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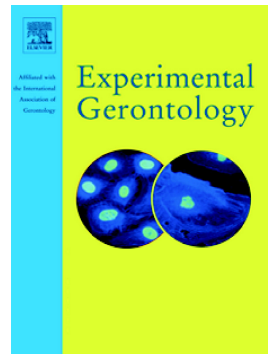
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# Accepted Manuscript

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# Mechanisms Driving the Ageing Heart

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## Abstract

Cardiovascular disease (CVD) is the leading cause of death globally. Although the number one risk factor for CVD is age, the detrimental biological processes that occur in the heart during ageing remain elusive. It is therefore vitally important to understand the fundamental mechanisms driving heart ageing to enable the development of preventions and treatments targeting these processes.

Cellular senescence has been described more than fifty years ago as the irreversible cell-cycle arrest which occurs in somatic cells. Emerging evidence suggests that cellular senescence plays a key role in heart ageing, however the cell-types involved and the underlying mechanisms are not yet elucidated.

In this review we discuss the current understanding of how mechanisms known to contribute to senescence impact on heart ageing and CVD. Finally, we will review recent data suggesting that targeting senescent cells may be a viable therapy to counteract the ageing of the heart.

**Key Words:**

Mitochondria, DNA Damage Response, Reactive Oxygen Species, Telomeres, Senescence.

**Introduction**

Over the last century, the global average life expectancy has risen significantly. This increase is largely attributable to improvements in both healthcare and living conditions. As a result, many countries are now experiencing a demographic shift towards having ageing populations, and this is thought to have contributed to a substantial increase in the prevalence of multiple age-related diseases, such as: cardiovascular disease (CVD), cancer and neurodegeneration. The single most common risk factor for the aforementioned diseases is age and therefore it seems justifiable to focus our research efforts on understanding the fundamental mechanisms which underlie the ageing process *per se*, with the hope of this knowledge being transferrable to a wide-array of age-related debilitating and life-threatening diseases. In this review, we will focus on research which has implicated drivers of cellular ageing, namely: dysfunctional mitochondria, DNA damage and telomere dysfunction, in driving cardiac ageing - with a focus on cellular senescence, a phenomenon evidenced to promote an age-dependent decline in numerous organs, including the heart.

**Cellular Senescence**

Cellular senescence was first described by Hayflick and Moorhead as the irreversible loss of proliferative potential of human somatic cells (Hayflick and Moorhead, 1961) and has traditionally been viewed as a mechanism to prevent uncontrolled proliferation of aged and damaged cells, thus acting as a tumour suppressor (Serrano et al., 1997) . However, cellular senescence has now been shown to be involved in other physiological processes such as embryonic development (Munoz-Espin et al., 2013; Storer et al., 2013), wound healing and tissue repair (Demaria et al., 2014). Contrary to these supposed beneficial roles, senescent cells have also been found to accumulate in several age-

related diseases and different tissues during ageing (Munoz-Espin and Serrano, 2014), and genetic and pharmacological clearance of senescent cells has been shown to ameliorate several age-related pathologies in different disease models and increase lifespan and healthspan in wild-type mice (Baar et al., 2017; Baker et al., 2016; Baker et al., 2011; Chang et al., 2016; Roos et al., 2016; Xu et al., 2015). Senescence can therefore be viewed as an example of antagonistic pleiotropy (Williams, 1957) – as a phenomenon which is beneficial in one setting, yet deleterious in another.

There are numerous theories as to why an accumulation of senescent cells may contribute to age-related pathologies.

At a cell-autonomous level, senescence has been observed to occur in cell populations required for tissue regeneration, for example, progenitor cells from fat tissue and skeletal muscle in progeroid mice are extremely susceptible to senescence (Baker et al., 2011), and therefore it is thought that an age-associated accumulation of senescent cells may lead to a decline in tissue homeostasis.

On a non-cell-autonomous level, a hallmark of cellular senescence is the induction of a pro-inflammatory secretome, known as the Senescence-Associated Secretory Phenotype (SASP) which involves the secretion of a number of factors, such as interleukins and inflammatory cytokines, into the extra-cellular environment (Coppe et al., 2008). Recent work has demonstrated that senescent cells are able to attract different immune cells such as natural killer cells, macrophages and T-lymphocytes which are involved in their specific clearance from tissues (Iannello and Raulet, 2013; Iannello et al., 2013; Kang et al., 2011; Krizhanovsky et al., 2008; Xue et al., 2007). However with age, it is thought that there is an increased induction of senescence in cells, coupled with a decline in the immune system's ability to clear senescent cells, leading to an accumulation and persistence of senescent cells and a chronic SASP. Evidence suggests that a chronic SASP can actually reinforce senescence (Acosta et al., 2013; Kuilman et al., 2008), and unexpectedly promote tumourigenesis in neighbouring cells (Krtolica et al., 2001). The SASP has been shown to elicit a so-called 'Bystander Effect', which can induce DNA damage and induce senescence in a paracrine nature in neighbouring

cells (Acosta et al., 2013; Hubackova et al., 2012). Senescent fibroblasts have also been shown to induce DNA damage in neighbouring cells by cell-to-cell contact via gap junctions, mediated by reactive oxygen species (ROS)(Nelson et al., 2012).

Senescence was first observed to occur following observations that cells would permanently exit the cell cycle after a reproducible number of cell divisions (Hayflick and Moorhead, 1961), which has now been termed 'replicative senescence', thought to occur as a result of attrition of the protective repetitive sequences of DNA at the end of each chromosome – telomeres - with each division, a topic discussed in greater depth below. Since this initial observation, numerous other stressors have been shown to induce senescence, including: aberrant ROS levels, DNA lesions, activated oncogenes, and other metabolic and mitogenic stressors (van Deursen, 2014). There appears to be cross-talk between different stressors, for example sustained activation of a DDR can lead to mitochondrial dysfunction, resulting in elevated ROS production, which can in turn lead to further DNA lesions (Passos et al., 2010). Furthermore, activated oncogenic RAS (Lee et al., 1999) as well as activation of p53/p21 (Macip et al., 2003; Macip et al., 2002), an important senescence effector pathway, can also elevate intracellular ROS levels. Moreover, the rate of telomere shortening has been shown to be accelerated by exposure to mild oxidative stress, thereby contributing to premature senescence (von Zglinicki, 2002).

Upon acute stress, cells may temporarily exit the cell cycle until conditions are more favourable for replication, however if the stress persists above tolerable levels, then cells may undergo senescence or apoptosis, a decision which varies depending on the nature/severity of the stressor and the cell type affected (Childs et al., 2014). In the induction of cellular senescence, the vast majority of senescence-inducing stressors have been shown to activate either or both the p53/p21 or p16INK4a/retinoblastoma protein pathways (van Deursen, 2014). However, despite most senescence-inducing stressors converging upon either the p53/p21 or p16INK4a/retinoblastoma protein pathways, the activation of either or both of these pathways does not provide conclusive

evidence that a cell is senescent. For example, p16 is not activated in all senescent cells, as it was observed that replication-induced senescent human fibroblasts activate p53/p21 but not p16 (Herbig et al., 2004). Furthermore, high levels of p16 expression have been observed in proliferating cancer cells (Nakao et al., 1997; Shapiro et al., 1995). Detection of elevated p21 levels also fail to specifically identify senescent cells, as p21 can be involved in transient cell cycle arrest in response to acute damage (Rodriguez and Meuth, 2006) and has been widely implicated in apoptosis regulation (Gartel and Tyner, 2002).

The induction of cellular senescence is therefore a multifarious phenomenon, which can be induced by numerous stressors in a cell-type dependent manner. Furthermore, the senescent phenotype also can vary dependent on mode of induction and cell type. For example, it has been shown that mitochondrial dysfunction-associated senescent cells have a distinct secretory phenotype when compared to senescent cells induced via genotoxic stress (Wiley et al., 2016). It is due to these reasons that the identification of a universal gold-standard marker for senescence marker remains hitherto elusive. To detect cellular senescence, a cocktail of various markers are therefore used to provide various information. For example, proliferating cells can be excluded from analysis by detecting proteins involved in the cell cycle i.e. Ki-67 and proliferating cell nuclear antigen (PCNA), or cells which have incorporated labelled nucleoside analogues into replicated DNA, however negativity for proliferation markers does not infer senescence, as non-proliferating quiescent cells will also be negative for these markers. Elevated senescence-associated beta galactosidase (SA- $\beta$ -Gal) activity at pH6 has been identified to occur in senescence (Dimri et al., 1995), however activity also increases in fibroblasts which have been cultured at confluence for prolonged amounts of time or in cells under serum starvation (Severino et al., 2000). Numerous other markers have been used to detect senescence including senescence-associated heterochromatin foci (SAHF) (Narita et al., 2003), senescence-associated distension of satellites (SADs) (Swanson et al., 2013), phosphorylated p38MAPK (Freund et al., 2011), however without exception, none are solely specific to senescence in every cell type, and thus our ability to detect cellular senescence *in vivo* is still wanting. Therefore,

understanding the intricacies of the impact of cellular senescence on the ageing of an organ such as the heart, which hosts numerous different cell types, each subjected to various stresses throughout life, will require an extensive research effort. There is however, mounting evidence that heart failure is associated with aberrant reactive oxygen species and elevated DNA damage, both of which have been heavily implicated in driving cellular senescence in numerous cell types and will be the topic of further discussion in this review.

## **Role of Senescence-Inducing Stressors in Cardiac Ageing**

### **Mitochondrial Dysfunction and Reactive Oxygen Species**

In the 1950s, Denham Harman proposed the free radical theory of ageing, which hypothesised that the ageing process is driven by an accumulation of free radical damage over time (Harman, 1956). In 1972, Harman updated his theory to implicate mitochondria as playing a central role in free radical production (Harman, 1972). Since its original formulation, a large amount of data has both supported and challenged this theory. However, more recently, senescent cells have been shown to be associated with dysfunctional mitochondria, leading to elevated ROS levels (Passos et al., 2007) and mitochondria have been shown to be essential in driving numerous of the pro-ageing features of the senescent phenotype, in particular the development of the SASP (Correia-Melo et al., 2016).

Free radicals are a class of atoms or molecules contain one or more unpaired electrons and as such are highly chemically reactive. Reactive Oxygen Species (ROS) is a generic term for a special class free radicals that contain oxygen and have biological functions. The outer shell of oxygen contains six electrons, therefore leaving two unpaired electrons, meaning that oxygen is highly susceptible to radical formation. ROS are generally short-lived, unstable and react with other molecules to attain electron stability. Their family comprises of molecules, ions, radicals including hydrogen peroxide, hypochlorite and hydroxyl radical respectively, as well as superoxide anions which are both an ion and a radical.



While cellular sources of ROS production include, mono-amine oxidase, xanthine oxidase, nitric oxide synthase and NADPH oxidases (NOX) (Holmstrom and Finkel, 2014), the main contributors to ROS production are the mitochondria as a by-product of oxidative phosphorylation-driven during respiration. ROS can also be generated from extra-cellular sources, for example ionising radiation can induce radiolysis of intra-cellular water, leading to production of ROS which can ionise biological molecules and disrupt redox homeostasis (Riley, 1994). Exposure to various environmental compounds including pesticides, herbicides and cigarette smoke can also generate aberrant ROS (Valavanidis et al., 2009). Physiologically, ROS can mediate signalling pathways associated with cell survival and proliferation and has mitogenic effects on cells (McCubrey et al., 2006). Moreover, phagocytic cells such as neutrophils and macrophages produce ROS, mostly via NOX, which are used as a mechanism to combat microbial pathogens (Dupre-Crochet et al., 2013). ROS signalling has been shown to play a key role in cardiac development, for instance, recent research has demonstrated that ROS-induced DNA damage contributes to cardiomyocyte cell cycle arrest and the transition of cardiomyocytes from the proliferative embryonic to postnatal post-mitotic state (Puente et al., 2014).

Despite these physiological roles for ROS, a cell experiencing unregulated levels of ROS can be said to be under 'oxidative stress', a state which can be harmful and can potentially cause irreversible damage to various molecules and structures within the cell (Cui et al., 2012). For example, lipid membranes are vulnerable to oxidative stress-induced lipid peroxidation. Oxidative damage to the unsaturated fatty acids in the lipid membranes is one of the main forms of lipid peroxidation, and can severely damage the structure of the membrane, decrease or alter the activity of membrane-bound enzymes and receptors respectively, and impact on membrane permeability (Brunk and Terman, 2002). In addition to lipid peroxidation, membrane proteins can also be targeted by ROS and result in undesirable crosslinking events between proteins and lipids. Nucleic acids are also highly susceptible to ROS, which can lead to harmful DNA mutations, which will be discussed in further-depth later in the review.

It is thought that oxidative damage to unsaturated fatty acids can contribute to the accumulation of the granular pigment 'lipofuscin', which is composed of an aggregation of oxidised lipids, along with covalently cross-linked proteins, transition metals and oligosaccharides, and is considered to be a marker of cellular ageing (Brunk and Terman, 2002). Post-mitotic cells are particularly prone to lipofuscin accumulation, and an age-dependent lipofuscin accumulation has been observed in numerous cells types such as: cardiomyocytes, neurons and retinal pigment epithelial cells (Brunk and Terman, 2002).

Another detrimental characteristic of ROS lies in their chain-reaction nature - as ROS will interact with a molecule and ionise it in order to stabilise its own electron configuration, and in the process form another free radical. However, this chain-reaction can be terminated, as cells have evolved mechanisms to maintain a homeostatic balance of ROS via the production of antioxidant enzymes including: catalase, superoxide dismutase (SOD), glutathione peroxidases (GSHPx) and glutathione reductase (GR) (Packer, 1995). SOD catalyses the reaction of two superoxide anions into oxygen and hydrogen peroxide, which can then be broken down into water and oxygen by either catalase or GSHPx. Furthermore non-enzymatic antioxidants such as ascorbic acid, vitamin E and glutathione, as well as a number of other small molecules also contribute to ROS homeostasis.

Despite this apparent abundance and redundancy in antioxidant systems, they are not infallible, and ROS levels can become unregulated under various conditions. Unregulated ROS have been implicated in a wide range of disease processes such as: fibrosis, inflammation and tumourigenesis (Cheresh et al., 2013; Ishikawa et al., 2008; Mittal et al., 2014). With regards to cardiac function specifically, cardiomyocytes have an extremely high energy demand resulting in the heart having a basal oxygen consumption rate of around 0.1mL O<sub>2</sub>/g per minute, which is higher than any other tissue in the human body (Goffart et al., 2004). As such, cardiomyocytes have an extremely high volume density of mitochondria and high ROS production (Iglewski et al., 2010). Furthermore, ROS have been associated with cardiac ischaemia-reperfusion injury, cardiac hypertrophy,

atherosclerosis, hypertension, and even cardiac failure (Braunersreuther and Jaquet, 2012; Mugge, 1998; Paravicini and Touyz, 2008). ROS damage can be chronic accumulating over time leading to various pathologies in an insidious manner, or acute for example in cardiac ischaemia reperfusion injury (Braunersreuther and Jaquet, 2012). Coronary heart disease as a result of atherosclerosis, blood clots or coronary artery spasm ultimately results in an ischaemic injury subsequently myocardial infarction. The most effective intervention is timely reperfusion of the myocardium via primary percutaneous coronary intervention (PPCI). Although PPCI can limit myocardial infarction, reperfusion can itself induce ischaemic reperfusion injury (IRI) resulting in myocardial remodelling and progression to heart failure. It is now known that an important component of IRI is the generation of reactive oxygen species as a result of tissue deoxygenation and thereby increased oxidative stress. Although the precise mechanism by which IRI contributes to myocardial remodelling and disease progression remains unclear.

As previously mentioned, mitochondria are one of the main sources of intracellular ROS production. Mitochondria are membrane bound organelles, ranging from around 0.5-1 $\mu$ m in diameter, which are responsible for producing the majority of a cell's chemical energy, via oxidative phosphorylation, in the form of adenosine triphosphate (ATP) (Friedman and Nunnari, 2014). Mitochondria are also responsible for an eclectic mix of other cellular activities such as cellular redox homeostasis, regulation of the membrane potential, ion homeostasis and regulation of apoptosis (programmed cell death), to name but a few (Friedman and Nunnari, 2014). Mitochondrial dysfunction can result in pathophysiological changes associated with ageing including the age-related decline in cardiac function (Ballinger et al., 2002) and as such has been implicated as a major driver of the ageing process. Mitochondria have their own genome which, in humans comprises 37 genes which encode for 13 polypeptides which form subunits of the various protein complexes involved in oxidative phosphorylation termed 'OxPhos' complexes. All other mitochondrial proteins are encoded for in the nuclear genome, which includes an additional 80 OxPhos genes, mitochondrial DNA polymerase gamma, and numerous other proteins. Due to the nature of mitochondria having their own genome,

they cannot be synthesised *de novo* by cells, and are only inherited from the maternal germline (Sato and Sato, 2013).

Within mitochondria, oxidative phosphorylation occurs via a series of redox reactions to produce ATP. Electrons are transported through electron transport chain complexes I-IV in the inner mitochondrial membrane. This involves their reduction by coenzymes such as NADH or FADH<sub>2</sub> and finally oxygen. The resultant protons are pumped out of the mitochondrial matrix creating pH gradient and the subsequent flow of protons back through the mitochondrial matrix provides energy for the synthesis of ATP. Despite the electron transport chain being highly efficient, electrons can escape and as a consequence oxygen is reduced to form superoxide anion (Jastroch et al., 2010).

Despite years of cumulative evidence suggesting ROS increase with age, it has only been relatively recently that studies have started to address questions regarding whether ROS have a causal role in ageing. Various studies have shown that manipulation of anti-oxidant enzymes, both over and under production, has no significant effect on lifespan (Doonan et al., 2008; Perez et al., 2009). Furthermore, neither nuclear- or peroxisome-specific overexpression of catalase had a significant effect on maximal lifespan (Schriner et al., 2005). Interestingly, mitochondria-specific overexpression of the antioxidant catalase was shown to result in a significant extension in both median and maximal lifespan in mice (Schriner et al., 2005). Furthermore, mice overexpressing mitochondrial-targeted catalase demonstrated a significant reduction in oxidative damage, mitochondrial DNA deletions, accumulation of mitochondrial protein oxidation, activation of the calcineurin-nuclear factor of activated T-cell pathway, and displayed a delay in range of age-related cardiac pathological processes including: hypertrophy, ventricular fibrosis and decreased cardiac SERCA2 protein expression (Dai et al., 2009). Independent studies have also demonstrated that mitochondria targeted the plastoquinone-containing antioxidant SkQ1 significantly increases in the lifespan of both mole-voles and hamsters (Anisimov et al., 2011) and mice deficient in mitochondrial superoxide dismutase display severely premature degeneration of neurons and cardiomyocytes, with around

10% mice exhibiting characteristics of dilated cardiomyopathy (Lebovitz et al., 1996). Aside from genetic manipulation of antioxidant enzymes, double knock out mice for *P66shc*, a gene encoding a redox enzyme which can generate hydrogen peroxide and act as a signalling molecule for mitochondrial-driven apoptosis (Giorgio et al., 2005), have an increased resistance to both ultraviolet light and hydrogen peroxide induced apoptosis, a reduction in ROS generation, enhanced resistance to ischaemia, and a significant lifespan extension (Galimov, 2010; Migliaccio et al., 1999). Monoamine oxidase A (MAO-A) is a protein also linked with driving oxidative stress - it is located at the outer mitochondrial membrane, involved in catalysing the oxidative deamination of monoamines, and produces hydrogen peroxide as one of its by-products (Villeneuve et al., 2013; Youdim and Bakhle, 2006). Furthermore, cardiomyocyte-specific over-expression of MAO-A in mice results in elevated ROS and drives cardiomyocyte necrosis and chronic ventricular dysfunction (Villeneuve et al., 2013). Together these data specifically support a specific role for mitochondrial dysfunction in cardiac ageing. Studies have shown that mitochondrial ROS production increases significantly with age in both the heart (Judge et al., 2005) and the vascular system (Ungvari et al., 2007) and this has been related to an increase in dysfunctional mitochondria as a direct result of reduced activity in electron transport complexes I and IV, resulting in increased electron leakage and thereby elevated mitochondrial ROS (Navarro and Boveris, 2007). Considering the observed beneficial effects of reduced mitochondrial ROS on cardiovascular health, it will be important to understand if these effects are a result of systemic or cardiac-specific mechanisms, and if the latter, what are the relative contributions of the different cardiac cell types to cardiovascular ageing.

A mitochondrial 'vicious cycle' theory has also been postulated which suggests that mutations to the mitochondrial genome lead to the production of abnormal electron transport chain proteins impairing the function of oxidative phosphorylation. This increases ROS production contributing to more mitochondrial dysfunction, a further increase in ROS production and a harmful positive feedback loop (Bandy and Davison, 1990). In opposition to this theory, research showed that embryonic fibroblasts from a transgenic knock-in mouse model for a proof-reading-deficient version

of mitochondrial DNA polymerase subunit PolgA, despite having significantly elevated levels of mitochondrial DNA mutations and a dysfunctional respiratory chain, had no significant differences in ROS levels (Trifunovic et al., 2005). However more recently, it was shown, using the mitochondria-targeted mass spectrometry probe MitoB, a significantly more sensitive technique, that hydrogen peroxide levels are indeed elevated in the mitochondria of the same mouse model (Logan et al., 2014). The group went on to demonstrate that although there was no detectable difference in mitochondrial hydrogen peroxide levels in young mice, in mice older than 35 weeks hydrogen peroxide levels are significantly higher in both the kidney and the heart.

The mitochondrial genome may also be more sensitive to oxidative stress, as frequency of the oxidised base 8-hydroxydeoxyguanosine (Oh8dG), was observed to be around 16 times higher, per base pair, in mitochondrial DNA compared to nuclear DNA (Richter et al., 1988). Explanations for this could include the proximity of the mitochondrial genome to sites of ROS production, or the lack of histones to provide protection (Richter et al., 1988). Interestingly, levels of Oh8dG in mtDNA were found to have an inverse correlation with maximum lifespan when examined in both the brain and heart of several mammalian species (Barja and Herrero, 2000).

Mounting evidence has implicated the role of oxidative stress in the development and progression of heart failure (Tsutsui et al., 2001), and electron spin resonance (ESR) spectroscopy detected elevated hydroxyl radicals in the failing myocardium in human patients (Hill and Singal, 1996; Ide et al., 2000), however no decreases in antioxidant activity have been observed in the failing heart (Tsutsui et al., 2001), suggesting elevated ROS production is the cause. However, cumulative data demonstrates that interfering with ROS levels as a means of therapy is extremely complicated, particularly due to the roles ROS plays under physiological conditions. For example, it has even been suggested that the beneficial effects of exercise could be partly due to exercise-induced ROS production, which could stimulate upregulation of DNA repair proteins, protein degrading enzymes and antioxidants (Radak et al., 2005). This supports the mitochondrial hormesis (mitohormesis) theory which stipulates that

low level ROS stimulates an upregulation of stress resistance mechanisms which manage to reduce oxidative stress in the long term (Ristow and Schmeisser, 2014). One possible mechanism for the mitohormetic response is via the transcriptional co-activator PGC-1 $\alpha$ , which is upregulated by a number of stresses such as ROS, reactive nitrogen species and cold-exposure, as well as endurance exercise (Pilegaard et al., 2003). Moreover, PGC-1 $\alpha$  has been shown to be responsible for up-regulation of a number of antioxidant enzymes such as SOD2 and GPx1 (St-Pierre et al., 2006). PGC-1 $\alpha$  is also involved in mitochondrial biogenesis, and thus could be contributing to maintenance of healthy mitochondria (Wu et al., 1999).

### **DNA Damage and Impaired Repair**

Our DNA is subjected to constant insults, both by endogenous sources such as ROS or exogenous sources such as UV irradiation, and DNA damage has been shown to accumulate with age and negatively affect essential biological processes such as transcription and replication. If DNA damage reaches a critical threshold, cells can undergo programmed cell death (apoptosis) or irreversible cell cycle arrest (senescence), which can heavily impact upon tissue homeostasis and function (Elmore, 2007). As such, DNA damage is associated with numerous genetic disorders, tumorigenesis and implicated in driving the ageing process (Garinis et al., 2008). Multiple cardiac pathologies, such as coronary artery disease, atherosclerosis and heart failure have also been associated with DNA damage (Kovacic et al., 2011). For example, patients who have been exposed to large doses of radiation, a known DNA damaging agent, have elevated risk of developing cardiovascular disease (Adams et al., 2003; Little, 2010; Mone et al., 2004; Shimizu et al., 2010). Furthermore, women undergoing radiotherapy for left breast cancer have a higher incidence of cardiovascular disease (Darby et al., 2003; Taylor et al., 2009; Taylor et al., 2008) even when compared to those who receive radiotherapy for right breast cancer, suggesting a causal role for ionising radiation in damaging the heart specifically, as opposed to a systemic response to radiation.

ROS is responsible for causing numerous types of DNA damage, including: intra-/inter-strand DNA crosslinks, protein-DNA adducts, single strand breaks, abasic sites, as well as non-bulky damage such

as formamidopyrimidine and 8-oxoguanine and bulky base modifications including etheno-DNA adducts and cyclopurine lesions. Indeed it has been calculated that intra-cellular ROS is responsible for the genetic modification of tens of thousands of bases of DNA per cell every day (Rao, 1993). If the damage is left unrepaired, modifications such as 8-oxoguanine can drive mutations upon DNA replication as DNA polymerase can incorrectly pair adenine to pair with 8-oxoguanine, leading to mutations which can impact on cellular function by altering both gene regulation and protein function.

To combat the accumulation of DNA damage, cells have evolved a multifaceted DNA Damage Response (DDR) system. The DDR comprises a number of intricate and complicated mechanisms which are essential for maintaining genomic integrity in the face of constant damage-inducing agents and processes. A dysfunctional DDR has been associated with cardiac pathologies such as ischaemia-reperfusion injury and atherosclerosis, as well as a plethora of other diseases including tumourigenesis, ataxia telangiectasia, xeroderma pigmentosum, and progeria diseases such as Werner syndrome, Rothmund-Thomson syndrome and Bloom syndrome (Cervelli et al., 2012; O'Driscoll, 2012).

In response to DNA damage, members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family can be activated and subsequently amplify the DNA damage signal, allowing downstream effector proteins to take the necessary action in relation to halting the cell cycle, the restructuring of chromatin and repairing the DNA damage (Rouse and Jackson, 2002). Ataxia-telangiectasia Mutated (ATM) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) mainly respond to double strand breaks, whereas Ataxia-telangiectasia and Rad3-related (ATR) is activated by stalled DNA replication forks and single-stranded DNA damage (Falck et al., 2005). Upon induction of a double strand DNA break, autophosphorylation occurs on Ser<sup>1981</sup> of the ATM dimer, resulting in dimer dissociation, which consequently frees the kinase domain of monomeric ATM, thus allowing the phosphorylation of downstream substrates containing the ATM consensus target



sequence (Bakkenist and Kastan, 2003). Independently of ATM activation, multiple proteins get recruited to the site of damage, including 53BP1, BRCA1 and the MRN complex (Kastan, 2008). ATM recruitment to double strand breaks has been shown to be facilitated by the NBS1 protein of the evolutionary conserved Mre11-Rad50-Nbs1 (MRN) complex (Carson et al., 2003). This activated ATM kinase can then phosphorylate substrates such as BRCA1, 53BP1, NBS1 and histone H2AX, which all play a role in the DDR (Kastan, 2008). Ataxia telangiectasia is an autosomal recessive disease resulting from mutation in the ATM gene, causing a severe lack of DNA repair capability, with homozygous sufferers presenting severe neurodegenerative disorders. Furthermore, even heterozygous carriers have an increased risk of breast cancer and are at higher risk of developing ischaemic heart disease, which is coupled with an accelerated onset of around 11 years when compared to the average onset of ischaemic heart disease in non-ATM-carriers (Su and Swift, 2000).

Base excision repair is also an important DDR process and is responsible for removing and replacing nitrogenous bases which have become damaged, usually due to alkylation, deamination or oxidation. This requires accurate recognition and excision of the damaged base via DNA glycosylase, leaving an abasic site, which is followed by a cleavage in the DNA backbone and allows access for DNA polymerase and DNA ligase to replace the missing base. Base excision repair has been shown to be significantly upregulated as a result of induced MI (Yndestad et al., 2009), thus highlighting that increased genomic stress is part of the pathophysiology of ischaemic injury.

Another important DDR protein that has been implicated in cardiovascular disease is Poly (ADP-ribose) polymerase (PARP). The main function of PARP is the recognition of single- and double-strand breaks and subsequently signalling for repair. PARP catalyses the cleavage of NAD<sup>+</sup> into ADP-ribose and nicotinamide, with polymers of ADP-ribose forming that can bind to and give a negative charge to histones. This process creates an electro-static repulsion between the DNA and the histones, which alters the configuration of the chromatin to allow accessibility to DNA repair proteins. Under physiological conditions of mild oxidative stress, PARP plays an important role in

DNA damage repair (Luo and Kraus, 2012). However, in more severe stress situations, over-activation of PARP has been shown to deplete its substrate NAD<sup>+</sup>, which has a knock-on effect on oxidative phosphorylation and reduces ATP production (Szabo et al., 2004). To prevent the cell from undergoing necrosis due to complete ATP ablation, PARP is cleaved by caspases preventing NAD<sup>+</sup> decline thereby allowing damaged cells to produce enough energy to undergo apoptosis instead of necrosis. Apoptosis is a much more favourable mechanism than necrosis as it is highly regulated and reduces the net damage to the surrounding tissue. The activation of PARP has been observed in cardiac pathologies, including ischaemia/reperfusion and circulatory shock (de Boer et al., 2000; Pacher et al., 2002). As a result the potential of using PARP inhibitors therapeutically, to prevent long term damage after myocardial infarction, is an area of intensive research. Early data is promising and it has already been demonstrated that both genetic and small molecule inhibition of PARP can attenuate myocardial remodelling in the aortic banding model (Xiao et al., 2005).

### **Telomere Dysfunction**

Eukaryotic organisms have evolved specialised structures, known as telomeres, to protect the ends of chromosomes. Telomeres are repetitive sequences of DNA located on both ends of each chromosome in eukaryotic organisms. In vertebrates, the sequence consists of a 5' – 3' hexameric repeat of the nucleotides TTAGGG, which in mice are thought to be up to 30 kilobases in length (Doksani et al., 2013). To shelter the exposed end from eliciting a DDR, telomeres have a single stranded 3' overhang of around 150 to 200 nucleotides, which can invade, displace, and bind to upstream telomeric DNA, thus forming a lariat-like structure known as the t-loop. The t-loop was first observed via electron microscopy of purified telomeric restriction fragments (Griffith et al., 1999), and has more recently been confirmed via super-resolution fluorescence microscopy (Doksani et al., 2013). The formation of the t-loop structure is facilitated by the association of a protein complex, known as 'shelterin', which associates with the telomeric repeat region. The proteins: TRF1, TRF2 and POT1, all have telomeric repeat recognition domains, and they are associated with a number of other proteins such as TIN2, TPP1 and Rap1. TRF2 has been shown to be quintessential

for the formation of the t-loop, as its deletion results in linear, or 'uncapped', telomeres, whereas deletion of TRF1, POT1, or Rap1 does not affect the frequency of observed t-loops (Doksani et al., 2013). In addition to its role in facilitating the formation of the t-loop, TRF2 has also been shown to inhibit non-homologous end joining, thus increasing the level of protection of telomeres being recognised as DNA damage (Bae and Baumann, 2007).

Due to the nature of DNA polymerase only being capable of synthesising DNA in the 5'-3' direction, there is an intrinsic inability for the replication machinery to fully replicate both strands of each telomere, and thus with each cell division, the telomeres get progressively shorter. Moreover, DNA replication is undertaken at replication forks, which form via DNA helicase unwinding double stranded DNA to create a two-pronged fork structure consisting of two single-stranded DNA sequences, which act as templates for replication. On the 5' strand, known as the 'leading strand', DNA polymerase can bind via one RNA primer and then continue to synthesise DNA continuously as DNA helicase unwinds further DNA template for replication. However, on the 3' strand DNA polymerase cannot synthesise DNA in the same direction as the replication fork is moving. Therefore replication occurs in short fragments, known as 'Okazaki fragments', in the opposite direction to the movement of the replication fork which has been coined the 'lagging strand'. In short, the enzyme primase synthesises an RNA primer, which is laid down to act as template for DNA polymerase III to bind to and then synthesise new DNA in the 5' to 3' direction. DNA polymerase III continues until it reaches the previously laid RNA primer downstream. DNA polymerase I then removes the RNA primer and replaced the ribonucleotides with deoxyribonucleotide and DNA ligase then joins together the fragments. Once the end of the chromosome is reached, the terminal RNA primer cannot be replaced with DNA as there is no further DNA template in the 5' direction. The RNA primer is then degraded which results in a short-single stranded 3' overhang, thus shortening the telomere, an attrition known as the "end replication problem", first theorised to occur years prior to the discovery of telomeres (Olovnikov, 1971, 1973; Watson, 1972). Telomeres also appear to be are more susceptible to oxidative stress and similar to the mitochondrial DNA discussed above,

demonstrate a higher rate for the accumulation of single strand breaks as result of reactive oxygen species stress (Sitte et al., 2000; von Zglinicki et al., 1995). Together, these two processes lead to an erosion of around 50-200 base pairs each cell division from the telomere inwards (Harley et al., 1990). Once telomeres reach a critical length, it is thought that the Shelterin complex can be disrupted and the telomere recognised as DNA damage, which leads to the recruitment of various DNA damage response proteins, activation of a DNA damage response and can result in a permanent cell cycle arrest known as 'replicative senescence' (d'Adda di Fagagna et al., 2003; Hayflick, 1965). It has been proposed that a threshold of 5 telomeric DNA damage foci can predict the onset of senescence in human fibroblast cells (Kaul et al., 2012). Multiple human progeria syndromes, such as dyskeratosis congenita, Werner syndrome and ataxia telangiectasia have been shown to display accelerated telomere shortening (Alter et al., 2012; Metcalfe et al., 1996; Wyllie et al., 2000).

Due to negligible proliferation of cardiomyocytes throughout an organism's lifespan (Bergmann et al., 2009), it is unlikely that they will undergo significant end-replication-associated telomere shortening. However, models of severe telomere shortening, for example late generation TERC<sup>-/-</sup> mice, have been associated with an increased risk of heart dysfunction and have significantly lower numbers of cardiomyocytes (Fuster and Andres, 2006). Contrary to this, the mammary artery has significantly larger telomeres than other arteries, and displays a formidable resistance to developing atherosclerosis (Fuster and Andres, 2006).

Telomere attrition can be avoided via ectopic expression of telomerase, which replenishes the telomeric DNA and thus maintains the telomeres from reaching a critically short length (Bodnar et al., 1998). Telomerase is a reverse transcriptase, which consists of a catalytic domain, known as Telomerase Reverse transcriptase (TERT), which recognises the 3'-OH group at the end of the G-rich overhang and elongates the telomeric DNA (Blasco, 2005). Moreover, this elongation is made possible by the Telomerase RNA Component (TERC), which also binds to the 3' overhang and creates a template for TERT to add complementary nucleotides (Blackburn, 2001). Telomerase expression

can be viewed as a double-edged sword in that, while telomere elongation is beneficial in maintaining telomere length in cells such as stem cells and germ cells, aberrantly upregulated telomerase expression occurs in the vast majority of tumour cells to avoid telomere-shortening mitotic catastrophe occurring (Jafri et al., 2016). A telomerase-independent mechanism for telomere elongation, known as alternate-lengthening of telomeres (ALT), is observed in most telomerase negative tumour cells (Bryan et al., 1997), thus highlighting the importance for telomere maintenance in tumour cells.

As telomerase activity is low or absent in the majority of human somatic cells telomere shortening and replicative senescence can occur. TERC knock out mice have an inability to elongate telomeres, this is inclusive of the germline, and thus if TERC knock out mice are bred with one another, each generation is born with progressively shorter telomeres than the last. As a result, early generations of TERC knock-out mice are relatively healthy, although later generations show increased chromosome end-to-end fusions, resulting from uncapped telomeres, which is accompanied with a number of age-related health issues such as reduced leukocyte numbers, spleen and small intestine atrophy, impaired wound healing and a significantly shorter lifespan than wild type mice (Samper et al., 2001). Late generation TERC knock-out mice have also been shown to undergo myocardial remodelling and cardiac dysfunction (Wong et al., 2009). Patients from families with dyskeratosis congenital due to mutations in either TERC or TERT have accelerated telomere shortening and are more susceptible to cardiac fibrosis, dilated cardiomyopathy and myocardial infarction (Basel-Vanagaite et al., 2008; Vulliamy et al., 2004). An elevated risk for coronary artery disease has also been observed in patients with SNPs in loci resulting in aberrant telomere length (Ding et al., 2014; Maubaret et al., 2013). However, the use of TERC knock-out mice to fully understand telomere-associated ageing is questionable for the population at large, as the degree of telomere shortening in late generation mice far exceeds the shortening experienced during normal ageing (Rudolph et al., 1999), and especially in cell types such as cardiomyocytes which have extremely low levels of cell division throughout life (Bergmann et al., 2009; Bergmann et al., 2015; Richardson et al., 2015;

Senyo et al., 2014) and thus should not be associated with excessive end-replication-associated telomere shortening. We have previously demonstrated that in the murine heart, a number of cell populations express telomerase including a rare subset of cardiomyocytes and in response to injury telomerase activity is increased (Richardson et al., 2012). Subsequent studies have demonstrated that elevated telomerase activity has a protective effect on the myocardium following ischaemic injury (Fuster and Andres, 2006). Furthermore, it has been shown that telomerase is essential for heart regeneration in zebrafish, with telomerase-mutant zebrafish presenting a severe lack in cardiomyocyte proliferation following cryo injury, which is associated with chronic cardiac dysfunction (Bednarek et al., 2015). Together these studies hint at an important role of telomerase in the heart and potentially in cardiomyocyte function, although again given the rare cycling nature of the cardiomyocytes and the observations that multiple generations of TERC knockout are required to elicit a cardiac phenotype this is unlikely to be simply the maintenance of telomeres.

Telomere damage may also occur independently of telomere length, with the proposed model suggesting this is the consequence of a physical double-strand break which then elicits a DDR within the telomeric region (Fumagalli et al., 2012; Hewitt et al., 2012). This form of telomere damage was coined 'Telomere Associated DNA Damage Foci (TAF) (Hewitt et al., 2012), to distinguish from 'Telomere-induced-foci' (TIF), thought to arise as a result of t-loop uncapping. TAF were initially thought to be irreparable due to their persistence following induction, with evidence suggesting that non-homologous end joining (NHEJ) is inhibited at telomeres due to the shelterin proteins TRF2 and RAP1 preventing the action of DNA-PK and ligase IV (Bae and Baumann, 2007). Contrary to this however, it has recently been demonstrated that TAF can be repaired either via homologous recombination, or by a PARP1/Lig3-Dependent pathway (Doksani and de Lange, 2016; Mao et al., 2016). However, both of these mechanisms elicit repair during S-phase of the cell cycle, which may explain the persistence and assumed irreparability of telomeres in cells which have been subjected to insult great enough to induce permanent exit from the cell cycle *vis-a-vis* cellular senescence, a topic discussed in more depth below.

The studies above highlight the requirement for future research to address a number of questions regarding telomere biology relating to cardiovascular health, and in particular cardiomyocytes. For example, are cardiomyocytes susceptible to TAF, and if so, due to negligible proliferation and unlikelihood of entering S-phase, would this damage remain persistent, and if so, what would be the downstream effect of this damage on individual cells and on cardiac function in general?

### **Evidence for Cardiac Senescence**

Recent research has shown that there is an increase in senescent markers in cardiac tissue with age (Baker et al., 2016). Smooth muscle cells of the aortic root wall along with both the atrial and ventricular surface of the heart display an age-dependent increase of SA- $\beta$ -Gal activity, with transmission electron microscopy detecting X-Gal crystals in both fibroblasts and ciliated epithelial cells within the pericardium (Baker et al., 2016). Furthermore, the chemotherapy drug doxorubicin, which is known to be cardio-toxic and contributes to cardiac dysfunction in a growing number of cancer patients, can induce DNA damage and cardiac senescence (Maejima et al., 2008). Among patients who have undergone radiotherapy treatment for breast cancer, those which were treated for left breast cancer have an increased incidence of cardiac disease compared to those treated for right breast cancer (Darby et al., 2003; Taylor et al., 2009; Taylor et al., 2008). Moreover, the mechanism for this is not understood, however, X-irradiation is known to cause senescence in numerous cell types, and it will therefore be important to investigate if senescence is occurring and contributing to disease progression.

Monocytes have an increased affinity to bind to senescent endothelial cells, as shown in X-ray-induced or replication-induced senescent endothelial cells, which display elevated SA- $\beta$ -Gal activity (Lowe and Raj, 2014). These data could provide an explanation as to why exposure to ionising radiation can lead to cardiovascular disease, as the initial stages of atherosclerosis involve the binding of circulating monocytes to endothelial cells, followed by their infiltration into the intima, subsequent transformation into macrophages and then absorption of cholesterol. This effect was

shown to be attenuated by down-regulation of the cell-surface glycoprotein CD44, which is increased in senescent endothelial cells, and thus provides a possible therapeutic target.

### **Cardiomyocyte Senescence?**

Traditionally, senescence has been viewed as a mechanism to prevent uncontrolled replication in proliferative cells, thus acting as a tumour suppressor mechanism. However, research has begun to emerge that cellular senescence is not restricted to proliferative cell types and that post-mitotic cells can also develop a senescent-like phenotype. For example, both murine cortical and purkinje neurones display an age-dependent increase in numerous senescent markers, including increased  $\gamma$ H2AX, IL-6, 4-HNE, activated p38 MAPK and SA- $\beta$ -Gal activity (Jurk et al., 2012). These senescent-like changes were also observed in neurones from late generation TERC<sup>-/-</sup> mice, however were attenuated in mice following deletion of p21 (Jurk et al., 2012). Furthermore, a senescent-like phenotype has also been observed in non-proliferative adipocytes from mice either fed on a high calorie diet, or transgenic G4TERC<sup>-/-</sup> mice, including an up-regulation of pro-inflammatory cytokines, elevated p53 expression and increased SA- $\beta$ -Gal activity (Minamino et al., 2009), which can be rescued in mice by deletion of p53. Post-mitotic osteocytes have also recently been shown to display an age-dependent increase in p16 expression, coupled with various senescent markers, including telomere dysfunction, SASD and develop the SASP (Farr et al., 2016). Therefore, a senescent-like phenotype can be induced in post-mitotic cells by both the p16/Rb and p21/p53 effector pathways, similar to that of proliferative cells.

It may seem counter-intuitive that a mechanism to prevent replication, *vis-à-vis* senescence, would have evolved to occur in cells which have already permanently exited the cell cycle or organs such as the heart which are essentially resistant to cancer. However, the evolutionary origin of cellular senescence may not have been to serve as a tumour suppressor mechanism. Moreover, senescence has been observed to play a vital role in mammalian embryonic development, by contributing to morphological development by promoting tissue remodelling (Munoz-Espin et al., 2013), and therefore stress-induced senescence in adult organisms may be a re-ignition of a developmental



program, as opposed to an independently arisen phenomenon. It is clear that this mechanism would give an evolutionary advantage for organisms to repress tumourigenesis, whilst perhaps not being subjected to evolutionary pressure to turn the mechanism off in post-mitotic cells in aged organisms. Therefore, it may be that senescence hasn't evolved to occur in post-mitotic cells *per se*, rather organisms simply have not evolved to prevent its induction following stress.

Although once thought to be a post-mitotic cell type, mounting evidence shows that cardiomyocytes do retain proliferative potential, albeit limited. Shortly following birth, cardiomyocytes have been shown to undergo mass cell cycle arrest due to activation of a DDR following exposure to the oxygen-rich postnatal environment (Puentes et al., 2014). However, contrary to initial beliefs that all the cardiomyocytes a human will ever have are present at birth, new cardiomyocyte generation was shown to occur, following the observation that carbon-14, produced as a consequence of the Cold War nuclear bomb testing, was incorporated into the DNA of cardiomyocytes in adult patients, thus revealing new cardiomyocyte generation (Bergmann et al., 2009). Subsequently, it has been shown that cardiomyocytes themselves can undergo mitosis, as opposed to their generation being reliant upon progenitor cell types such as cardiac stem cells (Malliaras et al., 2013; Senyo et al., 2014). Cardiomyocytes have also been observed to elicit a regenerative response (Bergmann et al., 2015; Hsieh et al., 2007; Richardson et al., 2015; Senyo et al., 2014), albeit limited, in response to cardiomyocyte loss, which involves cardiomyocyte proliferation (Hsieh et al., 2007). In more recent studies it has been demonstrated that cardiomyocyte turnover declines with age in both human and mice (Bergmann et al., 2015; Richardson et al., 2015). Moreover regenerative potential may also be compromised in the elderly (Richardson et al., 2015). Hitherto, the reason for this decline in cardiomyocyte turnover is poorly understood. Cardiomyocyte senescence is one possible explanation, indeed cardiomyocytes treated with doxorubicin activate classical senescence phenotypes such as upregulation of the cyclin-dependent kinase inhibitors p16 and p21, as well as increased SA-beta-gal activity (Spallarossa et al., 2009). However whether cardiomyocyte senescence exists *in vivo* is still a questionable and most studies providing evidence for senescence

markers have relied on using whole heart tissue due to the difficulty in isolating pure cardiomyocytes. One study shows age-related increases in p16 levels in murine cardiomyocytes *in vivo* (Torella et al., 2004), however myocytes undergoing apoptosis or necrosis were also positive for p16, thus highlighting the need for more specific senescence markers to fully elucidate if cardiomyocyte senescence *in vivo* truly exists.

If cardiomyocyte senescence was conclusively shown *in vivo*, there are numerous key questions which would need to be addressed. Firstly, what would the mechanism for cardiomyocyte senescence *in vivo* be, as canonical replicative senescence would seem an unlikely explanation due to the due to low level of cardiomyocyte proliferation through mammalian lifespan. Secondly, considering the low level of cell division, would cessation of proliferation impact on tissue homeostasis and regeneration. It would also be important to determine if these cells are associated with a SASP and if this was involved in cardiomyocyte clearance and/or capable of inducing a detrimental bystander effect in neighbouring cells.

### **Combatting Senescence**

Senescent cells have long been associated with the ageing process and recently been implicated in driving it, as inducible clearance of p16Ink4a-positive senescent cells in the BubR1 knock-out progeria mouse model, delays the onset of many age-related pathologies (Baker et al., 2011), and more recently the same model has been used to show that clearance of p16Ink4a-positive senescent cells can delay age-related diseases associated with physiological ageing (Baker et al., 2016; Xu et al., 2015). In the mouse heart, the authors show an upregulation in gene expression of numerous cyclin-dependent kinase inhibitors, such as p16, p19 and p21, between the ages of 12 and 18 months in mice hearts. However, if transgenic mice were treated with bi-weekly doses of AP20187 to specifically induce apoptosis in p16INK4a expressing cells, the increase in the expression level of these genes is attenuated between these ages, which correlates with a reduction in cardiac hypertrophy and rescue of stress tolerance in aged mice (Baker et al., 2016). Global deletion of *P53*,

a master transcriptional regulator of both senescence and apoptosis, has also been shown to protect against induced heart failure (Liu et al., 2004; Shizukuda et al., 2005).

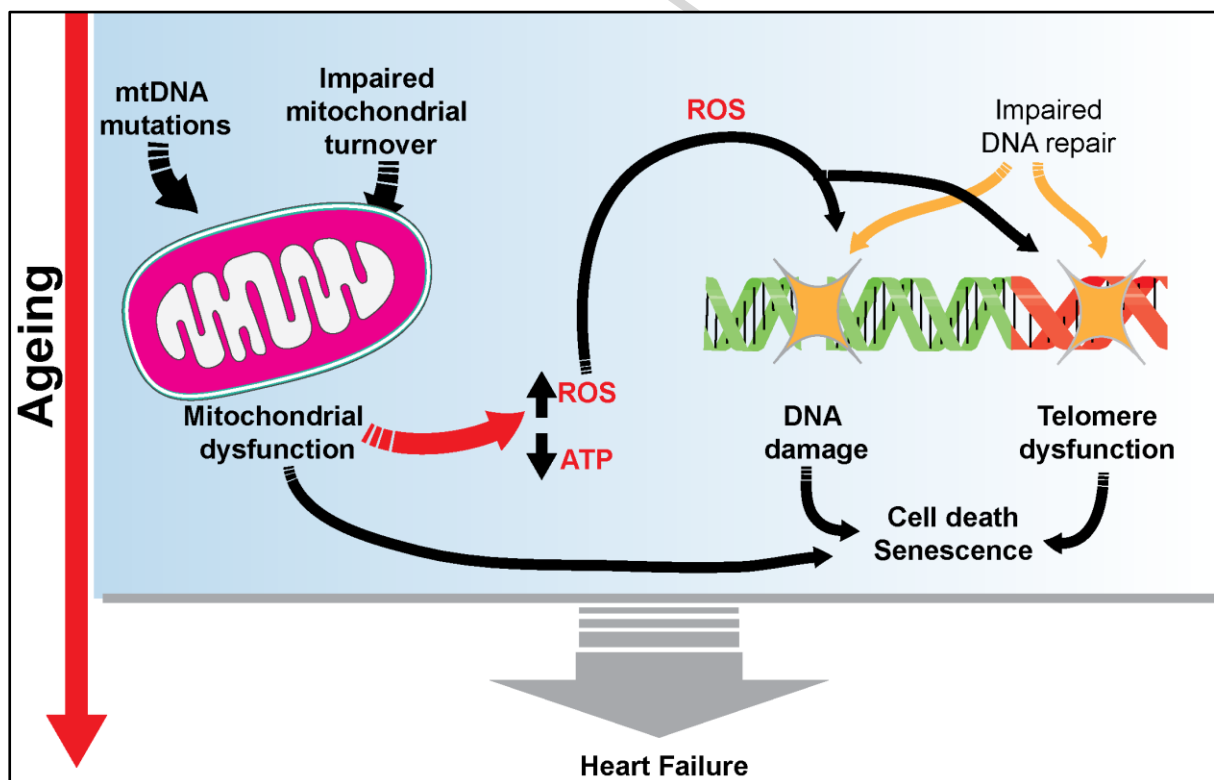
Due to causal evidence arising that senescent cells can contribute to driving age-related diseases, research efforts are now focusing on the possibilities of therapeutically targeting senescent cells to either kill them or attenuate pro-ageing associated features such as the SASP, a field which has been coined 'senotherapy'. Interestingly, drugs that can specifically eliminate senescent cells commonly known as 'senolytics' have been shown to have beneficial effects during heart ageing or upon exposure to stresses which induce heart dysfunction, therefore highlighting the great importance for investigation into the role of senescence in the ageing heart. Dasatinib and quercetin (D+Q) when given in combination have been shown to reduce senescent cells in aged, radiation-exposed, progeroid, high-fat diet mice improving several parameters of Healthspan (Ogrodnik et al., 2017; Zhu et al., 2015). In old mice, D+Q was shown to ameliorate several parameters of heart dysfunction including left ventricle ejection fraction and fractional shortening. Moreover, D+Q administration was shown to improve vasomotor function in aged and hypercholesterolaemic mice (Roos et al., 2016). Inhibitors of the BCL-2 family such as navitoclax have been shown to reduce senescent hematopoietic and muscle stem cells in radiation-exposed and aged mice (Chang et al., 2016). A FOXO4 peptide which perturbs the FOXO4 interaction with p53 was recently shown to clear senescent cells and improve several healthspan parameters in aged and progeroid mice, however its impact in the heart has not been investigated (Baar et al., 2017).

Whilst there are promising signs for the removal of senescent cells leading to an improvement of cardiac function, further research is required to elucidate whether these beneficial effects are due to the specific removal of senescent cells within the heart, or whether these are systemic effects resulting from total organismal removal and not cardiac-specific. Furthermore, what remains an important question to address is specifically which cell types within the heart become senescent, and what the relative contribution of these is.

## Summary

As human lifespan continues to increase and countries experience demographic shifts towards having ageing populations, it is likely that incidence of CVD will continue to rise. Evidence has implicated both DNA damage and mitochondrial dysfunction in contributing to an age-related decline in heart function (Figure 1). Moreover, it is of paramount importance that we continue to research the basic mechanisms which drive age-related heart dysfunction, in order to further understand the risk factors and develop interventions to attenuate disease initiation and progression.

## Figure & Legend



**Figure 1 - Linking Mitochondrial Dysfunction with DNA Damage.** Both mitochondrial DNA mutations and impaired mitochondrial turnover can lead to mitochondrial dysfunction, which can result in low ATP production and increased ROS. Elevated mitochondrial ROS can cause further mitochondrial dysfunction, leading to further increases in ROS production, thus forming a 'vicious cycle'. Both

elevated ROS and impaired DNA damage repair can lead to telomere dysfunction and DNA damage, which if persistent can result in the subsequent initiation of senescence or apoptosis pathways.

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