomponent Complement C3 Complement component C7 Complement factor B Complement factor H Connective tissue growth factor C-type mannose receptor 2 Cystatin-A Cystatin-B Cystatin-C Cysteine and glycine-rich protein 1 Cysteine-rich motor neuron 1 protein Cytochrome c

Cytosol aminopeptidase Cytosolic non-specific dipeptidase D-dopachrome decarboxylase Decorin Deoxyribonuclease-2-alpha Desmoplakin Dickkopf-related protein 3 Dihydrolipoyl dehydrogenase, mitochondrial Dihydropteridine reductase Dihydropyrimidinase-related protein 2 Di-N-acetylchitobiase Dipeptidyl peptidase 1 Dipeptidyl peptidase 2 Dipeptidyl peptidase 3 Dipeptidyl peptidase 4 Disintegrin and metalloproteinase domain-containing protein 9 DNA-(apurinic or apyrimidinic site) lyase Dystroglycan EGF-containing fibulin-like extracellular matrix protein 1 Elongation factor 1-alpha 2 Elongation factor 2 EMILIN-2 Endoplasmic reticulum resident protein 29 Endoplasmin Endosialin Endothelial protein C receptor Enolase-phosphatase E1 Epididymal secretory protein E1 Epididymis-specific alpha-mannosidase Exostosin-1

Extracellular matrix protein 1 Extracellular superoxide dismutase [Cu-Zn] Ezrin F-actin-capping protein subunit alpha-1 Farnesyl pyrophosphate synthase Fascin Fatty acid-binding protein, epidermal Fibrillin-1 Fibromodulin Fibronectin Fibulin-1 Filamin-A Filamin-C Follistatin-related protein 1 Fructose-bisphosphate aldolase C Fumarate hydratase, mitochondrial Fumarylacetoacetase Galectin-1 Galectin-3 Galectin-3-binding protein Gamma-enolase Gamma-glutamyl hydrolase GDH/6PGL endoplasmic bifunctional protein **GDP-L-fucose** synthase Gelsolin Glucosamine-6-phosphate isomerase 1 Glucosidase 2 subunit beta Glutamate dehydrogenase 1, mitochondrial Glutaredoxin-1 Glutathione peroxidase 3

Glutathione reductase. mitochondrial Glutathione S-transferase omega-1 Glutathione S-transferase P Glutathione synthetase Glyceraldehyde-3-phosphate dehydrogenase Glycine--tRNA ligase Glycogenin-1 Glyoxalase domain-containing protein 4 Glypican-1 Golgi membrane protein 1 Granulins Gremlin-1 Haloacid dehalogenase-like hydrolase domain-containing protein 2 Heat shock 70 kDa protein 1A/1B Heat shock protein beta-6 Heme-binding protein 2 Hemicentin-1 Heterogeneous nuclear ribonucleoprotein A1 Heterogeneous nuclear ribonucleoprotein A3 Heterogeneous nuclear ribonucleoproteins A2/B1 High mobility group protein HMGI-C Histone H2A type 1-B/E HLA class I histocompatibility antigen, Cw-17 alpha chain Hornerin Hydroxyacylglutathione hydrolase, mitochondrial Immunoglobulin superfamily containing leucine-rich repeat protein Insulin-like growth factor-binding protein 4 Insulin-like growth factor-binding protein 5 Insulin-like growth factor-binding protein 6 Insulin-like growth factor-binding protein 7

Integrin alpha-7 Integrin beta-like protein 1 Inter-alpha-trypsin inhibitor heavy chain H2 Interstitial collagenase Intraflagellar transport protein 25 homolog Isocitrate dehydrogenase [NADP] cytoplasmic Isoform 15 of Fibronectin Isoform 2 of Acid ceramidase Isoform 2 of Annexin A2 Isoform 2 of Calsyntenin-1 Isoform 2 of Clusterin Isoform 2 of Coiled-coil domain-containing protein 80 Isoform 2 of Dermcidin Isoform 2 of Discoidin, CUB and LCCL domain-containing protein 2 Isoform 2 of Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 Isoform 2 of Endoplasmic reticulum aminopeptidase 1 Isoform 2 of Eukaryotic initiation factor 4A-II Isoform 2 of Eukaryotic translation initiation factor 5A-1 Isoform 2 of Fructose-bisphosphate aldolase A Isoform 2 of Glucose-6-phosphate isomerase Isoform 2 of Glyoxalase domain-containing protein 4 Isoform 2 of Histone H2A.J Isoform 2 of Histone H2B type 2-F Isoform 2 of HLA class I histocompatibility antigen, A-11 alpha chain Isoform 2 of Inactive serine protease PAMR1 Isoform 2 of Low molecular weight phosphotyrosine protein phosphatase Isoform 2 of Lysosomal Pro-X carboxypeptidase Isoform 2 of Matrix-remodeling-associated protein 8 Isoform 2 of Microfibril-associated glycoprotein 4

Isoform 2 of N-acetyl-D-glucosamine kinase Isoform 2 of Nucleoside diphosphate kinase A Isoform 2 of Pregnancy-specific beta-1-glycoprotein 1 Isoform 2 of Protein disulfide-isomerase A6 Isoform 2 of Protein-L-isoaspartate(D-aspartate) O-methyltransferase Isoform 2 of Signal peptide, CUB and EGF-like domain-containing protein 3 Isoform 2 of Transketolase Isoform 2 of Tropomyosin alpha-3 chain Isoform 2 of Tyrosine-protein phosphatase non-receptor type substrate 1 Isoform 3 of Calumenin Isoform 3 of Glia-derived nexin Isoform 3 of L-lactate dehydrogenase A chain Isoform 3 of Malate dehydrogenase, cytoplasmic Isoform 3 of Nucleoside diphosphate kinase B Isoform 3 of Phosphoacetylglucosamine mutase Isoform 3 of Sulfatase-modifying factor 2 Isoform 3 of Ubiquitin-conjugating enzyme E2 L3 Isoform 4 of Alpha-actinin-1 Isoform 4 of Cadherin-13 Isoform 4 of Caldesmon Isoform 4 of Calumenin Isoform 4 of Extracellular matrix protein 1 Isoform 5 of Acyl-CoA-binding protein Isoform 5 of Branched-chain-amino-acid aminotransferase, cytosolic Isoform 5 of Cysteine-rich with EGF-like domain protein 2 Isoform 5 of Radixin Isoform 6 of Calpastatin Isoform 6 of Cellular nucleic acid-binding protein Isoform 6 of Inactive tyrosine-protein kinase 7

Isoform 7 of Tumor protein D54 Isoform C of Fibulin-1 Isoform Delta of Stromal cell-derived factor 1 Isoform HMG-R of High mobility group protein HMG-I/HMG-Y Isoform LAMP-2C of Lysosome-associated membrane glycoprotein 2 Isoform M1 of Pyruvate kinase PKM Isoform MBP-1 of Alpha-enolase Isoform Sap-mu-9 of Prosaposin Keratin, type I cytoskeletal 10 Keratin, type I cytoskeletal 13 Keratin, type I cytoskeletal 14 Keratin, type I cytoskeletal 16 Keratin, type I cytoskeletal 17 Keratin, type I cytoskeletal 9 Keratin, type II cytoskeletal 1 Keratin, type II cytoskeletal 1b Keratin, type II cytoskeletal 2 epidermal Keratin, type II cytoskeletal 5 Keratin, type II cytoskeletal 6A Keratin, type II cytoskeletal 6B Keratin, type II cytoskeletal 73 Kynurenine--oxoglutarate transaminase 3 Lactovlglutathione lyase Laminin subunit alpha-1 Laminin subunit alpha-4 Laminin subunit alpha-5 Laminin subunit beta-1 Laminin subunit beta-2 Laminin subunit gamma-1 Latent-transforming growth factor beta-binding protein 2

Latexin

Leukotriene A-4 hydrolase LIM and SH3 domain protein 1 LIM domain only protein 7 L-lactate dehydrogenase B chain Low molecular weight phosphotyrosine protein phosphatase Low-density lipoprotein receptor Lumican Lysosomal alpha-glucosidase Lysosomal alpha-mannosidase Lysosomal protective protein Lysosome membrane protein 2 Lysosome-associated membrane glycoprotein 1 Lysyl oxidase homolog 2 Lysyl oxidase homolog 3 Macrophage colony-stimulating factor 1 Malate dehydrogenase, mitochondrial Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA Matrix metalloproteinase-14 Mesencephalic astrocyte-derived neurotrophic factor Metalloproteinase inhibitor 1 Metalloproteinase inhibitor 2 Metallothionein-1E Metallothionein-1X Metallothionein-2 Microtubule-associated protein 1B Microtubule-associated protein 4 Microtubule-associated proteins 1A/1B light chain 3 beta 2 Moesin Myosin-9

Myristoylated alanine-rich C-kinase substrate N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 N(G),N(G)-dimethylarginine dimethylaminohydrolase 2 N-acetylgalactosamine-6-sulfatase N-acetylglucosamine-6-sulfatase NAD(P)H-hydrate epimerase Nestin Neudesin Neuroblast differentiation-associated protein AHNAK Neurofascin Neurofilament medium polypeptide Neuronal pentraxin-1 Neuropilin-1 Neutral alpha-glucosidase AB Nicotinate-nucleotide pyrophosphorylase [carboxylating] Nidogen-1 Nidogen-2 N-sulphoglucosamine sulphohydrolase Nuclear transport factor 2 Nucleobindin-1 Obg-like ATPase 1 Palmitoyl-protein thioesterase 1 Pappalysin-1 PDZ and LIM domain protein 5 Pentraxin-related protein PTX3 Peptidyl-prolyl cis-trans isomerase A Peptidyl-prolyl cis-trans isomerase B Peptidyl-prolyl cis-trans isomerase FKBP10 Peptidyl-prolyl cis-trans isomerase FKBP14

Peptidyl-prolyl cis-trans isomerase FKBP7 Peptidyl-prolyl cis-trans isomerase FKBP9 Peptidyl-prolyl cis-trans isomerase-like 1 Periostin Peroxiredoxin-1 Peroxiredoxin-2 Peroxiredoxin-6 PEST proteolytic signal-containing nuclear protein Phosphatidylethanolamine-binding protein 1 Phosphoglucomutase-1 Phosphoglycerate kinase 1 Phosphoglycerate mutase 1 Phospholipid transfer protein Phosphoserine aminotransferase Phostensin Pigment epithelium-derived factor Plasma alpha-L-fucosidase Plasma protease C1 inhibitor Plasminogen activator inhibitor 1 Plastin-3 Platelet-derived growth factor D Plectin Poliovirus receptor Polypeptide N-acetylgalactosaminyltransferase 2 POTE ankyrin domain family member J Pregnancy zone protein Prelamin-A/C Procollagen C-endopeptidase enhancer 1 Procollagen C-endopeptidase enhancer 2 Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1

Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3 Profilin-1 Prolargin Prolow-density lipoprotein receptor-related protein 1 Prosaposin Prostaglandin-H2 D-isomerase Protein canopy homolog 2 Protein CDV3 homolog Protein CYR61 Protein disulfide-isomerase Protein disulfide-isomerase A3 Protein DJ-1 Protein FAM3C Protein LYRIC Protein NOV homolog Protein S100-A11 Protein S100-A13 Protein S100-A6 Protein-lysine 6-oxidase Purine nucleoside phosphorylase Puromycin-sensitive aminopeptidase Putative 40S ribosomal protein S10-like Putative GTP cyclohydrolase 1 type 2 NIF3L1 Putative hydroxypyruvate isomerase Putative phospholipase B-like 2 Putative trypsin-6 Pyruvate kinase PKM Quinone oxidoreductase Quinone oxidoreductase PIG3 Rab GDP dissociation inhibitor alpha

Rab GDP dissociation inhibitor beta Ras suppressor protein 1 Reticulocalbin-1 **Reticulocalbin-3 Reticulon-4** Rho GDP-dissociation inhibitor 1 **Ribonuclease T2** Ribosome-binding protein 1 S-adenosylmethionine synthase isoform type-2 Secreted frizzled-related protein 4 Selenium-binding protein 1 Semaphorin-7A Seprase Septin-9 Serpin B6 Serpin H1 Serum albumin Serum deprivation-response protein SH3 domain-binding glutamic acid-rich-like protein SH3 domain-binding glutamic acid-rich-like protein 3 Sialate O-acetylesterase Soluble scavenger receptor cysteine-rich domain-containing protein SSC5D SPARC Src substrate cortactin Stanniocalcin-2 Sulfhydryl oxidase 1 Superoxide dismutase [Cu-Zn] Superoxide dismutase [Mn], mitochondrial Suprabasin

Sushi repeat-containing protein SRPX Sushi repeat-containing protein SRPX2 **Target of Nesh-SH3** Testican-1 Tetranectin Thioredoxin Thioredoxin domain-containing protein 12 Thioredoxin domain-containing protein 17 Thioredoxin domain-containing protein 5 Thioredoxin reductase 1, cytoplasmic Thrombospondin-1 Thrombospondin-2 Thrombospondin-3 Thrombospondin-4 Thy-1 membrane glycoprotein Thymosin beta-10 Thymosin beta-4 Tight junction protein ZO-1 Tissue alpha-L-fucosidase TPT1-like protein Transaldolase Transforming growth factor-beta-induced protein ig-h3 Transgelin Transgelin-2 Trans-Golgi network integral membrane protein 2 Translationally-controlled tumor protein Transmembrane glycoprotein NMB Triosephosphate isomerase Tripeptidyl-peptidase 1 Tropomyosin alpha-4 chain

Tryptophan--tRNA ligase, cytoplasmic Tubulin alpha-1B chain Tubulin-specific chaperone A Tubulointerstitial nephritis antigen-like Tyrosine-protein kinase receptor UFO Ubiquitin carboxyl-terminal hydrolase isozyme L1 Ubiquitin carboxyl-terminal hydrolase isozyme L3 Ubiquitin-40S ribosomal protein S27a UMP-CMP kinase UPF0556 protein C19orf10 UPF0587 protein C1orf123 Urokinase plasminogen activator surface receptor UV excision repair protein RAD23 homolog B Vacuolar protein sorting-associated protein 26A Vasorin Versican core protein Vimentin Vinculin V-type proton ATPase subunit S1 WD repeat-containing protein 1 Xaa-Pro dipeptidase Zyxin

Abstracts

Morufat Abisola Sanusi¹ and Karl E. Herbert (2014). Staying Young at Heart. East Midlands Student Conference, University of Leicester

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SFRBM 2015 Posters

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Mitochondria-derived Oxidants and Cellular Senescence

Morufat Abisola Sanusi and Karl E. Herbert, University of Leicester, United Kingdom





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